Conversion of hydroxycinnamic acids by *Furfurilactobacillus milii* in sorghum fermentations: impact on profile of phenolic compounds in sorghum and on ecological fitness of *Ff. milii*.

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Abstract

The conversion of phenolic compounds by lactobacilli in food fermentations contributes to food quality. The metabolism of phenolics by lactobacilli has been elucidated in the past years but information on the contribution of specific enzymes in food fermentations remains scarce. This study aimed to address this gap by disruption of genes coding for the hydroxycimmanic acid reductase Par1, the hydroxycinnamic acid decarboxylase Pad, the hydrocinnamic esterase EstR, and strains with disruption of all three genes in *Furfurilactobacillus milii* FUA3583. The conversion of phenolics by *Ff. milii* and its isogenic mutants in sorghum fermentations was studied by LC-UV and LC-UV-MS/MS analyses. *Ff. milii* FUA3583 converted hydroxycinnamic acids predominantly with Par1. Vinylphenols were detected only in mutants lacking *par1*. A phenotype for the *estR* defective mutant was not identified. The formation of pyrano-3-deoxyanthocyanidins was observed only after fermentation with strains expressing Pad. Specifically, formation of these compounds was low with *Ff. milii* FUA3583, substantially increased in the Par1 mutant and abolished in all mutants with disrupted *pad*. Competition experiments with *Ff. milii* FUA3583 and its isogenic mutants demonstrated that expression of one of the two metabolic pathways for hydroxycinnamic acids increases the ecological fitness of the strain. Disruption of EstR in a Δ*par1*Δ*par2*Δ*pad* background improved ecological fitness, indirectly demonstrating a phenotype of the esterase in *Ff. milii*. The documentation of the functionality of genes coding for conversion of hydroxycinnamic acids may support the selection of starter cultures for improved quality of fermented cereal products.

Keywords. *Lactobacillus,* sorghum, deoxyanthocyanidin, pyranoanthocyanidin, sourdough, polyphenols, phenolic acid.

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 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 (Gaur et al., 2020; 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 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 and this 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., 2016, 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 is a major cereal crop in Africa and Asia (Xiong et al., 2019). 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). Depending on the sorghum, cultivar, the total concentration of hydroxycinnamic acids ranges from 0.3 to 1 g / kg and hydroxycinnamic acids are mainly bound to cell wall polysaccharides (Awika and Rooney, 2004). Red sorghum varieties are more pest resistant than white sorghum varieties but their bitter taste has to be

mitigated by fermentation or malting (Kobue-Lekalake et al., 2007; Wu et al., 2019). Pest resistance as well as bitterness of red and black sorghum relates to the high content of 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 also been associated with health benefits (Hullings et al., 2020; Xiong et al., 2019). Phenolic compounds with antimicrobial activity also select for fermentation organisms including *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum* and *Furfurilactobacillus milii* 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; Svensson et al., 2010). Red sorghum varieties exhibit a stronger antimicrobial activity than white sorghum varieties but prior studies did not identify the contribution of specific phenolic compounds to the overall antimicrobial activity (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 occur in back-slopped type II sourdoughs, and *Lactiplantibacillus* or *Pediococcus* species 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 (Corsetti et al., 2005; Pswarayi and Gänzle, 2019; Ripari et al., 2016; Zheng et al., 2020). The metabolism of phenolic acids by *Ff. milii* (Simpson et al., 2022) is well characterized (Gaur et al., 2020); however, the phenolic acid metabolism in laboratory media does necessarily match the metabolic pathways observed in food fermentations (Filannino et al., 2015). In addition, the genotype of

Lactobacillaceae does not necessarily match the phenotype because alternative pathways of metabolism are available (Gaur et al., 2020; Ripari et al., 2019). This work therefore aimed to study the influence of genetic determinants of hydroxycinnamic acid metabolism on metabolite conversion in sorghum sourdoughs. We constructed 5 isogenic mutants of *Furfurilactobacillus milii* FUA3583 lacking genes involved in hydroxycinnamic acid metabolism and quantified the free phenolic acid profile in different pure cultivar sorghum sourdoughs. Additionally, qualitative analysis of red sorghum sourdough was performed to identify the profiles of flavonoids and deoxyanthocyanidins. We also assessed the role of hydroxycinnamic acid metabolism in ecological fitness by performing competition experiments with mutant and wild type (WT) strains in sorghum.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 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 5g/L cysteine under microaerophilic conditions at 30° or 37°C.

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) or Millipore Sigma (St. Louis, MO, USA).

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 (Gaur et al., 2020) 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, the fast track genome editing approach described 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 2.

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 24 h (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 applied on B: 0 min, 10%; 6 min, 15%; 14 min, 100%. Compounds were analyzed using a UV detector at 280 nm to quantify substrates and metabolites with external standards (Gaur et al., 2020).

2.5 Sorghum sourdough fermentation and determination of pH and cell counts

Sourdoughs were prepared in biological triplicates using pure cultivar sorghum grains or brown sorghum flour. 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 mixed with 10 g of grounded pure cultivars and commercial flour and incubated at 30°C for 24 h. Initial cell counts of the inoculum for all the cultures was 10^8 log CFU/g. To account for activities of sorghum enzymes in absence of microbial metabolism during

sourdough fermentation, chemically acidified dough were 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 (1g) 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 48-72h. Observation of a uniform colony morphology matching the inoculum and pH value were used to verify the absence of contamination. Remaining fermented sourdoughs were freeze dried and stored at -20°C for further analysis.

2.6 Quantification of free phenolic acids and metabolites in fermented sorghum sourdoughs using HPLC

Free dried sourdough samples were used for the extraction of free phenolics using the protocol by Ripari et al. (2019). Briefly, 250mg 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 500µl of 2% acetic acid adjusted to pH 2 using 12M hydrochloric acid. Samples were extracted twice using 500µl of ethyl acetate followed by evaporation under nitrogen and addition of 200µl 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.

2.7 Characterization of phenolic compounds in red sorghum sourdoughs using LC-MS

The samples were extracted using the procedure described by Bai et al., (2014) with some modifications. Briefly, 1 g of sorghum was extracted with a mixture of methanol, water and formic acid (70/29/1; v/v/v) for 1 h and then sonicated for 20 min. After centrifugation (10947 rcf) the supernatant was collected and the extraction procedure was repeated with the residue. The combined supernatants were evaporated under reduced pressure at 40 °C. Ethyl acetate (4 mL) was added to the residue and sonicated for 20 min. After centrifugation (10947 rcf) the supernatant was collected and the extraction procedure was repeated with the residue. The combined supernatants were evaporated under reduced pressure at 30 °C. The obtained residue was redissolved in 500 µL methanol, water and formic acid (70/29/1; v/v/v). After microfiltration (regenerated cellulose, 0.2 µm), 5 µL of the samples were analyzed using liquid chromatography coupled with a linear ion trap mass spectrometer.

UHPLC analysis of phenolic compounds was performed on an Acquity UPLC I-Class system (Waters, Milford, MA) consisting of a binary pump, a sample manager cooled at 20 °C, a column oven set at 40 °C and a diode array detector scanning from 190 to 700 nm. Chromatographic

separation was performed on an Acquity HSS-T3 RP18 column (150 x 2.1 mm; 1.8 µm), connected with a precolumn Acquity UPLC HSS T3 VanGuard (100 Å, 2.1 x 5 mm, 1.8 µm) (Waters, Milford, MA). The flow rate was set at 0.5 mL/min; eluents were water (A) and acetonitrile (B), both acidified with 3% (v/v) formic acid. Gradient applied on B was as follows: 0 min, 1%; 18 min, 25%; 23 min, 100 %.

The UHPLC was coupled to an LTQ-XL ion trap mass spectrometer, equipped with an electrospray interface operating in both positive and negative ion mode. Source parameters were as follows: source voltage (4 kV), capillary temperature (350 °C), capillary voltage (22 V) and tube lens (75 V) for positive ion mode; capillary voltage (−19 V) and tube lens (−85 V) for negative ion mode. Nitrogen was used as sheath, auxiliary, and sweep gas at a flow of 70, 10, 1 arb respectively for positive ion mode and 40, 5, 1 arb respectively for negative ion mode.

Ion mass spectra were recorded in the range of m/z 110-2000. Three consecutive scans were conducted using helium as the collision gas: full mass scan, an MS2 scan of the most abundant ion from the first scan using a normalized collision energy (CE) of 55%, and an MS3 of the most abundant ion in MS2 with a CE of 60%. Data evaluation was performed with Xcalibur (2.2SP1.48, Thermo Scientific, Inc.,Waltham, MA).

2.8 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 1g 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 (1.9-2g) from cycles 1, 4, 7 and 10 were homogenized with 25mL of 0.8% (Wt/V) saline (NaCl) solution and centrifuged at 500 rcf for 6 min to remove the solids. Cells were harvested by centrifugation at 5300 rcf 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.

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

Four sets of primers and probes listed in Table 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 1µl of 100-600pg of template DNA was added to the reaction mixture. Each 20µl reaction contained 10µl of 2X ddPCR supermix for probes (Bio-Rad Laboratories Inc., USA) along with 500nM of each

probe and 460nM of each forward and reverse primer. After the reaction set up in 96 well plates, 20µl 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. 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 WT vs double, triple and quadruple mutant 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 using QuantaSoft software version 1.3.2 (Bio-Rad Laboratories Inc.). Positive and negative controls were analyzed for both the 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.

2.10 Statistical analysis

The slope of the ratio of wild type to mutants over the number 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.). Significant differences of the slope of the ratio of wild

type to mutants were confirmed by two-way ANOVA analysis of the ratios of wild type to mutants observed after a specific number of fermentation cycles (data not shown). 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.

3. Results

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 (Gaur et al., 2020). 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 1).

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). 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).

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 5). Fermentation of white and commercial (brown) sorghum flour 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 ($P<0.001$). The ethanol concentration was highest in pure cultivar white sorghum sourdoughs and lowest in sourdoughs prepared with commercial white sorghum flour ($P<0.001$).

3.3 Identification of phenolic acid and flavonoid profile in red sorghum sourdough

To determine the impact of specific genes encoding for metabolism of hydroxycinnamic acids in *Ff. milii* on conversion of phenolic compounds in sorghum, the profile of phenolic acids and flavonoids in sorghum fermented with *Ff. milii* FUA3583 and its isogenic mutants was qualitatively assessed by LC-MS/MS. In fermented sorghum, phenolic acids and flavone aglycones luteolin (14) and apigenin (20) were identified by comparing retention times, UV-spectra and MS^n fragmentation pattern with reference substances (Table 6). Two flavanones, eriodictyol (13) and naringenin (17) were identified by comparison of the elution time and MS^n

fragmentation pattern with literature data (Svensson et al., 2010; Tsimogiannis et al., 2007). Similarly, compounds (8-11) were identified as 3-deoxyanthocyanidins namely, luteolinidin (8), apigeninidin (9), methoxyluteolinidin (10) and methoxyapigeninidin (11) (Bai et al., 2014).

Two anthocyanidins dimers, apigeninidin-flavene dimer (16) and apigeninidin-methylflavene dimer (27) were also identified based on their molecular masses of *m/z* 509 and *m/z* 523 respectively (Bai et al. 2014). Fragmentation of compound 16 led to the product ion m/z 255, indicating the cleavage of the interflavan 4→8 linkage, along with the release of a quinone methide intermediate and apigeninidin. The fragments m/z 384 corresponds to a heterocyclic ring fission with the loss of $[M+H-125]^+$, corresponding to a phloroglucinol moiety (Geera et al., 2012). Compound 27 exhibited similar fragment ions at m/z 384 and 255, with an additional fragment ion at m/z 269 indicating a methoxylated quinone methide unit (Geera et al., 2012). In total, 27 phenolic compounds were detected and 23 of them were tentatively identified. Retention times, UV maxima, and MS^n data are shown in Table 6.

Five pyrano and vinylphenol adducts were also tentatively identified in fermented sorghum with compounds 18, 21 and 25 identified as pyrano-apigenidin 4-(3'-hydroxyvinylphenol), pyrano-apigeninidin 4-vinylphenol and pyrano-methoxyapigeninidin 4-vinylphenol, respectively (Bai et al., 2014). The available spectrometric data could not differentiate between pyrano-apigeninidin 4-(3'methoxyvinylphenol) and pyrano-methoxyapigeninidin 4-(3'-hydroxyvinylphenol) for the identification of compound 23. The chromatographic behavior and UV spectra of compound 15 indicated a luteolinidin core and therefore was tentatively identified as pyrano-luteolinidin 4-hydroxyvinylphenol. In sorghum fermented with Δ*par1*Δ*par2* mutant, the intensities of pyrano and vinylphenol adducts of 3-deoxyanthocyanidins were substantially higher in comparison to the wild type, the Δ*pad2* mutant (Figure 2) and the chemically acidified control (data not shown),

correlating increased decarboxylation of phenolic acids to formation of pyrano and vinylphenol adducts of 3-deoxyanthocyanidins (Figures 2, 3 and 4). Likewise, adducts of 3-deoxyanthocyanidins were not detected in sourdoughs fermented with *Ff. milii* FUA3583 Δ*par1*/Δ*par2*/Δ*pad* or *Ff. milii* FUA3583 Δ*par1*/Δ*par2*/Δ*pad*/Δ*estR* (data not shown). Of note, LC-MS/MS analyses did not identify a phenotype for any of the two *ΔestR* mutants of *F. milii* FUA3583.

3.4 Hydroxycinnamic acid metabolism in sorghum sourdoughs

Hydroxycinnamic acids and their metabolites were quantified in sorghum sourdoughs fermented with *Ff. milii* FUA3583 and its isogenic mutant derivatives by reverse phase HPLC. The concentration of phenolic acids and metabolites in red, and white and commercial brown flour are shown in Figures 3 and 4, 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 3 and 4). HPLC-UV analysis detected low concentrations of dihydrocaffeic and phloretic acid in unfermented sorghum and in chemically acidified controls (Figures 3 and 4). Because these microbial metabolites are not present in intact seeds and their presence was not confirmed by LC-MS/MS (Table S1), these peaks are attributable to 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 (Table S1). 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 3 and 4). *Ff. milii* FUA3583 metabolised phenolic acids almost exclusively by reduction to the corresponding dihydro-derivatives. In most samples, products of decarboxylation were below the detection limit of 1.5

mg/kg (4-vinylphenol) and 8 mg/kg (4-vinylguaiacol). Vinylderivatives were also below the detection limit of the LC-MS/MS assay (Table S1). Deletion of reductase genes abolished the production of dihydro-derivatives; the concentration of dihydrocaffeic acid in all sourdoughs fermented with the *Δpar1Δ par2* reductase mutant was comparable to the chemically acidified control. Deletion of the reductases increased the concentration of 4-vinylcatechol ($P<0.001$) 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 limited impact on the spectrum of metabolites; the same deletion in a Δ*par1*Δ*par2* background abolished formation of 4-vinylcatechol and 4-vinylphenol. 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 (Figures 2 and 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 3 and 4).

3.5 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 5). 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 slope of the linear regression lines were used as a measure of the ecological fitness (Figure 5). 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 5). The wild type strain outcompeted the Δ*par1*Δ*par2*Δ*pad* and Δ*par1*Δ*par2*Δ*pad*Δ*estR* mutants (Figure 5). 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 5). 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.

4. Discussion

Genetic determinants encoding enzymes for the reduction and decarboxylation of hydroxycinnamic acids have been characterized in several species of the *Lactobacillaceae* (Cavin et al., 1997; Rodríguez et al., 2008; Santamaría et al., 2018a) but owing to the presence of two metabolic pathways their presence/absence does not always match the phenotype (Filannino et al., 2015; Gaur et al., 2020; Ripari et al., 2019). This study identified the role of genes coding for hydroxycinnamic acid metabolism in sorghum fermentations to better understand their role in metabolite production and ecological fitness in phenolic rich fermented foods.

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; Svensson et al., 2010). During fermentation, microbial metabolism converts phenolic compounds in sorghum by esterases, conversion of phenolic acids, and by hydrolysis of flavonoid glycosides (Svensson et al., 2010). 3-Deoxyanthocyanidins are converted by reaction with vinyl-derivatives of hydrocinnamic acids

399 while conversion of proanthocyanidins has not been described (Bai et al., 2014; Svensson et al.,
400 2010). Fermentation greatly increased the total free hydroxycinnamic acid content in sorghum
401 sourdoughs (this study) which is consistent with phenolic profiles in rye and wheat sourdoughs
402 (Ripari et al., 2019; Skrajda-Brdak et al., 2019) and spontaneous sorghum sourdough (Ravisankar
403 et al., 2021).

404 Phenolic acids act as external electron acceptors in heterofermentative lactobacilli resulting in
405 higher acetate production and ATP generation via the phosphoketolase pathway (Filannino et al.,
406 2016, 2014). Despite the high concentrations of hydroxycinnamic acids in sorghum, deletion of
407 reductase and/or decarboxylase genes did not result in any significant differences in acetate
408 production, however, the experimental error for quantification of acetate, ranging from 3 to
409 10mmol/kg, is larger than the concentration of hydroxycinnamic acids, ranging from 1 to
410 2mmol/kg (Figure 3, 4 and Tab. 5).

411 Despite a comprehensive analysis of phenolic compounds in fermented sorghum by LC-MS/MS,
412 the current study could not identify a phenotype for the hydroxycinnamic esterase mutant of *Ff.*
413 *milii*. Hydroxycinnamic acid esterases that were characterized in *Lp. plantarum* and *Lactobacillus*
414 *johnsonii* have shown a diverse substrate specificity (Esteban-Torres et al., 2015, 2013; Lai et al.,
415 2009).

416 Many *Lactobacillaceae*, including furfurilactobacilli, possess alternative pathways for metabolism
417 of hydroxycinnamic acids, decarboxylation, or reduction. NADH-dependent reduction of
418 hydroxycinnamic acids leads to production of phenylpropionic acids while decarboxylation to
419 vinyl derivatives consumes intracellular protons (Gänzle, 2015; Sánchez-Maldonado et al., 2011).
420 Both phenylpropionic acid and vinyl derivatives have a lower antimicrobial activity than the
421 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 (Gaur et al., 2020). The metabolism of hydroxycinnamic acid in 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 (Gaur et al., 2020), but reduction was by far major pathway observed in sorghum fermentations (Figure 3 and 4). *Ff. milii* FUA3583 over-expressed both *par1* and *pad* in response to addition of the substrates (Gaur et al., 2020). Quantification of gene expression in *Lp. plantarum* and *Lm. fermentum* in cereal substrates demonstrated, however, 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 impact gene expression in lactobacilli.

Dihydro-derivatives of phenolic acids were present only in the samples fermented with *par1* expressing strains. Deletion of phenolic acid reductase genes lead to an increased vinyl derivative production that may impact the flavour of fermented foods (Muñoz et al., 2017; Shahidi and Yeo, 2018). In sorghum, vinyl-derivatives of hydrocinnamic acid were additionally reported to react with 3-deoxyanthocyanidins to form pyrano and vinylphenol adducts of 3-deoxyanthocyanidins (Bai et al., 2014). Results obtained in this study confirm and extend these prior observations. Prior studies compared two wild type strains, which does not exclude a contribution of microbial, enzymatic or chemical conversions other than the microbial decarboxylation of hydroxycinnamic acids (Bai et al., 2014). This study used the comparison of the wild type strain with an isogenic Δ*par1*Δ*par2* mutant to demonstrate that an increased flux through the decarboxylase pathway also

445 increased the formation of pyrano and vinylphenol adducts of 3-deoxyanthocyanidins. Conversely,
446 deletion of the decarboxylase *pad* either in a wild type or in a Δ*par1*Δ*par2* background abolished
447 the formation of pyrano and vinylphenol adducts of 3-deoxyanthocyanidins (Figure 2 and data not
448 shown). This result demonstrates that microbial decarboxylation of hydroxycinnamic acid to
449 vinylphenols is the major contributor to the formation of pyrano-deoxyanthocyanidins and results
450 in conversion of a substantial proportion of 3-desoxyanthocyanidins (this study). This finding may
451 also explain the formation of pyranoanthocyanidins during fermentation of red wine (De Freitas
452 and Mateus, 2011), which invariably includes *Saccharomyces cerevisiae* as hydroxycinnamic acid
453 decarboxylase positive organism (Dzialo et al., 2017).

454 Competition experiments quantified the ecological fitness of strains with deletions of genes coding
455 for metabolism of hydroxycinnamic acids relative to the wild type in sorghum fermentations.
456 Antimicrobial activity of phenolic acids can act as a selective pressure in phenolic rich
457 environments (Gänzle, 2014). *Fl. sanfranciscensis* is a predominant organism in wheat and rye
458 sourdoughs and handily outcompetes other lactobacilli in wheat and rye sourdoughs that are back-
459 slopped frequently at ambient conditions (Dinardo et al., 2019; Meroth et al., 2003; Ripari et al.,
460 2016). Conversely, *Fl. sanfranciscensis* is outcompeted by other lactobacilli in sorghum
461 sourdoughs or in wheat sourdough with addition of ferulic acid (Dinardo et al., 2019; Sekwati-
462 Monang et al., 2012) and has not been isolated from sourdoughs produced with sorghum, millet
463 or other gluten free flours (Van Kerrebroeck et al., 2017). The present study is the first to document
464 the role of individual genes related to metabolism of phenolic compounds on the competitiveness
465 of lactobacilli in food fermentations. Strains that maintained only the reductase pathway were as
466 competitive as strains that maintained only the decarboxylase pathway, indicating that this
467 contribution to ecological fitness relates to NADH+ recycling (Filannino et al., 2014) or to proton

consumption through decarboxylation (Gänzle, 2015). The competition experiments also suggested an ecological role for the hydroxycinnamic acid esterase EstR as deletion of *estR* in a Δ*par1*Δ*par2*Δ*pad* background increased ecological fitness (Figure 5). In the wild type strain, hydroxycinnamic acids that are released by intracellular esterase activity (Esteban-Torres et al., 2013) are further converted by reductase and decarboxylase activities. In the strain *Ff. milii* FUA3583 Δ*par1*Δ*par2*Δ*pad*, hydroxycinnamic acids that are released by esterases are not detoxified. This explanation remains speculative, however, as analysis of phenolic compounds analysis did not identify differences between esterase mutants and their cognate isogenic strains.

The isogenic mutant strains of *F. milii* FUA3583 that were generated in this study allow production of fermentates or even purified compounds that contain substrates or products of metabolism and are thus suitable tools to explore chemical properties or biological activities of metabolites of hydroxycinnamic acids. Initial studies indicate that phenylpropionic acid metabolites supress or inhibit cell proliferation *in vitro* in human Caco-2 and SW480 carcinoma cell lines (Ekbatan et al., 2018; Martini et al., 2019) but their biological activities are not as well described as those of hydroxcinnamic acids. Biological or technological properties of pyrano- or vinylphenol adducts of 3-deoxyanthocyanidins are completely unexplored. Because African and Asian societies consume food produced from red sorghum preferably after malting and / or lactic fermentation (Gänzle, 2022; Pswarayi and Gänzle, 2022), these compounds are as relevant for human nutrition and human health as the precursor compounds, 3-desoxyanthocyanidins (Awika and Rooney, 2004). Cereal fermentations are thus an attractive proposition for production of bioactive phenolics and functional foods by selectively metabolizing hydroxycinnamic acids to different end products.

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Figure legends

Figure 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 (Gaur et al., 2020); *par2* – homolog of par1; *pad*- phenolic acid decarboxylase (Rodríguez et al., 2008). Gene locus tag numbers from NCBI are shown in the parenthesis.

Figure 2. UHPLC-UV-MS/MS chromatograms of red sorghum sourdoughs fermented with single strains of *Ff. milii* FUA3583 (wild type, middle black line) and its isogenic mutants Δ*pad* (bottom orange line) and ΔΔ*par1*/*par2* (upper blue line). Shown is the absorbance at 480 nm to specifically depict 3-deoxyanthocyanidins, eluting between 12 and 17 min, and their vinylphenol- or pyrano-adducts, eluting between 19 and 23 min. The numbers to designate peak identity correspond to Table 6. Chromatographic traces are representative for three independent fermentations.

Figure 3. Concentration of free hydroxycinnamic acids and their metabolites in pure cultivar red sorghum (Mahube variety) sourdough, fermented for 24 h with single strains of WT and mutant *Ff. milii* FUA3583. Extracted samples were analyzed using HPLC. Yellow bars, ferulic acid; orange bars, caffeic acids; blue bars, *p*-coumaric acid; diagonally hatched bars with colors matching the respective substrates: dihydroferulic acid, dihydrocaffeic acid and phloretic acid; horizontally hatched bars with colors matching the respective substrates, vinylcatechol and vinylphenol. Vinylcatechol concentrations are represented as % of relative peak area. Data are shown as mean + standard deviation of three independent experiments.

Figure 4. Concentration of free hydroxycinnamic acids and their metabolites in pure cultivar and commercial sorghum, fermented for 24 h with single strains of WT and mutant *Ff. milii* FUA3583.

a) Cultivar Segaolane (White) b) Commercial flour (Brown). Extracted samples were analyzed using HPLC. Yellow bars, ferulic acid; orange bars, caffeic acids; blue bars, *p*-coumaric acid; diagonally hatched bars with colors matching the respective substrates: dihydroferulic acid, dihydrocaffeic acid and phloretic acid; horizontally hatched bars with colors matching the respective substrates, vinylcatechol and vinylphenol. Vinylcatechol concentrations are represented as % of relative peak area. Data are shown as mean + standard deviation for three independent experiments.

Figure 5. 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). The mutant strains are designated as follows: □, Δ*estR*; *, Δ*pad;* □, ΔΔ*par1/par2;* □, ΔΔΔ*par1/par2/pad;* □, ΔΔΔΔ*par1/par2/pad/estR.* 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 ($P<0.05$).

Table 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	(Gaur et al., 2020)
Ff. milii FUA3583 Δ*par2*	Single deletion mutant of *Ff. milii* FUA3583 lacking *par2* gene	(Gaur et al., 2020)
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*/Δ*par2*/Δ*pad*	Tripe deletion mutant of *Ff. milii* FUA3583 lacking *par1*, *par2* and *pad* genes	This study
Ff. milii FUA3583 Δ*par1*/Δ*par2*/Δ*pad*/Δ*estR*	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)
Plasmids		
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	(Gaur et al., 2020)
pVPL3002/Δpad	pVPL 3002 containing *pad* flanking regions, Em^R	This study
pVPL3002/ΔestR	pVPL 3002 containing *estR* flanking regions, Em^R	This study

Table 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 (Gaur et al., 2020)	upstream flanking region of *par1* in *Ff. milii* FUA3583	GCAGCCAGATAGCCTGAAAC
par1 U/S R		CGACTGGCAGTTGCGCCAGCTGCGC
par1 D/S F	downstream flanking region of *par1*	AAGACGTTGGTCGTAAGGCCGTG
par1 D/S R	in *Ff. milii* FUA3583	CATAGCGGCAGTGAACTTGA
par1 BO1	LCR bridging oligo for pVPL3002/Δpar1	AAACGACGGCCAGTGAATTCGAGCTCGGTAGCAGCCAGAT AGCCTGAAACAATTCGTTGG
par1 BO2	LCR bridging oligo for pVPL3002/Δpar1	CTTTGGCGCAGCTGGCGCAACTGCCAGTCGAAGACGTTGGT CGTAAGGCCGTGGAGGAGA
par1 BO3	LCR bridging oligo for pVPL3002/Δpar1	GAATCCTTCATCAAGTTCACTGCCGCTATGATCCTCTAGAG TCGACCTGCAGGCATGCAA
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	AAACGACGGCCAGTGAATTCGAGCTCGGTAGTTGATTCTGG ACGGACGATTTACCAAAAC
pad BO2	LCR bridging oligo for pVPL3002/Δpad	TCACTTTATTTACACGTACGACAATGGCTGCCATACGATGG GATGACTGATGATATTCGC
pad BO3	LCR bridging oligo for pVPL3002/Δpad	ACCTCTATTTCTGCTTACGAGCCTGTCGTTATCCTCTAGAGT CGACCTGCAGGCATGCAA
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	AAACGACGGCCAGTGAATTCGAGCTCGGTAGGCCGACCAA TGCTCTATTAATTGGTGTTC
estR BO2	LCR bridging oligo for pVPL3002/ΔestR	AATTCATTTTCGTCAACCAGAACCACTGTAGCATCACCAAT TGCAAACAGACGTTGAAGC
estR BO3	LCR bridging oligo for pVPL3002/ΔestR	GGAAGTATTGGATGAAGCCAATGCAATTCCATCCTCTAGAG TCGACCTGCAGGCATGCAA
estR DCO F	DCO screening for Δ*estR* in *Ff. milii* FUA3583	GGCGATTCTCTTGATTACGG
estR DCO R		CGCGGTCAGTCAGATAAACA

Table 3. Primer 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/

Table 4. Phenotypic characterization of hydroxycinnamic acid metabolism of *Ff. milii* FUA3583 wild type and isogenic mutant strains after incubation with 1mM of different 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*/Δ*par2*	-	-	+	-	+	-	+
Ff. milii FUA3583 Δ*pad*	+	+	-	+	-	+	-
Ff. milii FUA3583 Δ*estR*	+	+	-	+	+	+	+
Ff. milii FUA3583 Δ*par1*/Δ*par2*/Δ*pad*	-	-	-	-	-	-	-
Ff. milii FUA3583 Δ*par1*/Δ*par2*/Δ*pad*/Δ*estR*	-	-	-	-	-	-	-

Table 5. Metabolite concentrations, pH and cell counts in sorghum sourdoughs fermented for 24h with single strains. Data are shown as mean ± standard deviation (n=3). Nd- Not determined.

Sourdough samples	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
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 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

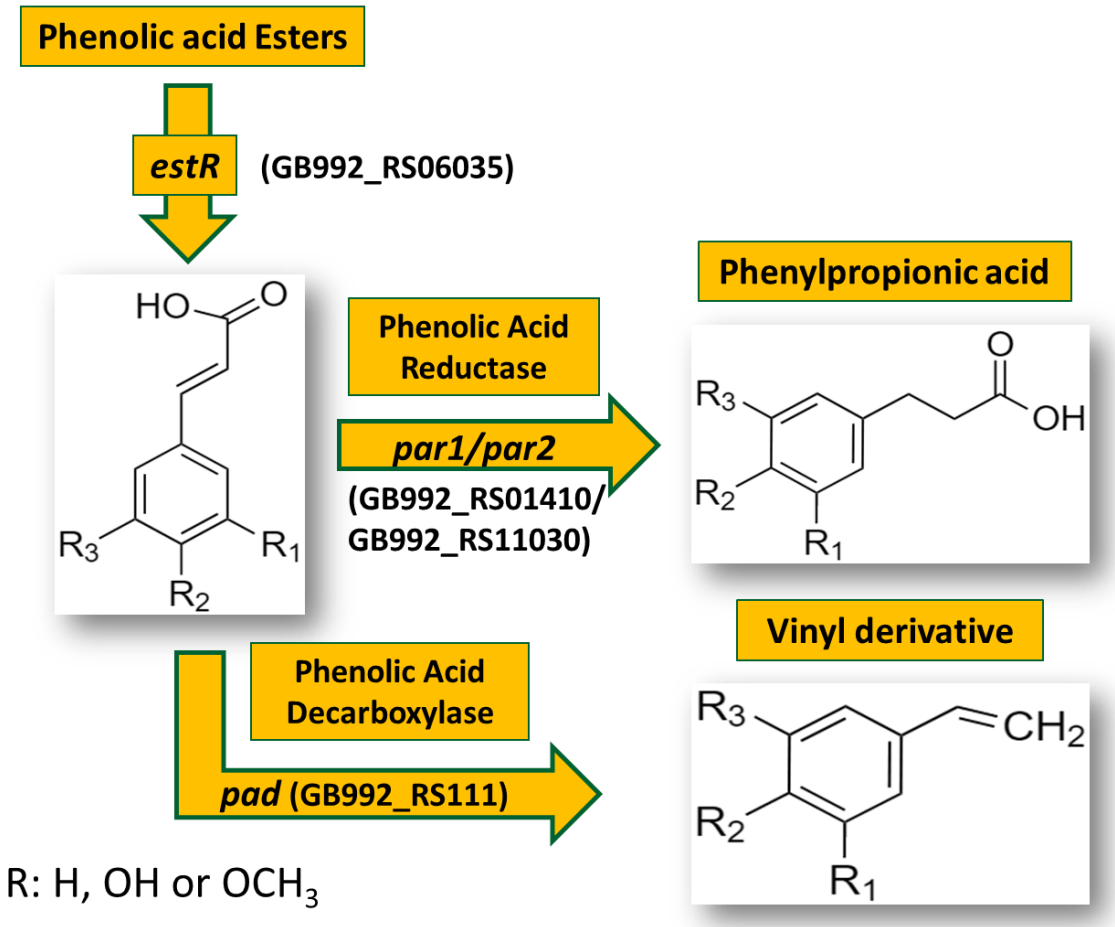
Table 6. LC-MS Identification of phenolic compounds in unfermented and fermented red (PAN 8609) sorghum.

Peak	RT (min)	UV_{max} (nm)	$[M-H]^-$	$[M+H]^+$	$[M]^+$	MS^n *m/z*	Compound	Reference
1	6.25	256		139		MS^2: 121 MS^3[-]	4-Hydroxybenzoic acid	Standard
2	7.55	296/325	181			MS^2: 137, 113, 119 MS^3[-]:	Dihydrocaffeic acid	Standard
3	8.37	296/325		181		MS^2: 163 MS^3[-]:	Caffeic acid	Standard
4	8.37	296/325		169		MS^2: 125,151 MS^3[-]:	Vanillic acid	Standard
5	9.58	288/322		199		MS^2: - MS^3[-]:	Syringic acid	Standard
6	10.94	312		165		MS^2: 147 MS^3[147]: 119, 147	*p*-Coumaric acid	Standard
7	12.63	296sh/325		195		MS^2: 177 MS^3[177]: 145	Ferulic acid	Standard
8	13.1	282/311/490			271	MS^2: 271,229,243,197,225,253,169, 187,235,230,215 MS^3[253]: 235, 123, 225, 171, 217, 165, 192	Luteolinidin	(Bai et al., 2014)
9	14.5	277/322/415/ 475			255	MS^2: 213,255,185,214,227,237,174, 223,163,157,187 MS^3[213]: 171	Apigeninidin	(Bai et al., 2014)
10	14.98	282/490			285	MS^2: 270,271,285,242,243,257,229 MS^3[270]: 242, 243	Methoxyluteolinidin	(Bai et al., 2014)
11	16.53	279/308sh/47 3			269	MS^2: 254,255,226,269,228 MS^3[254]: 226	Methoxyapigeninidin	(Bai et al., 2014)
12	17.19	288/340sh		723		MS^2: 435,561,273,417,409,297 MS^3[435]: 417,339,299,399,381,315,273	5,7,3´,4´-tetrahydroxyflavan-5-O-glucosyl-4,8-eriodictyol	(Gujer et al., 1986)
13	18.41	289/336sh		289		MS^2: 163,179,153,271,145,253,187, 205 MS^3[163]: 145, 135	Eriodictyol	(Tsimogiannis et al., 2007)
14	19.6	269/351 (Coelution)		287		MS^2: 153,287,259,241,245,185,177, 219,137,179,269,270,121 MS^3[-]:	Luteolin	Standard
15	19.82				403	MS^2: 403,385,375,367,376,357,283		-

		283/343/465/ 494			MS^3[385]: 367,337,211,251,207	Pyrano-luteolinidin 4-hydroxyvinylphenol	
16	20.32	283/482		509	MS^2: 255, 384 MS^3[255]:213,227,181,237,211,170,141	Apigeninidin-flavene dimer 4-8 linked	(Bai et al., 2014)
17	20.49	Coelution		273	MS^2: 153, 147, 231, 189, 179 MS^3[153]: 67, 111, 129, 109, 127	Naringenin	(Svensson et al., 2010)
18	20.50	291/331/ 491		387	MS^2: 387, 359 MS^3[359]: 296	Pyrano-apigenidin 4-(3'-hydroxyvinylphenol)	(Bai et al., 2022, 2014)
19	20.86	281/485		509	MS^2: 371, 384, 509 MS^3[371]: 371, 343, 249	Apigeninidin-flavene dimer 4-6 linked	-
20	20.92	269/336	271		MS^2: 271,153,229,225,145,253,230, 121,119,226,131,203 MS^3[-]:	Apigenin	Standard
21	21.04	272/393/ 460/486		371	MS^2: 371,343,354,344,350,341 MS^3[343]: 315	Pyrano-apigeninidin 4-vinylphenol	(Bai et al., 2014)
22	21.28	290/350/ 484 (Coelution)		523	MS^2: 371,383,508,523,491,479,269, 255 MS^3[371]: 371, 343, 250	unknown	
23	21.44	296/312/432sh/493		401	MS^2: 386, 401, 373 MS^3[386]: 358	Pyrano-apigeninidin 4-(3'methoxyvinylphenol) or Pyrano-methoxyapigeninidin 4-(3'-hydroxyvinylphenol)	(Bai et al., 2014)
24	21.78	290/324/ 484 (Coelution)		523	MS^2: 385,523,397,370,439 MS^3[385]: 370, 385, 357	unknown	
25	22.03	275/399/ 461/490		385	MS^2: 370,385,371,357,342,367,211, 339,353 MS^3[370]: 342, 343	Pyrano-methoxyapigeninidin 4-vinylphenol	(Bai et al., 2014)
26	22.22	283/486		537	MS^2: 385,521,397,537,505,429,370 MS^3[385]:370, 385, 357	unknown	
27	22.72	281/490		523	MS^2: 384, 269, 255 MS^3[383]: 355,383,291,366,263,339, 327	Apigeninidin-methylflavene dimer	(Bai et al., 2014)

Standard: Identified by comparison to reference compounds

Figure 1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O
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R1
R2
Phenolic Acid Reductase
par1/par2
(GB992_RS01410/
GB992_RS11030)
Phenylpropionic acid
O
OH
R3
R2
R1
Phenolic Acid Decarboxylase
pad (GB992_RS111)
Vinyl derivative
R3
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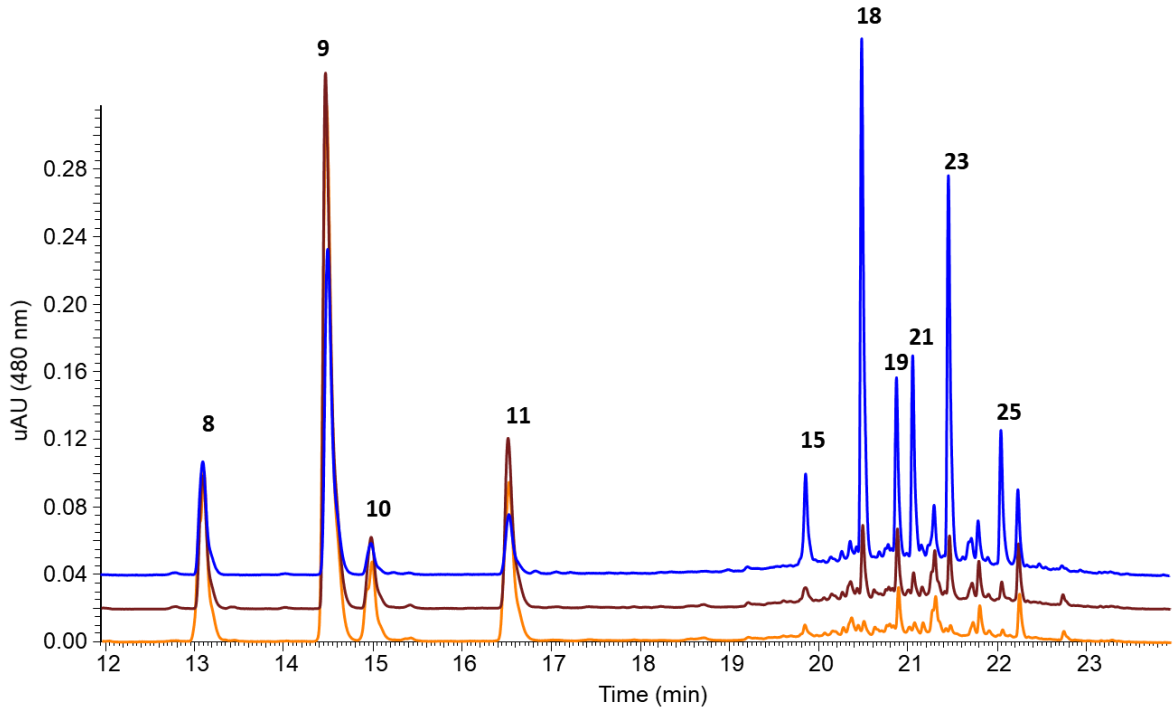
R2
R1
R: H, OH or OCH3

Figure 2.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23
21
19
25
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0.04
0.00
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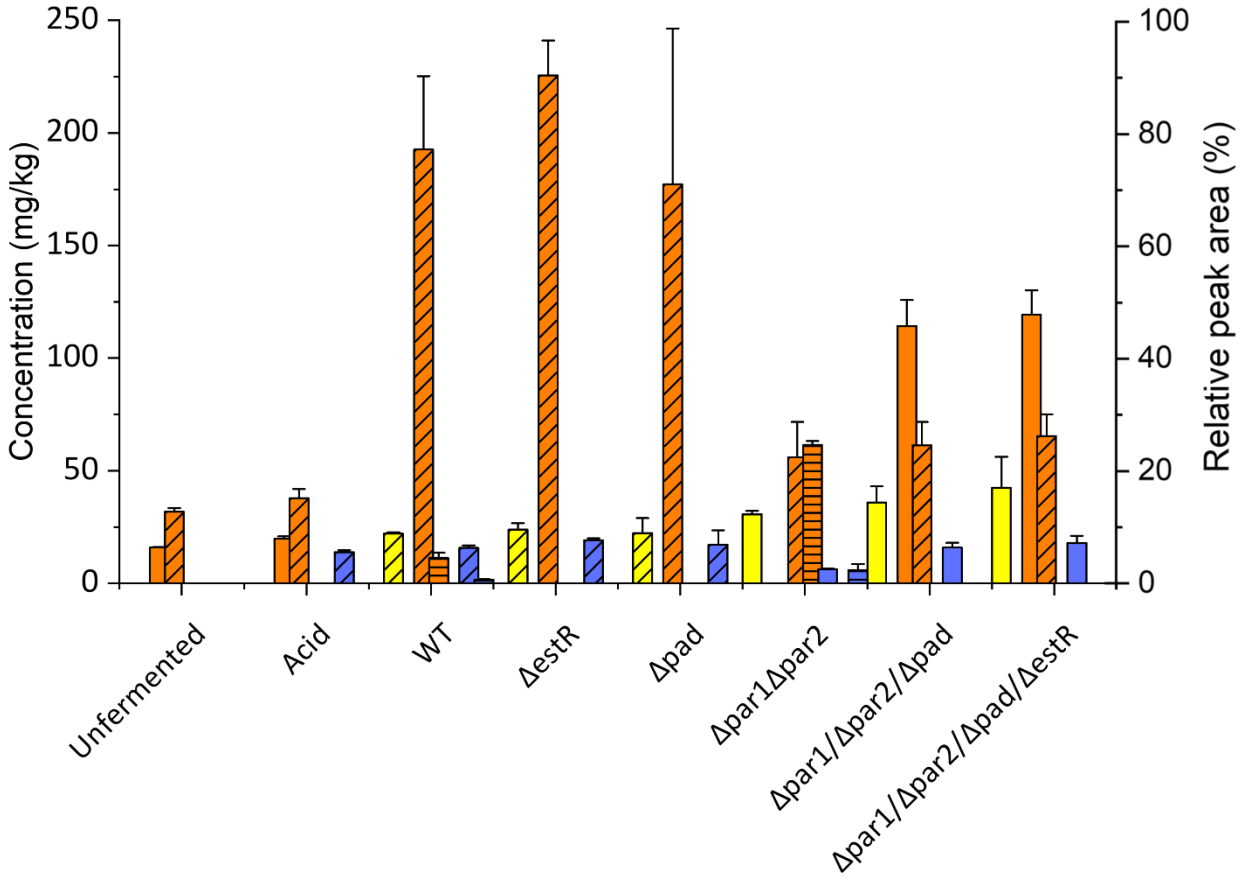
13
14
15
16
17
18
19
20
21
22
23
Time (min)

Figure 3.


Concentration (mg/kg)
Relative peak area (%)
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200
150
100
50
0
100
80
60
40
20
0
Unfermented
Acid
WT
ΔestR
Δpad
Δpar1Δpar2
Δpar1/Δpar2/Δpad
Δpar1/Δpar2/Δpad/ΔestR

Figure 4.

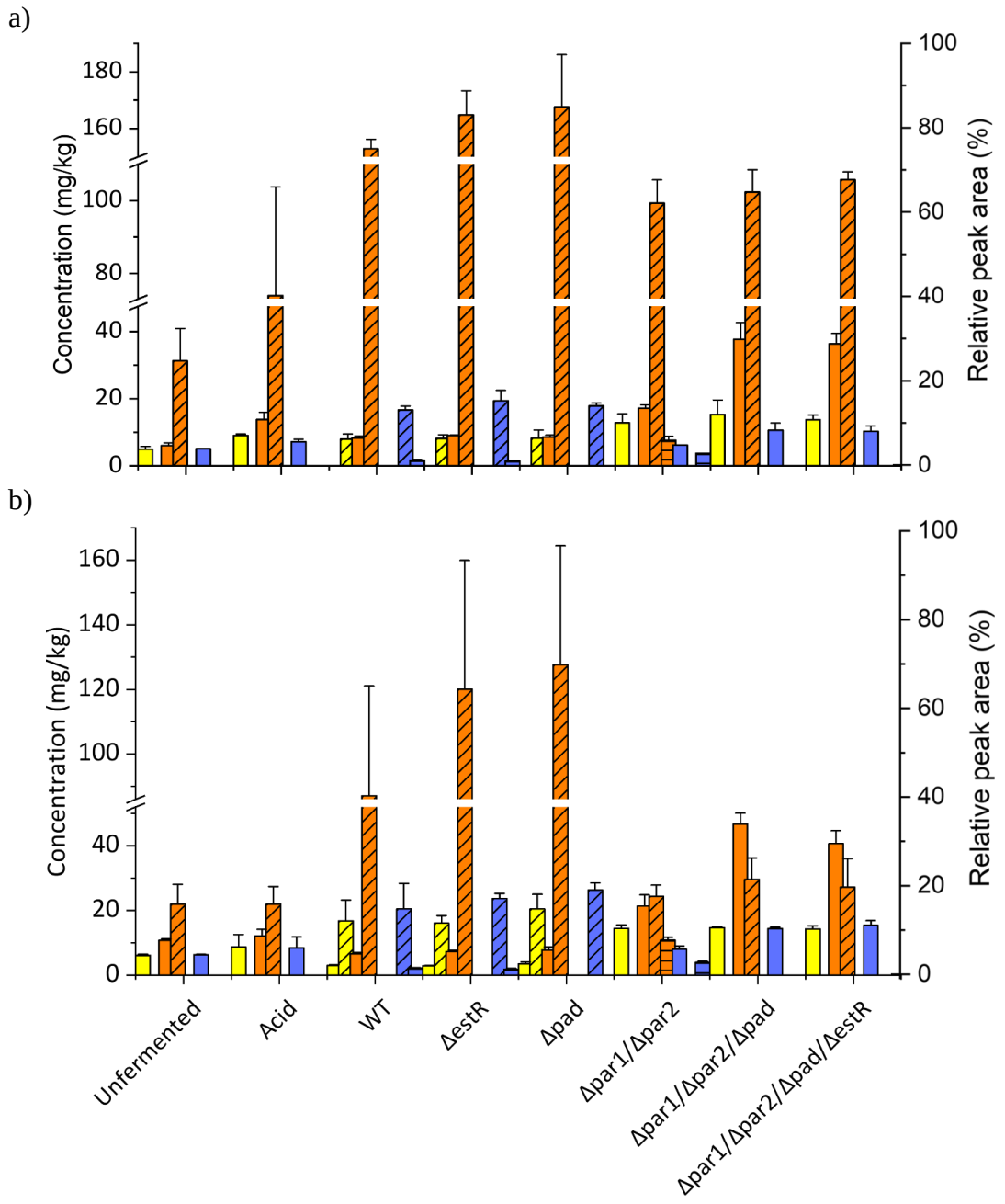
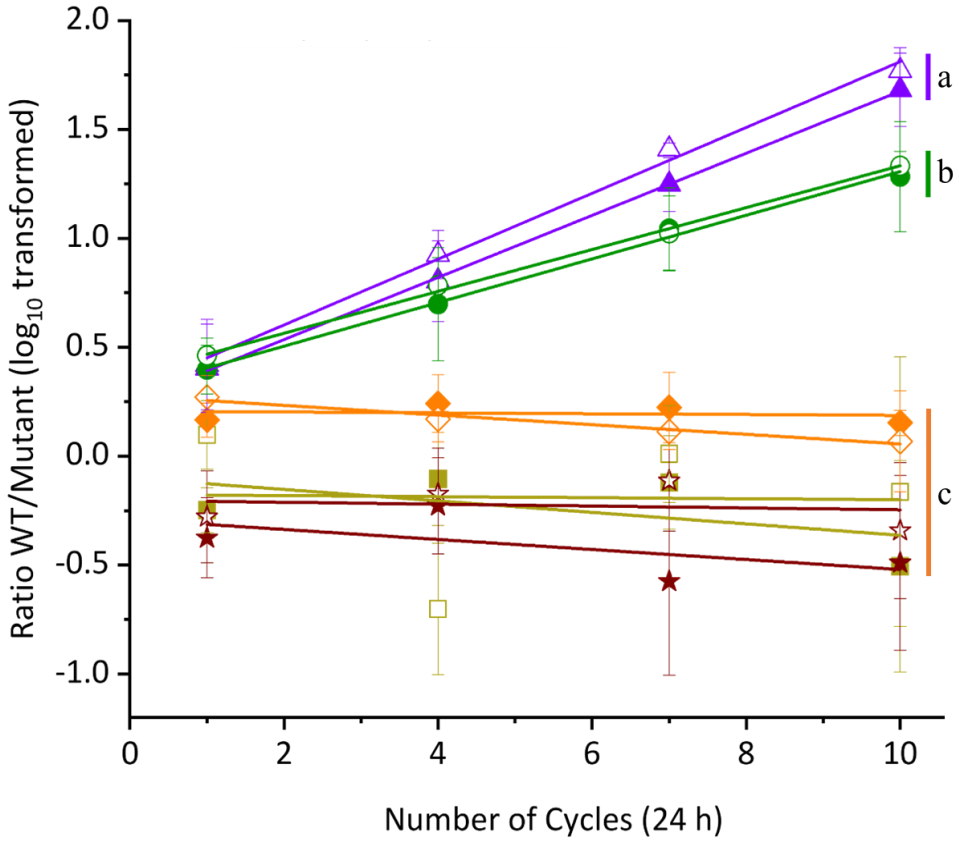

a)
b)
Concentration (mg/kg)
Relative peak area (%)
Unfermented
Acid
WT
ΔestR
Δpad
Δpar1/Δpar2
Δpar1/Δpar2/Δpad
Δpar1/Δpar2/Δpad/ΔestR

Figure 5.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0.0
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-1.0
Ratio WT/Mutant (log10 transformed)
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2
4
6
8
10
Number of Cycles (24 h)
a
b
c

Online supplementary material to

Conversion of hydroxycinnamic acids by *Furfurilactobacillus milii* in sorghum fermentations: impact on profile of phenolic compounds in sorghum and on ecological fitness of *Ff. milii*.

Gautam Gaur, Sandra Damm, Maike Passon, Hiu Kwan Lo, Andreas Schieber, Michael G. Gänzle

Table S1. Presence of hydroxycinnamic acids and their metabolites in unfermented and fermented red (PAN 8609) sorghum.

Table S1. Presence of hydroxycinnamic acids and their metabolites in unfermented and fermented red (PAN 8609) sorghum. Qualitative analysis was performed for 3 independent biological replicates using LC-MS/MS. The LC-MS/MS assay did not detect sinapic acid, dihydrosinapic acid and vinyl-derivatives of hydroxycinnamic acid in any of the samples.

Sample	Caffeic acid	Dihydro- caffeic acid	Ferulic acid	Dihydro-ferulic acid	*p*-Couma-ric acid	Phloretic acid
Unfermented	++	-	++	-	++	-
Acid	++	-	++	-	++	-
WT	-	++	-	+	-	+
Δ*est*	-	++	-	+	-	+
Δ*pad*	-	++	-	+	-	+
ΔΔ*par*	++	-	++	-	++	-
3Δ	++	-	++	-	++	-
4Δ	++	-	++	-	++	-

++: detected; +: detected but MS signal intensity was too low to confirm peak identity; -: not detected;