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Sourdough yeast-bacteria interactions can change ferulic acid metabolism during fermentation

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ABSTRACT

The metabolism of ferulic acid (FA) was studied during fermentation with different species and strains of lactic acid bacteria (LAB) and yeasts, in synthetic sourdough medium. Yeast strains of *Kazachstania humilis, Kazachstania bulderi*, and *Saccharomyces cerevisiae*, as well as lactic acid bacteria strains of *Fructilactobacillus sanfranciscensis*, *Lactiplantibacillus plantarum*, *Lactiplantibacillus xiangfangensis*, *Levilactobacillus hammesii*, *Latilactobacillus curvatus* and *Latilactobacillus sakei* were selected from French natural sourdoughs. Fermentation in presence or absence of FA was carried out in LAB and yeasts monocultures, as well as in LAB/yeast co-cultures. Our results indicated that FA was mainly metabolized into 4-vinylguaiacol (4-VG) by *S. cerevisiae* strains, and into dihydroferulic acid (DHFA) and 4-VG in the case of LAB. Interactions of LAB and yeasts led to the modification of FA metabolism, with a major formation of DHFA, even by the strains that do not produce it in monoculture. Interestingly, FA was almost completely consumed by the *F. sanfranciscensis* bFs17 *and K. humilis* yKh17 pair and converted into DHFA in 89.5 ± 19.6% yield, while neither bFs17, nor yKh17 strains assimilated FA in monoculture.

1. Introduction

Bread is one of the most widely cereal products consumed worldwide. Unlike unleavened or yeast bread, sourdough bread has an important role in the diet thanks to its appreciated sensory and nutritional qualities (Cappelle et al., 2013; Gobbetti et al., 2014, 2019; Gänzle and Ripari, 2016; Pétel et al., 2017). The preparation of this bread requires a "sourdough" which consists of a mixture of flour and water, naturally fermented by lactic acid bacteria (LAB) and yeasts (De Vuyst et al., 2017; Carbonetto et al., 2018; Gobbetti et al., 2019). Over the world, a wide diversity of microbial species has been identified in sourdoughs, with more than 60 species of LAB and 30 species of yeast (De Vuyst et al., 2014; Carbonetto et al., 2018). Usually only one or two abundant species of each microbial type (LAB and yeast) is detected per sourdough. Most of the sourdough's bacteria belong to genera *Fructilactobacillus*, *Lactiplantibacillus*, *Levilactobacillus*, *Latilactobacillus* with an abundance of species *Fructilactobacillus sanfranciscensis* (heterofermentative), but *Levilactobacillus brevis*, *Limosilactobacillus fermentum*, *Levilactobacillus hammesii* (heterofermentative), *Latilactobacillus sakei, Companilactobacillus kimchi, Latilactobacillus curvatus*, *Lactiplantibacillus plantarum* (homofermentative), can also be found (Gobbetti, 1998; De Vuyst et al., 2014; Zheng et al., 2020). Yeast species described in sourdoughs belong to genera *Saccharomyces*, *Kazachstania*, *Wickerhamomyces*, *Torulaspora*, *and Pichia* (Lhomme et al., 2015, 2016; Michel et al., 2016; Van Kerrebroeck et al., 2017). The most abundant species are *Saccharomyces cerevisiae*, *Kazachstania humilis, Kazachstania exigua*, *Pichia kudriavzevii*, *Wickerhamomyces anomalus,* and *Torulaspora delbrueckii* (Carbonetto et al., 2018).

Sourdough yeasts ferment flour carbohydrates to mainly produce ethanol and CO_2, which allows the raising of dough. They also generate other metabolites which bring new flavors to the bread, such as organic acids and aroma (Hazelwood et al., 2008; Pico et al., 2015; Pétel et al., 2017). LAB present in sourdough are either homofermentative or heterofermentative. The former metabolize hexoses into lactic acid only; while the latter convert hexoses into lactic acid, acetic acid, ethanol and CO_2 (Gänzle, 2015). The production of acids is generally responsible of the sour taste of breads; it also contributes to increase their shelf-life. Associated to yeasts, LAB could modify their metabolic pathway and vice versa. In co-culture of *K. humilis* and heterofermentative LAB, the amount of the consumed maltose is higher than in LAB monocultures, while *K. humilis* is maltose-negative and maltose consumption by heterofermentative LAB is very low (Carbonetto et al., 2020). The

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production of organic acids may also be altered by the yeast/LAB interactions (Collar, 1996; Gobbetti, 1998). The volatile profile of breads turned out to be richer when fermentation occurs with a mixture of yeast and LAB (Xu et al., 2019b). Indeed, a higher content of 2,3-methyl-1-butanol, 2-methyl-propanoic acid, 3-methyl-butanoic acid and 2-phenylethanol are obtained in co-cultures of *F. sanfranciscensis* or *L. plantarum* with *S. cerevisiae* (Gobbetti et al., 1995; Damiani et al., 1996; Gobbetti, 1998).

Beside carbohydrates, other metabolites present in wheat, may potentially contribute to enhance health benefits and organoleptic properties of sourdough breads. Phenolic acids known to be precursors of antioxidant and aromatic compounds, are divided into two subgroups, hydroxybenzoic acids (gallic acid, vanillic acid, syringic acid, etc.) and hydroxycinnamic acids (*p*-coumaric acid, caffeic acid, ferulic acid, etc.) (Li et al., 2008). Ferulic acid (FA) is the major hydroxycinnamic acid present in wheat, accounting for almost 90% of total phenolic compounds (Boz, 2015) and it is mainly located in bran. It varies between 1.36 mg/g and 2.8 mg/g in wheat bran depending on the wheat variety, the terroir, the growing conditions and the extraction methods (Verma et al., 2009; Boudaoud et al., 2020; Ferri et al., 2020; Sharma et al., 2020). FA is widely known for its antioxidant, antimicrobial and anti-inflammatory properties (Mancuso and Santangelo, 2014; Dędek et al., 2019). Through fermentation, FA is transformed into different derivatives of nutritional and organoleptic interest such as 4-vinylguaiacol or dihydroferulic acid (Fig. 1) (Coghe et al., 2004; De Las Rivas et al., 2009; Filannino et al., 2014; Adeboye et al., 2015).

The potential of metabolizing FA by sourdough microorganisms has been particularly investigated (separately) for *L. plantarum* and *S. cerevisiae,* with mainly two metabolic pathways. The first involves the decarboxylase activity, responsible of transforming FA into 4-VG. This activity is encoded by genes *pdc1* and *pdc2*, with a lower activity for *pdc2 in L. plantarum* (Barthelmebs et al., 2000) and by genes *PAD1* and *FDC1* in *S. cerevisiae* (Mukai et al., 2010). The second pathway is the reduction of FA into DHFA through the action of phenolic acid reductase. The reductase activity is linked to the expression of genes *hcrAB*, and particularly to *hcrB* gene in *L. plantarum* (Santamaría et al., 2018). Homologs of *HcrB* have been recently identified in other LAB species (Gaur et al., 2020). To our knowledge this activity has not been documented in *S. cerevisiae*. In bacteria, the ratio between decarboxylase and reductase activities depends on both environmental conditions and strains. In a medium with 20% ammonium sulfate or 20% sodium chloride, the decarboxylase activity is weak. Reductase activity is rather induced in a medium rich in glucose (20 mM) (Barthelmebs et al., 2000). In *L. plantarum* strains that combine reductase and decarboxylase activities, DHFA is produced in a higher amount than 4-VG (Ripari et al., 2019). The only study dealing with the association of sourdough yeast and bacteria on the metabolism of FA was conducted by Koistinen et al. (2018). In this work, two synthetic sourdoughs made from wheat and rye whole meal flours were inoculated with *L. brevis* and *L. plantarum* bacteria combined with *K. humilis* yeast. After fermentation, they identified 118 compounds with increased levels in the sourdoughs, including microbial metabolites of phenolic acids (dihydroferulic acid, dihydrocaffeic acid and dihydrosinapic acid) (Koistinen et al., 2018). However, this was done for a single synthetic starter and the diversity of LAB/yeasts interactions in metabolizing FA, expressed by the differences in the behavior of microorganisms in mono- and co-cultures has never been studied.

In order to shed light on yeast-bacteria interactions effect, this study investigate the metabolism of FA in a synthetic sourdough medium, in the presence of LAB, yeasts, as well as LAB-yeast associations. Strains were isolated from natural sourdoughs and selected among the commonly found sourdough microbial species. LAB strains belong to a wide diversity of species found in sourdoughs (Michel et al., 2016). Yeast strains include *S. cerevisiae* but also *K. bulderi* and *K. humilis* strains. *K. bulderi* has been frequently found in French farmer-baker sourdoughs (Urien et al., 2019; Michel et al., 2019), while *K. humilis* has been detected in worldwide artisanal sourdoughs (Carbonetto et al., 2018).

2. Materials and methods

2.1. Chemicals

Wheat Peptone, potassium dihydrogenphosphate (KH_2PO_4), Dipotassium hydrogen orthophosphate (K_2HPO_4), Manganese (II) sulfate tetrahydrate ($MnSO_4{\cdot}4H_2O$), TWEEN® 80 solution, Glucose, Maltose, Sodium chloride (NaCl), Potassium chloride (KCl), Disodium phosphate (Na_2HPO_4), *trans*-ferulic acid and dihydroferulic acid were purchased from Sigma-Aldrich. 4-vinylguaiacol was synthesized according to (Zago et al., 2016). Ammonium chloride (ClH_4N) and Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) were purchased from Fluka™. Tryptone was obtained from Becton Dickinson and Company. Meat extract and yeast extract were supplied by BIOKAR Diagnostics. Sodium acetate ($C_2H_3NaO_2$), Methanol (CH_3OH), Ethanol (C_2H_5OH), Acetonitrile (CH_3CN), Formic acid (HCOOH), were purchased from VWR chemicals.

2.2. Strains, synthetic sourdough medium, and growth conditions

2.2.1. Microbial strains

LAB strains were propagated anaerobically at 24 °C in MRS-5 medium plates (De Man, Rogosa and Sharp medium, prepared according to (Meroth et al., 2003), for 48 h. Yeast strains were propagated at 28 °C in

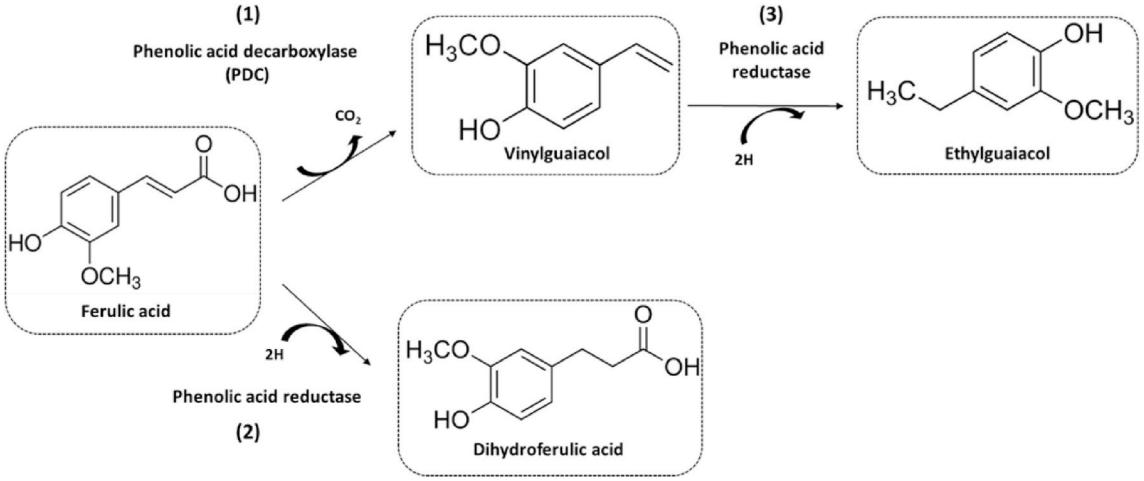

Fig. 1. Schematic representation of the general metabolic pathways of FA by LAB. Modified from (Filannino et al., 2014).

YEPD medium plates (Yeast Peptone Dextrose medium, prepared according to Xu et al. (2019a), for 24 h. LAB and yeast strains data are described in Table 1. These strains were used in both mono- and co-cultures.

2.2.2. Synthetic sourdough medium (SSM)

SSM preparation was adapted from Vrancken et al. (2008). It contains per L: wheat peptone, 24 g; $MgSO_4.7H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.05 g; KH_2PO_4, 4 g; K_2HPO_4, 4 g; Tween 80, 1 mL. milliQ water (0.899 L) was added to the mixture and pH was adjusted to 4.5 with citric acid at 3 M. The solution was autoclaved (pressure = 0.5 bar; temperature = 120 °C) using autoclave (LEQUEUX-1983). The sterilized SSM was then completed with 100 mL of sugars mixture solution (glucose 150 g/L, maltose 350 g/L, filtrated over 0.22 μm filter to remove microorganisms) to reach a final concentration of 15 g/L of glucose and 35 g/L of maltose. Then, 1 mL of filtrated (over 0.22 μm filter) vitamin solution containing 200 mg/L of each of the following vitamins (cobalamin, nicotinic acid, folic acid, pantothenic acid, pyridoxal-phosphate, thiamine) was added to complete 1 L of SSM. This synthetic sourdough medium was used as control. SSM-FA containing FA was prepared as follows: *trans*-FA was added to SSM at a concentration of 25 mg/L. The solution was stirred for 1 h to completely dissolve FA and then sterilized by steam pasteurization for 15 min. After pasteurization, the FA concentration remained unchanged.

2.2.3. Preparation of microorganism cultures

Pre-cultures were prepared as follows: a colony of each strain was grown in 20 mL of MRS-5 medium for LAB, and in 6 mL of YEPD medium for yeasts. All strains were incubated overnight at 28 °C with stirring. These pre-cultures were centrifuged (4500 rpm for 5 min at 20 °C) and cells were suspended in 1 mL of SSM. Cells concentration was determined by means of C6 flow cytometer (Accuri™, BD Biosciences). In monoculture system, 15 mL of SSM or SSM-FA with either 10^6 cells/mL of yeasts or 10^8 cells/mL of LAB were placed in 20 mL glass tubes, equipped with filter tip to allow CO_2 release. Co-cultures were prepared by mixing 10^6 cells/mL of yeast strain with 10^8 cells/mL LAB strain in 15 mL of SSM or SSM-FA.

Table 1
Genera, species and strains of lactic acid bacteria (LAB) and yeasts.

Genus	Species	Strain's Code	Reference
Lactic acid bacteria (LAB)			
Fructilactobacillus	*sanfranciscensis*	bFs17	Michel et al. (2016)
		bFs15	Michel et al. (2016)
		bFs9	Michel et al. (2016)
		bFs12	Michel et al. (2016)
Lactiplantibacillus	*plantarum*	bLp6	Michel et al. (2016)
		bLp16	Michel et al. (2016)
		bLp20	Michel et al. (2016)
	xiangfangensis	bLx29	Michel et al. (2016)
Levilactobacillus	*hammessii*	bLh5	Lhomme et al. (2015)
Latilactobacillus	*curvatus*	bLc16	Michel et al. (2016)
	sakei	bLsk4	Lhomme et al. (2015)
Yeast			
Saccharomyces	*cerevisiae*	ySc10	Lhomme et al. (2015)
		ySc16	Michel et al. (2019)
		ySc29	Michel et al. (2019)
		ySc9	Urien et al. (2019)
		ySc32	Michel et al. (2019)
Kazachstania	*bulderi*	yKb12	Urien et al. (2019)
		yKb15	Urien et al. (2019)
		yKb20	Michel et al. (2019)
		yKb4	Urien et al. (2019)
	humilis	yKh17	Michel et al. (2019)
		yKh5	Urien et al. (2019)
		yKh6	Urien et al. (2019)

2.3. Automated fermentation

Fermentations were carried out over 37 h, with a constant stirring (300 rpm) at 24 °C, using an automated robotic system (PlateButler® Robotic System by Lab services) (Bloem et al., 2018). This system allowed online monitoring of the fermentation by measuring the weight loss of each sample every 50 min, which is correlated to CO_2 release. The online data are automatically inserted into the ALFIS (Alcoholic Fermentation Information System) software. Based on polynomial smoothing, four fermentation parameters were estimated from the CO_2 accumulation curve over time (Sablayrolles et al., 1987): the maximum CO_2 release (g/L), the fermentation latency-phase time (h) which is the time between inoculation and the beginning of the fermentation calculated as 1 g of CO_2 release, the maximum CO_2 production rate Vmax (g/L/h) and the time to reach the maximum CO_2 production rate tVmax (h). FA assimilation capacity was first tested in LAB and yeast monocultures. Then, three independent experimentations were carried out for each co-culture, while repeating monocultures as controls.

2.4. Cell count and viability

After 37 h, 200 μL of each sample were centrifuged (10,000 rpm for 5 min at 20 °C) and microbial cells were diluted in PBS (Phosphate-Buffered Saline, containing: NaCl; 137 mM, KCl; 2.7 mM; Na_2HPO_4, 10 mM; KH_2PO_4; 2 mM, at pH = 7.4, filtered through 0.2 μm filter) for flow cytometry analysis (C6 flow cytometer, Accuri, BD Biosciences). Population size and cell viability were determined as described in Delobel et al. (2012).

2.5. Characterization of FA metabolism after fermentation

Characterization and quantification of FA consumed after fermentation, as well as its corresponding metabolites were performed using ultra pressure liquid chromatography (UPLC). The fermentation tubes were centrifuged (4500 rpm for 10 min at 4 °C) and 300 μL of supernatant were then filtered through 0.2 μm filter to remove all the residues from the solution. Supernatants were injected on an Acquity UPLC (Waters, Milford, MA) equipped with a photodiode array detector (DAD). The Waters C18 column was 100 mm × 2.1 mm, HSS T3, with particles size of 1.8μm. solvents used were A (99.9% H_2O and 0.1% HCOOH v/v) and B (100% CH_3CN) and the flow rate was 0,55 mL/min. The gradient conditions were as follows: from 0 to 4 min, 99%–70% A; from 4 min to 7 min, 70%–20% A; from 7 min to 8 min, 20% A; from 8 min to 9 min, 20%–99% A. The injection volume was 2 μL and DAD was set at 280 nm (λ_{max} of phenolic compounds) (adapted from Rouméas et al., 2018). FA and its identified metabolites, namely 4-vinylguaiacol (4-VG) and dihydroferulic acid (DHFA) were quantified after calibration with standard compounds dissolved in SSM for FA and in methanol for 4-VG and DHFA. The material balance of FA conversion reaction was determined according to (Filannino et al., 2014).

2.6. In silico analysis of putative ferulic acid reductase and decarboxylase in F. sanfranciscensis

According to the work of Gaur et al. (2020), three different genes from Lactobacillaceae species were considered as reference ferulic acid reductase, namely *hcrB* (*L. plantarum*, UniProt: F9UNH3, 812 aa), *hcrF* (*L. fermentum*, UniProt: A0A158SNB3, 617 aa), and *par1* (*F. rossiae*, UniProt: A0A0R1RH44, 614 aa). For ferulic acid decarboxylase activity, *pdc1* (*L. plantarum*, UniProt: P94900, 174 aa) was used as reference gene (Barthelmebs et al., 2000). In order to identify putative ferulic acid reductase and decarboxylase genes in *F. sanfranciscensis*, an *in silico* analysis was performed on all sequenced strains of this species available on public databases. The complete genome sequences and gene annotations of 30 different strains were retrieved at the NCBI (see Table S22 for the strain names and the corresponding accession numbers). In a first

step, BLAST searches (version 2.10.0+) of four genes cited above on the 30 strains of *F. sanfranciscensis* at both the protein level (BLASTp) and the whole chromosome level (tBLASTn) were performed. Then, putative protein domain signatures for all the proteins of the 30 strains (39,264 proteins) were predicted with InterProScan (version 5.47–82.0) and compared with the protein domain signatures from the four reference genes.

2.7. Statistical analysis

All experiments were carried out in three replicates. All statistical tests were performed using R software, version 4.0.3. To test the effect of FA on bacteria and yeast growth, the population size per mL was analyzed after 37 h fermentation in a synthetic sourdough. The population size was expressed in Log 10 to meet the condition of application of statistical linear models. Also, the effect of FA on the four fermentation parameters was tested. First, we analyzed the variation of each quantitative variable (Population size, Vmax, tVmax, CO_2max and Latency) in LAB and yeast monocultures in the same way. For bacteria, we used a linear mixed model with presence of FA in the medium, LAB strains and their interaction as fixed effect and experimental block as random effect. When there was no bloc effect, we delete the random block effect from the model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_{ij} + D_k + \varepsilon_{ijkl},$$

where Y_{ijk} is the quantitative variable, α_i is the fixed LAB strains effect, β_j is the fixed FA effect, γ_{ij} is the interaction effect between FA and LAB strains, D_k is the random block effect and ε_{ijk}, the residual error.

For yeast, we used the same model except that we include the yeast species fixed effect and a strain random effect. This was not possible for LAB as some LAB species were represented by a single strain only:

$$Y_{ijklm} = \mu + \alpha_i + \beta_j + \gamma_{ij} + D_k + \varepsilon_{ijklm},$$

where Y_{ijklm} is the parameter variable, α_i is the fixed yeast species effect, β_j is the fixed FA effect, γ_{ij} is the interaction effect between FA and yeast species, D_k is the random strains effect, F_l is the random block effect and ε_{ijklm}, the residual error. When residues deviated from normality (LAB population size), we also ran non parametric tests, *i.e.* Kruskal-Wallis tests. Second, we compared the difference between mono and co-culture by adding to the previous models a fixed effect with two levels mono- or co-cultures. For this analysis, we only include monocultures of strains that were also tested in co-cultures.

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_{ij} + D_k + \varepsilon_{ijkl},$$

where Y_{ijk} is the parameter variable, α_i is the fixed culture type effect (monoculture vs co-culture), β_j is the fixed FA effect, γ_{ij} is the interaction effect between FA and culture type, D_k is the random block effect and ε_{ijk}, the residual error.

3. Results

3.1. Selection of LAB-Yeast couples for co-culture fermentations

Based on a preliminary test dealing with the assimilation of FA (monitored by liquid chromatography) in monoculture fermentations, LAB/yeast couples were formed according to three different scenarios: both microorganisms assimilate FA, one of them is able to do that, and none of them transform FA. For each scenario, at least three different LAB/yeast pairs of strains were chosen randomly. The different combinations are displayed in Fig. 2.

3.2. Effect of FA on microbial growth in mono- and co-culture

The presence of 25 mg/L of FA had no significant effect on the growth of LAB in average ($p = 0.73$), since the LAB population size was almost the same at the end of fermentation in the presence and absence of FA (8.6 ± 0.3 $\log_{10}$ cell/mL on average at [FA] = 0 mg/L, and 8.6 ± 0.5 $\log_{10}$ cell/mL at [FA] = 25 mg/L). For some specific strains, FA seems to decrease the LAB population size but this effect was only marginally significant ($p < 0.001$, Figure S1; Table S1). In the case of yeasts, FA had an inverse effect on the growth of *S. cerevisiae* and *K. bulderi*. While increasing the population size of the former ($p = 0.004$), it decreased the population size of the latter ($p = 0.04$) (Figure S2; Table S2). This resulted in a non-significant effect of FA on average yeast population size (8.2 ± 0.2 $\log_{10}$ cell/mL in the absence of

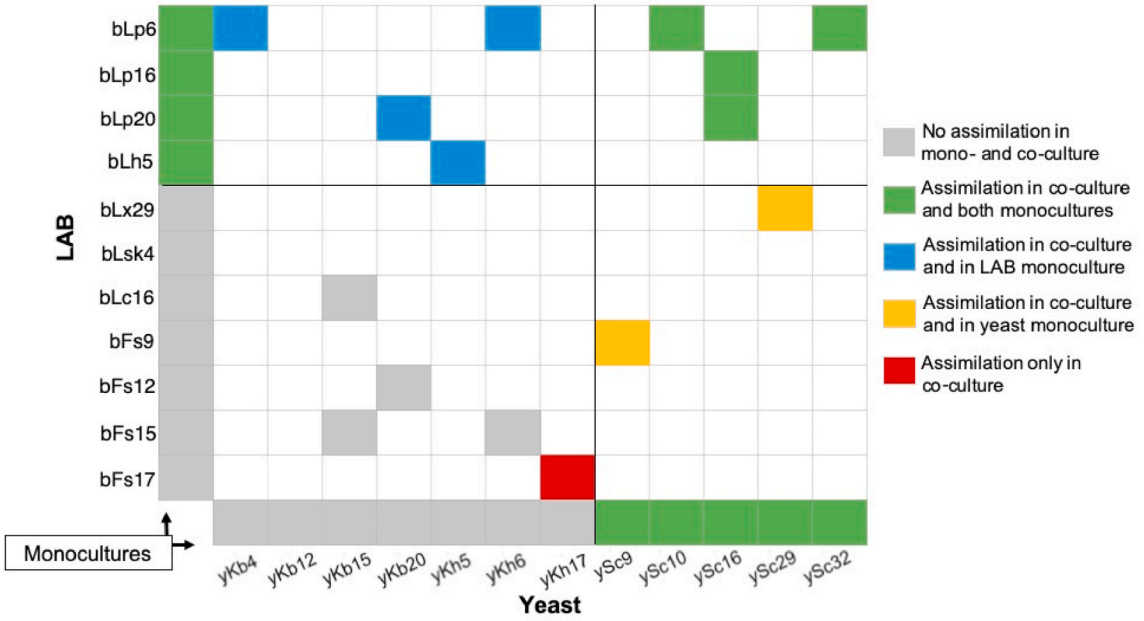

Fig. 2. Assimilation of FA (as function of its concentration at t0) after 37 h of fermentation in the presence of LAB and yeast strains in mono- and co-culture. The data are presented by a matrix whose colors indicates different cases of strains behavior in the assimilation of FA. The first column and line give monocultures results. The figure center shows co-cultures results. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

FA and 8.2 ± 0.2 log_{10} cell/mL in the presence of FA). Irrespective of the presence of FA, a variation on microbial growth was found among LAB strains ($p < 0.01$), where strains bLsk4, bLc16, bFs15 reached population sizes from 2 to 3,5 times lower than those of strains bLp6, bLp20 (Table S1); as well as among yeast species ($p = 0.003$; Table S2) with a lower growth of *K. humilis* compared to *K. bulderi* ($p = 0.002$) and *S. cerevisiae* ($p = 0.002$).

The absence of FA effect on microbial growth was also observed in the case of LAB and yeasts co-cultures (for LAB: FA effect $p = 0.71$, FA x Culture type interaction $p = 0.65$; for yeasts: $p = 0.18$, interaction effect $p = 0.59$). However, each type of microorganisms had a negative effect on the growth of the other. Thus, LAB reached a lower population size in co-culture (8.5 ± 0.4 log_{10} cell/mL) than in monoculture (8.6 ± 0.4 log_{10} cell/mL; $p < 0.001$, Figure S3; Table S3), and yeasts population size decreased from 8.2 ± 0.2 log_{10} cell/mL in monoculture to 8.1 ± 0.2 log_{10} cell/mL in co-culture ($p < 0.001$, Figure S4; Table S4).

3.3. Effect of FA on fermentation parameters of yeast and LAB in mono- and co-culture

For each strain fermented in mono- and co-culture, four parameters were analyzed in the presence and absence of FA, CO_2max, latency phase duration, Vmax, and tVmax. During bread making, these parameters provide information on the required fermentation time and the quality of the dough rise. Results showed that again, the differences in fermentation parameters were related to LAB strains and yeast species and not to FA effect. No significant effect of FA was observed on any of the four fermentation parameters.

As expected, heterofermentative LAB produced low amount of CO_2max. However, the CO_2max was significantly different between strains ($p < 0.001$, Figure S5; Table S5). The three-yeast species produced significantly different CO_2max ($p < 0.001$, Figure S6; Table S6), with a better performance of *S. cerevisiae* which produced 24.4 ± 0.7 g/L of CO_2max on average compared to *K. bulderi* and *K. humilis* with 7.7 ± 0.5 g/L and 8.0 ± 0.8 g/L respectively. CO_2max generated in co-culture was different from that produced in monoculture (yeasts in monoculture and co-culture with LAB: $p < 0.001$; and LAB in monoculture and co-culture with yeasts: $p < 0.001$, Figures S7 and S8; Tables S7 and S8).

While the maximum rate of CO_2 production (Vmax) did not significantly vary between LAB strains (Figure S5; Table S9), but varied among yeast species ($p < 0.001$, Figure S6; Table S10) and strains of *S. cerevisiae*. Significant differences in Vmax were observed between monoculture and co-culture for both LAB ($p < 0.001$, Figure S7; Table S11) and yeasts ($p < 0.001$, Figure S8; Table S12). The higher Vmax was obtained for *S. cerevisiae* monocultures, followed by yeast/LAB co-cultures, and then LAB monocultures.

The time to reach Vmax, tVmax, in LAB monocultures was shorter than in co-cultures (significant difference: $p < 0.001$, Figure S7; Tables S13 and S14). tVmax was significantly different between yeast species ($p < 0.001$, Figure S6; Table S15). *S. cerevisiae* had a significantly longer tVmax (18.0 ± 1.5 h), than *K. bulderi* (12.3 ± 1.4 h) which in turn displayed a significantly longer tVmax than that of *K. humilis* (10.2 ± 0.3 h). Compared to yeast monocultures, tVmax significantly decreased in the presence of LAB ($p < 0.001$, Figure S8; Table S16), especially for *K. humilis* and *K. bulderi* (in monoculture: 10.2 ± 0.3 h and 12.4 ± 1.3 h respectively, and in co-culture with LAB: 9.6 ± 1.9 h and 12.0 ± 1.4 h respectively).

Latency phase duration did not significantly vary neither between LAB strains, nor among yeast species, nor between yeast monocultures and co-cultures; but a significant difference was found between LAB monoculture and co-culture ($p = 0.001$, Figures S7 and S8; Tables S17 to S20). The latency time of LAB in monoculture lasted 12.8 ± 8.8 h, but the fermentation started more quickly when *K. humilis, K. bulderi* or *S. cerevisiae* yeast strain was added (7.4 ± 2.4 h, 8.2 ± 2.6 h and 8.6 ± 2.2 h respectively, Figures S7 and S8; Table S19).

In general, the presence of LAB had a little effect on the fermentation performance of certain yeast species, especially *S. cerevisiae*.

3.4. Consumption and conversion of FA by LAB and yeasts in mono- and co-culture

The FA consumption by microorganisms, as well as the formation of its derived metabolites, namely 4-vinylguaiacol (4-VG) and dihydroferulic acid (DHFA), during fermentation were monitored by UPLC (a representative chromatogram is depicted in Figure S9), and the material balance of conversion reactions was determined taking SSM as control. Results are presented in Figs. 2 and 3.

After 37 h of fermentation in synthetic sourdough medium, the assimilation of FA was detected in 4 out of 11 strains of LAB (Fig. 2 and Table S21). *L. hammesii* bLh5 strain transformed 86.0 ± 15.4% of FA into DHFA with a 98.5 ± 28.5% high yield. *L. plantarum* bLp20 and bLp16 strains assimilated respectively 57.6 ± 8.7% and 27.1 ± 4.5% of FA, and transformed it into 4-VG with 53.2 ± 13.0% and 33.8 ± 9.2% yield respectively. *L. plantarum* bLp6 strain was the only strain able of converting FA (91.3 ± 6.1%) into both DHFA and 4-VG, with a majority of DHFA (67.6 ± 14.4% of DHFA and 11.9 ± 1.0% of 4-VG) (Fig. 3).

Concerning yeasts, the assimilation and conversion of FA was only observed in *S. cerevisiae*, which transformed FA into 4-VG exclusively (Fig. 2 and Table S21). Strains ySc10 and ySc16 transformed respectively 77.6 ± 18.0% and 56.5 ± 3.1% of FA into 66.6 ± 14.7% and 60.3 ± 10.8% of 4-VG respectively, while strains ySc29, ySc9 and ySc32 have almost completely assimilated FA (91.0 ± 10.2%, 92.3 ± 6.0% and 91.7 ± 9.9% respectively) to produce approximately the same amount of 4-VG (60.7 ± 1.6%) (Fig. 3).

The conversion of FA into DHFA and 4-VG was also analyzed in yeast/LAB co-cultures. LAB and yeast strains assimilating FA in monoculture continued to do so in co-culture. However, a variation in the quantities of metabolites produced was noted (Fig. 3 and Table S21). Coupling of *S. cerevisiae* strains ySc10 or ySc32 (which converted FA into 4-VG exclusively in monoculture), with *L. plantarum* bLp6 (which produced mainly DHFA under the same conditions), resulted in the conversion of FA into DHFA in high yield (in average of 89.8 ± 6.6%), with a small amount of 4-VG (in average of 6.9 ± 0.8%). Interestingly, the co-culture of *L. plantarum* bLp16 with *S. cerevisiae* ySc16 strains which both produced only 4-VG (33.8 ± 9.2%, 60.3 ± 10.8% respectively) in monoculture, led to the assimilation of almost all FA (92.6 ± 9.1%) and the production of a large amount of DHFA (85.4 ± 9.5%). It was also the case for the strain pairs *L. plantarum* bLp20/*K. bulderi* yKb20 and *L. plantarum* bLp20/*S. cerevisiae* ySc16. The predominance of DHFA as metabolite of FA has also been observed in combinations involving strains able of assimilating FA in monoculture and strains unable of doing so. Indeed, the combination of *L. plantarum* bLp6 and *K. humilis* or *K. bulderi* which are not able to assimilate FA alone, gave rise to the conversion of FA in quantitative yield (92.7 ± 7.8% on average) into mainly DHFA (92.1 ± 8.8% on average). Association of *F. sanfranciscensis* bFs9 strain unable to assimilate FA in monoculture, with *S. cerevisiae* ySc9 strain, which transformed FA into only 4-VG (60.1 ± 8.5%), generated the formation of DHFA and 4-VG in equal amount (37.8 ± 12.3% DHFA and 35.0 ± 10.9 4-VG), after the assimilation of nearly all FA (92.0 ± 8.3%). Even when DHFA is not formed, the LAB-yeast association seems to reduce the amount of 4-VG, produced by yeast strain monoculture. *S. cerevisiae* ySc29 strain produced a lower amount of 4-VG (34.0 ± 3.62%) when it was associated to *L. xiangfangensis* bLx29 strain (which did not assimilate FA), compared to its activity alone (62.4 ± 6.3%). FA was not assimilated in most of yeast-LAB combinations made of yeast and LAB strains unable to metabolize it in monoculture. A surprising result was obtained from the combination of *F. sanfranciscensis* bFs17 *and K. humilis* yKh17 strains, where 87.3 ± 21.0% of FA was assimilated and a large amount of DHFA (89.5 ± 19.6%) was produced, while neither the LAB nor the yeast strains assimilated FA in monoculture. As *F. sanfranciscensis* strains tested in this study were not able to assimilate FA in monoculture, we

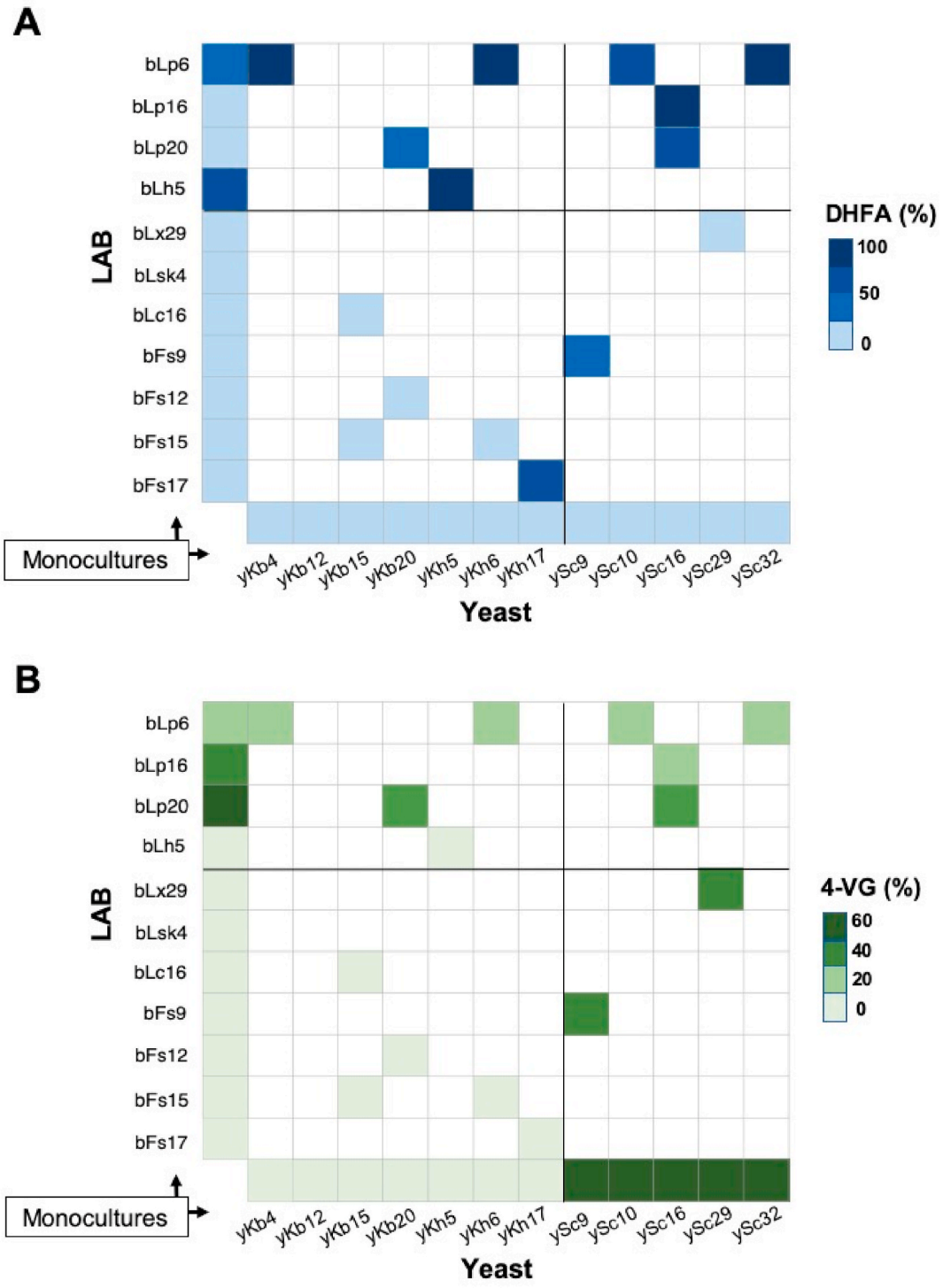

Fig. 3. Production of dihydroferulic acid (DHFA) (A) and 4-vinylguiacol (4-VG) (B) by LAB and yeast strains in mono- and co-culture after 37 h of fermentation. The data are presented by a matrix whose color intensity indicates the amount (in %) of DHFA (in A) or 4-VG (in B) formed from consumed FA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

investigated the presence of putative FA reductase genes, namely *hcrB*, *hcrF* and *par1,* as well as the FA decarboxylase encoding gene *pdc1* in the 30 sequenced strains of *F. sanfranciscensis* available. BLAST searches revealed no consistent homology among all the investigated strains. In addition, none of the protein signature domains from the four reference genes were found in the complete set of proteins from the 30 strains (on the basis of InterProScan predictions), except for the domain IPR005025 (NADP(H) binding domain). This latter is identified in a gene shared by the 30 compared strains that is a homologous gene of *hcrA* (*L. plantarum*) with no enzymatic activity (Gaur et al., 2020). As a consequence, no evidence of genes coding for a ferulic acid reductase or decarboxylase were found in sequenced *F. sanfranciscensis* strains. We could not investigate the presence/absence of decarboxylase (PAD1, FDC1) or putative reductase encoding genes in *K. humilis*, since no genome is currently available for this species.

All these results suggest that the behavior of LAB and yeast strains

when together is not easily predictable from their activity when alone. It is interesting to note that strains which were assumed to not assimilate FA or to produce only 4-VG in monocultures, consumed FA to convert it mainly into DHFA in co-cultures (except *L. xiangfangensis* bLx29), suggesting that yeast-LAB co-cultures facilitate the production of DHFA (bLp16/ySc16, bLp20/yKb20, bLp20/ySc16, bFs9/ySc9, bFs17/yKh17) and/or limit the production of 4-VG.

4. Discussion

4.1. Effect of FA on microbial growth in mono- and co-culture

With a concentration of 0.13 mM of FA, no inhibitory effect was observed neither for yeasts, nor for LAB. This is consistent with previous studies that showed that the effect of phenolic acids on microbial growth is concentration-dependent. In the case of *S. cerevisiae*, yeast biomass began to decrease at 0.25 mM FA (Hou et al., 2018) and FA inhibition threshold is about 1.8 mM (Adeboye et al., 2014). Sanchez-Maldonado et al. (2011) studied the minimum inhibitory concentrations (MIC) of 12 phenolic acids and their metabolites for several bacterial species. They observed that the negative effect of FA in bacteria started at concentration of 0.8 mM, with a better tolerance of lactobacilli (MIC 4.6–8.65 mM) (Sánchez-Maldonado et al., 2011).

Independently of the presence of FA, we have noticed a reduction in the average cell concentration of yeasts and LAB in co-cultures. This may result from a competition between yeast and LAB caused by limited substrates. Several studies showed that according to the yeast or LAB species, cell growth of one or both can be reduced in co-cultures (De Vuyst and Neysens, 2005; Mendoza et al., 2010; Freire et al., 2015; Carbonetto et al., 2020; Khan et al., 2020).

4.2. Fermentation of yeast and LAB in mono- and co-culture

Strains of *S. cerevisiae* produce three times more CO_2 than *K. humilis* and *K. bulderi*. Although the synthetic medium do not contain all sourdough metabolites (like ergosterol, fructose, arabinose, raffinose, FODMAP, …), our data are consistent with a previous study reporting a better leavening capacity of *S. cerevisiae* compared to *K. humilis* in experimental doughs (Carbonetto et al., 2020). Moreover, we observed a reduced CO_2 production in LAB/*S. cerevisiae* co-cultures. Competition for glucose and maltose may explain these observations. Yeasts have a preference for monosaccharides (Webster et al., 2019; Wilson et al., 2019), and the presence of glucose suppresses the molecular activities involved in the use of alternative carbon sources such as maltose (Paramithiotis et al., 2007; Kayikci and Nielsen, 2015). In the case of glucose depletion in co-cultures, yeasts need to metabolize more maltose, which could lead to a longer lag phase duration and lower CO_2 production. Additional experiments in sourdough are needed to compare fermentation performance between yeast species and between yeast and LAB-yeast starters.

4.3. Consumption and conversion of FA by LAB and yeasts in mono- and co-culture

At a certain concentration, FA, like other phenolic acids, inhibits the microbial growth (Hou et al., 2017, 2018). To survive in this quite toxic environment, microorganisms undertake a detoxification process that converts toxic phenolic compounds into less toxic derivatives as DHFA and 4-VG (Adeboye et al., 2015). In this study, we investigated the assimilation of FA and its conversion into 4-VG and DHFA by the selected yeasts and LAB. Metabolism of FA has been investigated for some LAB present in sourdough such as *L. plantarum*, *Levilactobacillus brevis, Limosilactobacillus fermentum, Furfurilactobacillus rossiae and Latilactobacillus curvatus* (Rodríguez et al., 2008; Filannino et al., 2014; Ripari et al., 2019; Gaur et al., 2020).

Depending on the presence/absence of reductase and decarboxylase encoding genes, their regulation and the substrate-specificity of their products, FA metabolism by LAB may vary. Among the 11 LAB strains used in this study, only four were able to convert FA into 4-VG, DHFA or both. All *L. plantarum* strains tested in this study metabolized FA and produced the corresponding metabolites in different ratios. For instance, *L. plantarum* bLp6 strain produced more DHFA than 4-VG while bLp20 strain does not produce any DHFA but produces 4-VG in monoculture. These data demonstrate that the transformation of FA by LAB is strain-specific (Van Beek and Priest, 2000; Curiel et al., 2010; Svensson et al., 2010; Gaur et al., 2020). Moreover, none of the *F. sanfranciscensis* strains tested were able to assimilate FA in monocultures. The *in silico* analysis revealed that it may be attributed to the absence of genes coding for FA reductase or decarboxylase activities in *F. sanfranciscensis*. In a future work, it will be interesting to characterize the genes encoding the metabolic enzymes of FA in the tested LAB strains to better understand the mechanisms involved in FA metabolism.

Among sourdough yeast species, FA conversion was widely studied for *S. cerevisiae* (Larsson et al., 2001; Mukai et al., 2010; Adeboye et al., 2015; Dzialo et al., 2017). This species generally lacks the reductase activity (Huang et al., 1993; Larsson et al., 2001; Steensels et al., 2015). However, it expresses two phenylacrylic acid decarboxylase and ferulic acid decarboxylase genes (*PAD1* and *FDC1* respectively) which act synergistically to decarboxylate hydroxycinnamic acids (Mukai et al., 2010; Bhuiya et al., 2015). The role of *PAD1* is to synthesize cofactor (modified flavin mononucleotide) required by *FDC1* to fulfill its decarboxylation task (Lin et al., 2015). In our experimental conditions, all *S. cerevisiae* strains assimilate FA and transform it into 4-VG in monocultures. *S. cerevisiae* strains which assimilated all FA gave the same amount of 4-VG as those which did not assimilate FA entirely, suggesting that decarboxylation is a limiting step in FA metabolism in our conditions. Previous studies suggested that the expression of *PAD1* gene constitute a stress response induced by phenolic acid and a resistance to their inhibitory effect (Goodey and Tubb, 1982; Clausen et al., 1994). Adeboye et al. (2015), showed that FA was completely metabolized after 72 h of fermentation with industrial strains of *S. cerevisiae*. They also found that FA was converted into ferulic acid isomer and DHFA during the first 2 h of cultivation (Adeboye et al., 2015). In order to determine if 4-VG results from other metabolites' transformation, FA fermentation in the presence of *S. cerevisiae* strains ySc16 and ySc9 was monitored every 2 h and products were identified by liquid chromatography (see M&M). It was observed that no intermediates were formed before the appearance of 4-VG. In contrast, Kazachstania strains were not able to assimilate FA in monocultures. However, in the absence of available genomes for *K. humilis* and *K. bulderi*, it becomes difficult to discuss their behavior.

Interestingly, in co-cultures, microorganisms behaved differently than in mono-cultures. The consumption of FA was higher, since 11 couples have entirely assimilated FA while the same microorganisms did not assimilate it or assimilated it partially in monocultures. As FA was totally consumed to be mainly converted into DHFA, reductase activity seems to be favored in these conditions. By combining yeasts with LAB, a modification of the extracellular conditions occurs, thus, inducing the reductase activity of LAB. Factors favoring the expression of LAB reduction gene in the presence of yeasts are still unknown and no obvious mechanism can be predicted at this stage. Further investigations must be undertaken in order to elucidate the impact of yeasts on LAB activities. In addition, production of DHFA was observed in two couples involving *F. sanfranciscensis* strains (bFs17/yKh17 and bFs9/ySc9) while there was no FA reduction in monocultures. As no FA reductase gene homologs were identified by *in silico* analysis of *F. sanfranciscensis* genomes, it might be suggested that FA reductase activity in yeast was induced by the presence of LAB.

Sourdough metabolites of FA, namely, DHFA and 4-VG are known for their nutritional and organoleptic benefits. DHFA is a better antioxidant, anti-inflammatory and neuroprotector agent than FA (Ordoudi et al., 2006; Larrosa et al., 2009; Verzelloni et al., 2011; Amić et al., 2018;

Ohue-Kitano et al., 2019). It is also suspected to be a vanillic acid precursor, even though the metabolic pathways of such transformation are not elucidated yet (Rechner et al., 2001). 4-VG is a volatile phenolic compound with a smoky flavor note and a clove/spicy aroma that can contribute to a typical bread flavor. It has also higher antioxidant properties than some other phenolic derivatives (4-vinylphenol, 4-vinylsyringol and 4-vinylcatechol) (Terpinc et al., 2011), and therefore it is considered as a good food preservative (Tańska et al., 2018).

The presence of FA (if it is not entirely assimilated), DHFA and 4-VG in bread can improve its nutritional and organoleptic quality. However, this very preliminary study does not inform us about the behavior of bran FA under real bread making conditions. Several questions remain outstanding and require further investigations. Among other, (i) the bioavailability of bran FA in flour and the capacity of sourdough microorganisms to release it and to transform it; (ii) the influence of bread making conditions including kneading, fermentation time and temperature, as well as baking on the transformation of FA and the availability of its metabolites at the end of the process (Angelino et al., 2017).

5. Conclusion

In this study, assimilation and transformation of FA by sourdough LAB and yeasts have been investigated. In the case of yeasts, *S. cerevisiae* metabolized FA into its decarboxylated product 4-VG. LAB, processing both decarboxylase and reductase activities, produced exclusively or mainly the reduction derivative of FA (DHFA). LAB-yeast combinations resulted in a modification of extracellular conditions, inducing thereby the reductase activity of LAB. Consequently, DHFA was the predominant fermentation product in co-cultures. Influence of yeasts on genes expression of LAB will be the subject of further investigations.

Declaration of competing interest

The authors declare that they have no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2021.103790.

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