International Journal of Food Microbiology 289 (2019) 88–105

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

The addition of citrate stimulates the production of acetoin and diacetyl by a citrate-positive *Lactobacillus crustorum* strain during wheat sourdough fermentation

Andrea Comasio, Henning Harth, Stefan Weckx, Luc De Vuyst*

Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel (VUB), Pleinlaan 2, B-1050 Brussels, Belgium

ARTICLE INFO

Keywords:
Sourdough
Lactobacillus crustorum
Citrate
Acetoin
Diacetyl

ABSTRACT

In traditional sourdough fermentation recipes of artisan bakeries, often extra ingredients are added to the flour-water mixture. This may accentuate the aroma and taste of the baked products produced from such sourdoughs. This is made possible, for instance, by stimulating certain microbial activities during fermentation. This study examined the effects of the addition of citrate (a food-grade organic acid present in milk and lemons) on wheat sourdough fermentation in the presence of a citrate-positive, homofermentative, lactic acid bacterial starter culture strain, namely *Lactobacillus crustorum* LMG 23699. Both liquid and firm wheat sourdoughs were produced. The starter culture strain was able to steer all wheat sourdough fermentations performed, as it always prevailed due to its competitiveness, as shown through culture-dependent microbiological plating and culture-independent bacterial community profiling. Moreover, it possessed all enzyme-encoding genes (as unraveled through genome mining) necessary to convert citrate into desirable compounds such as lactic acid, acetic acid, succinate, acetoin, diacetyl, and 2,3-butanediol. Indeed, citrate addition to the wheat flour-water mixture had an impact on the sourdough fermentation dynamics and thus on the aroma profile of the liquid and firm sourdoughs produced and breads made thereof. A higher final pH, higher total titratable acidity values, and low yeast counts were found in wheat sourdoughs produced with citrate. In particular, the starter culture strain added converted the supplemented citrate into more L-lactic acid as well as acetoin and diacetyl (buttery aroma compounds), which was independent of the dough yield. The buttery aroma compounds were also accentuated in the concomitant breads produced. Further, organic acid production was stimulated in the sourdoughs, whereas increased pyrazine concentrations occurred in the breads. Consequently, citrate supplementation to wheat sourdoughs could be of interest to produce baked goods with enhanced buttery aroma compounds and notes.

1. Introduction

Sourdough is the result of a spontaneous or starter culture-initiated fermentation of a mixture of flour and water, in which lactic acid bacteria (LAB) and yeast communities are predominantly active (De Vuyst et al., 2014, 2016, 2017; Gänzle and Ripari, 2016; Gobbetti et al., 2016; Minervini et al., 2014; Van Kerrebroeck et al., 2017). LAB species such as *Lactobacillus fermentum* and *Lactobacillus sanfranciscensis* and yeast species such as *Kazachstania humilis* and *Saccharomyces cerevisiae* often prevail; sometimes acetic acid bacteria (AAB) belong to the outgrowing background microbiota. Both the endogenous flour enzymes and microorganisms contribute to the breakdown of carbohydrates and proteins and the production of metabolites, including volatile compounds (Gänzle, 2014; Gänzle et al., 2007, 2008, 2009; Gobbetti et al., 2014). For instance, flour amylase activity continuously provides maltose and glucose for fermentation by LAB and yeasts; glucofructan breakdown by yeasts further provides fructose as an alternative external electron acceptor for heterofermentative LAB species, enabling enhanced acetic acid production (De Vuyst et al., 2017; Gänzle, 2014). Similarly, during sourdough fermentation, amino acid concentrations increase steadily, due to primary proteolysis by endogenous flour proteases at low pH (De Vuyst et al., 2017; Gänzle, 2014; Gänzle et al., 2008; Gobbetti et al., 2016). Although yeasts consume amino acids during growth, resulting in low amino acid concentrations in doughs fermented by yeasts solely, amino acid concentrations remain unchanged or are even higher in the presence of certain strains of LAB species, due to enhanced proteolysis by the flour proteases as a result of acidification, peptidase activity, and amino acid expulsion by LAB

* Corresponding author at: VUB, IMDO, Pleinlaan 2, B-1050 Brussels, Belgium
E-mail address: luc.de.vuyst@vub.be (L. De Vuyst).

https://doi.org/10.1016/j.ijfoodmicro.2018.08.030
Received 4 April 2018; Received in revised form 17 August 2018; Accepted 31 August 2018
Available online 04 September 2018
0168-1605/

(Gänzle et al., 2008; Thiele et al., 2002; Zhao et al., 2015).

In traditional sourdough fermentation recipes of artisan bakeries, often extra ingredients are added to the flour-water mixtures, such as adjunct carbohydrates, fruits, honey, milk, vinegar, and yoghurt to produce distinct sourdoughs (Aplevicz et al., 2014; Gordún et al., 2015; Minervini et al., 2016; Nutter et al., 2017; Palomba et al., 2011; Ripari et al., 2016b; Yu et al., 2018; Zhang et al., 2011). Those ingredients may be either the source or the stimulus of certain microbial communities. For instance, the occurrence of the yeast *Metschnikowia pulcherrima* in sourdoughs destined for the production of sweet baked products in an Italian bakery have been associated with fruits, honey, and sugars present in the bakery (Palomba et al., 2011). Similarly, the use of milk in Chinese sourdoughs is responsible for the occurrence of *Leuconostoc citreum* (Zhang et al., 2011). Alternatively, such ingredients may be used intentionally to accentuate the aroma and taste of bakery products produced from sourdoughs enriched with volatiles obtained through microbial activities on substrates of these ingredients. Substrates of these ingredients may indeed activate certain metabolic pathways of, for instance, LAB species, through the expression of their respective genes. This may result in the formation of additional flavours. For instance, it is well known that the addition of fructose to sourdoughs results in higher concentrations of acetate and lower concentrations of lactate. This is due to an increased activity of both mannitol dehydrogenase (reducing fructose into mannitol for redox balancing) and acetate kinase (converting acetyl-phosphate into acetate instead of ethanol for enhanced competitiveness through extra ATP generation) and a decreased activity of lactate dehydrogenase (less or no conversion of pyruvate into lactate), respectively. This has been shown during co-metabolism of maltose and fructose in strictly heterofermentative lactobacilli (Gobbetti et al., 1995; Stolz et al., 1995a, 1995b).

Similarly, citrate is a common constituent of milk, lemons, and limes and may act as substrate for microbial activities. Its impact on flavour formation *via* the production of C4 compounds, such as acetoin and diacetyl, during food fermentation processes is well known (Gänzle, 2015; Laëtitia et al., 2014). This is due to its conversion by several LAB species, thereby contributing to energy production and acid tolerance. Formation of diacetyl from pyruvate during sourdough fermentation has been particularly attributed to homofermentative lactobacilli (Damiani et al., 1996; Hansen et al., 1989). As pyruvate is, next to its conversion through the C4 flavour compound pathway, metabolized to lactate *via* the homofermentative pathway or to ethanol and/or acetate *via* the heterofermentative one, higher concentrations of internal pyruvate are required for enhanced flavour formation. This can be achieved through co-metabolism of carbohydrates and citrate (Gobbetti and Corsetti, 1996; Laëtitia et al., 2014).

Citrate conversion in LAB is initiated by its transport into the cell by a citrate permease, which is followed by citrate lyase activity, yielding acetate and oxaloacetate, the latter being further converted into succinate through the reductive branch of the citric acid cycle (to accommodate redox balancing) or pyruvate by a decarboxylase (to fuel pyruvate metabolism and hence contributing to redox balancing, acid tolerance, and energy production) (Gänzle, 2015; Laëtitia et al., 2014; Zaunmüller et al., 2006). Pyruvate is used by a synthase to form α-acetolactate, while releasing carbon dioxide, that can be converted into acetoin enzymatically (decarboxylation) or diacetyl non-enzymatically (oxidative decarboxylation). Diacetyl is a buttery flavour compound of the bread crumb, albeit usually made through yeast fermentation (Birch et al., 2013a, 2014; Cho and Peterson, 2010; Hansen and Hansen, 1996; Hansen and Schieberle, 2005; Pico et al., 2015). Although being produced during sourdough fermentation, its final concentration depends on the extent of its further reduction to less flavour-active compounds by both LAB and yeasts (Birch et al., 2013b, 2014). Alternatively, diacetyl can be formed and degraded during baking by Maillard-type reactions (Birch et al., 2014; Cho and Peterson, 2010; Pico et al., 2015). As such, the obligate heterofermentative *Lb. sanfranciscensis* co-metabolizes maltose and citrate, whereby the latter substrate is commonly converted into lactate and acetate (Gobbetti and Corsetti, 1996; Stolz et al., 1995a). However, enhanced diacetyl formation has not been shown. In contrast, diacetyl formation has been found when a *Lactobacillus crustorum* strain is applied as sourdough starter culture (Ravyts and De Vuyst, 2011; Ripari et al., 2016a).

Therefore, the present study aimed at the assessment of the addition of citrate to a wheat flour-water mixture fermented by *Lb. crustorum* LMG 23699, a citrate-positive homofermentative LAB strain, as well as to its impact on the sourdough fermentation dynamics and the flavour profiles of both the resulting sourdoughs (firm and liquid) and breads produced thereof.

2. Materials and methods

2.1. Sourdough production

2.1.1. Flour

Three batches (A, B, and C) of the same wheat flour were provided by Ceres (Vilvoorde, Belgium) with the following characteristics (averages, m/m): 14.4% moisture, 68.4% carbohydrates, 12.9% proteins, 0.6% ash, and falling number of 396.3 s.

2.1.2. Starter culture strain used and genome mining

The starter culture strain used throughout this study was the homofermentative, citrate-positive *Lb. crustorum* LMG 23699. It has been isolated from a traditional wheat sourdough of an artisan bakery in the province of East-Flanders, Belgium (Scheirlinck et al., 2007). The strain was stored at −80 °C in modified de Man-Rogosa-Sharpe-5 (mMRS-5) medium (Harth et al., 2016) containing 25% (v/v) glycerol. The citrate-positive behaviour of *Lb. crustorum* LMG 23699 was checked through genome mining in comparison with other *Lb. crustorum* genomes available (*Lb. crustorum* JCM 15951 and *Lb. crustorum* MN047 from wheat sourdough and koumiss, respectively) and the genomes of citrate-positive *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* IL1403 (from dairy origin) and *Lb. fermentum* 222 (originating from fermenting cocoa pulp-bean mass) (Bolotin et al., 2001; Illeghems et al., 2015; Sun et al., 2015; Yi et al., 2017; http://www.ncbi.nlm.nih.gov). Therefore, the protein sequences of all known enzymes of the citrate pathway in the two latter strains were aligned to all proteins from the *Lb. crustorum* strains, using blastP, resulting in identity/similarity percentages (Altschul et al., 1990). The resulting hits provided the protein accession number in *Lb. crustorum* LMG 23699 and, hence, this allowed to confirm the presence of the enzymes considered.

2.1.3. Inoculum build-up

To start up a sourdough production, the *Lb. crustorum* strain was first propagated at 30 °C in 10 mL of mMRS-5 medium overnight. A second overnight propagation was performed at 30 °C in 100 mL of mMRS-5 medium (transfer volume of 1%, v/v). In the case that the sourdough production was performed with the addition of citrate, the second propagation step was done in mMRS-5 medium containing 8 mM citric acid (Merck, Darmstadt, Germany). This concentration of citric acid is comparable with that in cows' milk. The stock citric acid solution used (80 mM) was adjusted to pH 6.0 with NaOH. The final inoculum was obtained by collecting the cells of the second overnight culture by centrifugation (4600 × *g* for 20 min at 4 °C). Then, the cells were resuspended in 10 mL of sterile saline (0.85%, m/v, NaCl) to achieve an initial concentration of 10^7 colony forming units (CFU) per mL of liquid sourdough or per g of firm sourdough at the start of the sourdough productions.

2.1.4. Sourdough productions

Both liquid and firm wheat sourdoughs were produced, whereby the wheat flour-water mixture was supplemented with citric acid to obtain an initial concentration of 8 mM. Therefore, 1 L of sodium citrate buffer (64 mM, pH 6.0) composed of anhydrous citric acid (Merck) and

Table 1
Genome mining concerning the citrate metabolism of *Lactobacillus crustorum* LMG 23699.

Enzyme in *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* IL 1403 and *Lactobacillus fermentum* 222	Gene	Identity/similarity (in %) of the alignment of *Lb. crustorum* LMG 23699 to *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* IL 1403 (Ll) and *Lb. fermentum* 222 (Lf) using blastp	Protein accession number in *Lb. crustorum* LMG 23699	Annotation in the genome of *Lb. crustorum* LMG 23699
Citrate lyase				
Citrate lyase subunit gamma	*citD*	Ll: 54/70 Lf: 61/75	WP_057868311.1	Citrate lyase acyl carrier protein
Citrate lyase subunit beta	*citE*	Ll: 67/83 Lf: 68/82	WP_057868310.1	Citrate (pro-3S)-lyase subunit beta
Citrate lyase subunit alpha	*citF*	Ll: 72/86 Lf: 72/85	WP_057868309.1	Citrate lyase subunit alpha
Accessory subunit	*citC*	Ll: 48/67 Lf: 50/68	WP_057868312.1	[citrate (pro-3S)-lyase] ligase
Accessory subunit	*citX*	Ll: 33/55 Lf:/	WP_057868308.1	Citrate lyase holo-[acyl-carrier protein] synthase
Accessory subunit	*citG*	Ll: 51/65 Lf: 44/59	WP_057868307.1	Triphosphoribosyl-dephospho-CoA synthase
Citrate lyase regulator	*citR*	Ll: 42/65 Lf: 41/65	WP_057868314.1	Hypothetical protein
Malate dehydrogenase (oxaloacetate-decarboxylating)	*mae*	Ll: 56/75 Lf: 62/77	WP_057868313.1	NADP-dependent malic enzyme
Alpha-acetolactate synthase	*als*	Ll: 56/69 Lf: 59/74	WP_057867717.1	Acetolactate synthase
Alpha-acetolactate decarboxylase	*aldB*	Ll: 35/56 Lf: 60/72	WP_057867716.1	Acetolactate decarboxylase
2,3-Butanediol dehydrogenase	*butB*	Ll: 37/55 Lf: 38/59	WP_057868200.1	Threonine dehydrogenase
Acetoin reductase	*butA*	Ll: 30/49 Lf: 37/54	WP_057866432.1	NAD(P)-dependent oxidoreductase
L-Lactate dehydrogenase	*ldh*	Ll: 57/76 Lf: 56/76	WP_057866644.1	L-Lactate dehydrogenase
Fumarate hydratase	*fumC*	Ll:/ Lf: 72/85	WP_057866698.1	Class II fumarate hydratase
Fumarate reductase	*frd*	Ll: 50/68 Lf: 63/77	WP_057866699.1	Flavocytochrome c

trisodium citrate dihydrate (Sigma-Aldrich, Saint-Louis, MO, USA) was used.

2.1.4.1. Liquid sourdough productions. The production of liquid sourdoughs was carried out in 15-L Biostat® C fermentors (Sartorius, Melsungen, Germany) that were previously sterilized (121 °C, 2.1 bar, 20 min) with water. Subsequently, 5 L of sterile water and 1 L of sterile sodium citrate buffer (added to the fermentor aseptically) or 6 L of sterile water (control fermentation) were mixed with 2 kg of wheat flour to obtain a final dough yield (DY) [(dough mass / flour mass) × 100] of 400. The fermentations were performed at 30 °C for 72 h, while the flour-water mixture was continuously stirred at 300 rpm and the fermentor headspace was flushed with 1 mL/min of sterile air. The fermentations were performed in triplicate and are further referred to as LC400-Cit1 (wheat flour batch A), LC400-Cit2 (A), and LC400-Cit3 (B) in the presence of citrate, and LC400-Ctl1 (B), LC400-Ctl2 (B) and LC400-Ctl3 (B) in its absence.

2.1.4.2. Firm sourdough productions. The production of firm sourdoughs was carried out in clean and decontaminated (with ethanol, 70%, v/v) 15 L plastic vessels covered with a lid. Therefore, 3 L of sterile water and 1 L of sterile sodium citrate buffer or 4 L of sterile water (control fermentation) were mixed with 4 kg of wheat flour (DY of 200) by means of a hand mixer (Bosch, Stuttgart, Germany) for 5 min. The flour-water mixtures were not stirred during fermentation. The fermentations were performed at 30 °C for 72 h in triplicate and are further referred to as LC200-Cit1 (wheat flour batch B), LC200-Cit2 (C), and LC200-Cit3 (C) in the presence of citrate, and LC200-Ctl1 (B), LC200-Ctl2 (C) and LC200-Ctl3 (C) in its absence.

2.1.4.3. Sampling. Samples were taken aseptically from the wheat flour-water mixtures (further referred to as time 0), after their inoculation with the starter culture strain (further referred to as time 0′), and after 8, 12, 24, 48, and 72 h of fermentation during both liquid and firm sourdough productions. Part of the samples was analysed immediately [sourdough pH and total titratable acidity (TTA) measurements and microbiological plating]. For the microbiological plating, 10 g of sample was mixed with 90 mL of sterile saline in a stomacher bag (Stomacher 400; Seward, Worthington, Leicestershire, UK) for 10 min. Another part (10 g) was supplemented with 90 mL of peptone-physiological solution [0.1%, m/v, bacteriological peptone (Oxoid, Basingstoke, Hampshire, UK) and 0.85%, m/v, NaCl (Merck)] and mixed in a stomacher bag (Stomacher 400) for 5 min. Fifty milliliter of this suspension was centrifuged (1000 × *g* for 5 min at 4 °C) to remove solid flour particles. The supernatant was collected and a second centrifugation (4600 × *g* for 20 min at 4 °C) yielded a cell pellet that was stored at −20 °C for culture-independent microbiological analysis (denaturing gradient gel electrophoresis or DGGE). Further, in the case of liquid sourdoughs, 50 *g* of sample was centrifuged (4600 × *g* for 20 min at 4 °C) and the supernatant was stored at −20 °C for metabolite target analysis. In the case of firm sourdoughs, 25 g of sample was mixed with 25 mL of ultrapure water (MilliQ; EMD Millipore, Billerica, MA, USA) by means of a rotator Stuart SB3 (Bibby Scientific, Stone, Staffordshire, UK) at 25 rpm for 20 min, before being centrifuged (4600 × *g* for 20 min at 4 °C) to store the supernatant at −20 °C.

2.1.5. Determination of pH and total titratable acidity

Sourdough pH and TTA were determined as described previously (Harth et al., 2016). The pH was measured with an InoLab 720 pH meter (WTW, Weilheim, Germany). The TTA value was measured by titration of a suspension of 10 g of sourdough in 100 mL of ultrapure water (0.015 µS/cm; MilliQ); the TTA value was expressed as the number of mL of 0.1 M NaOH used to titrate the sample to reach a final

Table 2
Evolution of the viable counts of lactic acid bacteria (LAB), yeasts, and acetic acid bacteria (AAB); pH; and total titratable acidity (TTA) during wheat sourdough productions with *Lactobacillus crustorum* LMG 23699 as starter culture strain. This was done in the presence (Cit) and absence (Ctl) of citrate for both liquid (dough yield of 400, LC400) and firm (dough yield of 200, LC200) wheat sourdough productions. The viable counts are expressed as mean values ± standard deviations in log (CFU/mL) (liquid wheat sourdoughs) or log (CFU/g) (firm wheat sourdoughs). A bar (−) indicates that the viable microbial counts were below the limit of detection. X_{max}, maximum viable LAB counts (modeled value).

Time (h)	LC400-Cit1					LC400-Cit2					LC400-Cit3				
	LAB	Yeasts	AAB	pH	TTA (mL)	LAB	Yeasts	AAB	pH	TTA (mL)	LAB	Yeasts	AAB	pH	TTA (mL)
0	–	–	–	5.99	3.15	–	–	–	5.98	3.10	–	–	–	6.00	3.10
0′	7.0 ± 0.1	–	–	5.99	3.15	6.8 ± 0.1	–	–	5.98	3.10	6.8 ± 0.1	–	–	6.00	3.10
8	7.8 ± 0.1	–	–	5.25	3.70	7.7 ± 0.1	–	–	5.20	3.60	7.8 ± 0.1	3.1 ± 0.1	–	5.32	3.70
12	8.6 ± 0.1	–	–	4.32	5.05	8.5 ± 0.1	–	–	4.40	5.10	8.7 ± 0.1	2.8 ± 0.1	–	4.40	4.35
24	9.3 ± 0.1	–	–	3.82	8.50	9.3 ± 0.1	–	–	3.79	8.25	9.6 ± 0.1	–	–	3.82	7.80
48	9.7 ± 0.1	–	–	3.70	11.50	9.6 ± 0.1	–	–	3.73	11.35	9.6 ± 0.1	–	–	3.75	11.20
72	9.7 ± 0.1	–	–	3.62	15.25	9.6 ± 0.1	–	–	3.61	15.10	9.5 ± 0.1	–	–	3.61	14.75
X_{max}	9.2	–	–	–	–	9.5	–	–	–	–	9.5	–	–	–	–

Time (h)	LC400-Ctl1					LC400-Ctl2					LC400-Ctl3				
	LAB	Yeast	AAB	pH	TTA (mL)	LAB	Yeast	AAB	pH	TTA (mL)	LAB	Yeast	AAB	pH	TTA (mL)
0	–	–	–	6.10	1.00	–	–	–	6.10	1.00	–	–	–	5.99	1.00
0′	6.8 ± 0.1	–	–	6.10	1.00	6.9 ± 0.1	–	–	6.10	1.00	6.8 ± 0.1	–	–	5.99	1.00
8	7.7 ± 0.1	–	–	5.56	1.60	7.7 ± 0.1	–	–	5.32	1.80	7.7 ± 0.1	–	–	5.41	1.85
12	8.6 ± 0.1	–	–	4.53	2.80	8.65 ± 0.1	–	–	4.35	3.10	8.7 ± 0.1	–	–	4.32	3.10
24	9.4 ± 0.1	2.0 ± 0.1	–	3.65	6.50	9.5 ± 0.1	2.0 ± 0.1	–	3.72	6.75	9.6 ± 0.1	–	–	3.81	6.90
48	9.4 ± 0.2	4.2 ± 0.1	–	3.53	10.20	9.5 ± 0.1	4.4 ± 0.1	–	3.55	10.35	9.6 ± 0.1	4.5 ± 0.1	3.5 ± 0.1	3.56	10.45
72	9.3 ± 0.1	5.1 ± 0.5	–	3.50	13.00	9.5 ± 0.1	5.6 ± 0.1	–	3.50	12.85	9.5 ± 0.1	5.9 ± 0.1	6.8 ± 0.1	3.50	13.05
X_{max}	9.4	–	–	–	–	9.5	–	–	–	–	9.5	–	–	–	–

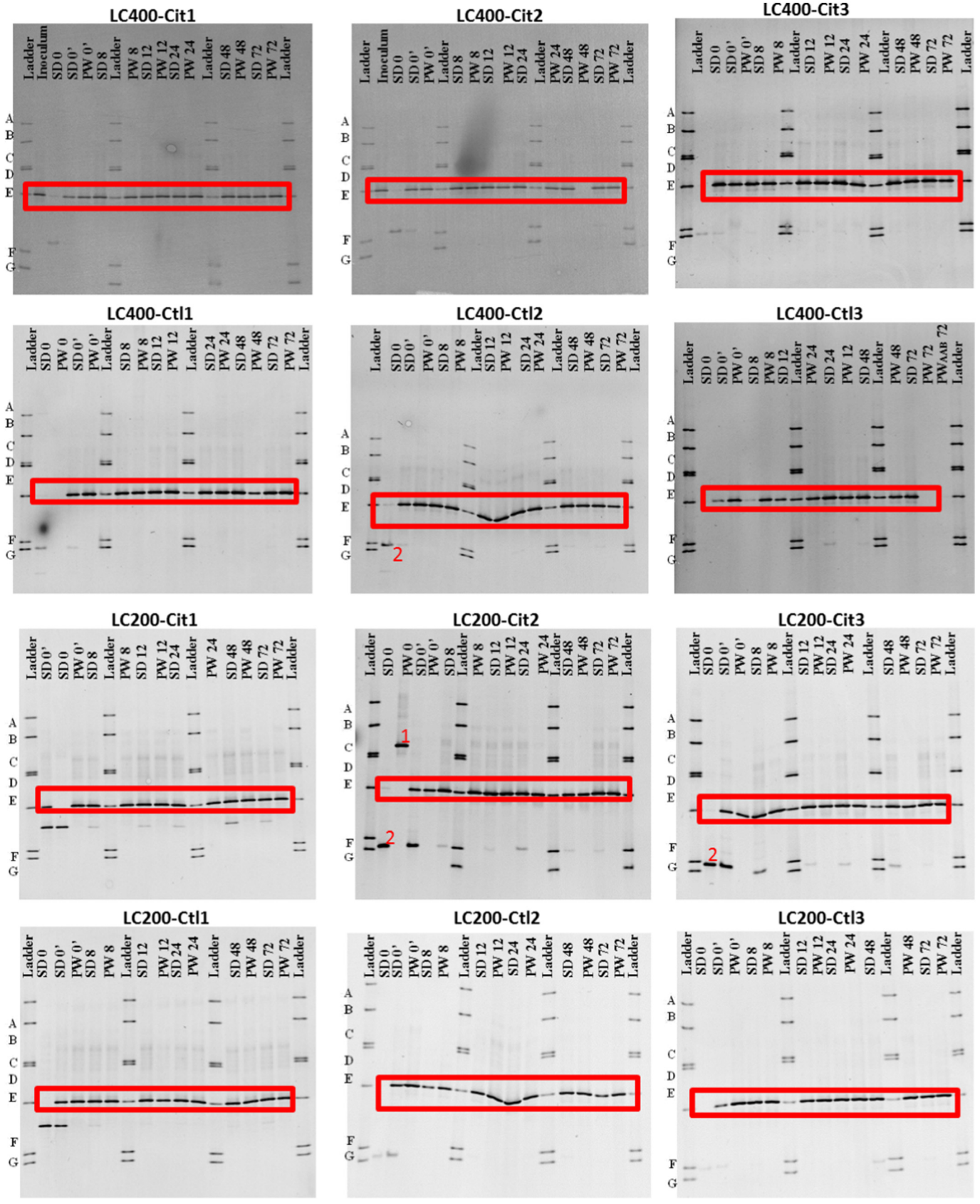
Time (h)	LC200-Cit1					LC200-Cit2					LC200-Cit3				
	LAB	Yeast	AAB	pH	TTA (mL)	LAB	Yeast	AAB	pH	TTA (mL)	LAB	Yeast	AAB	pH	TTA (mL)
0	–	–	–	6.12	1.55	1.5 ± 1.2	–	–	6.18	2.20	–	–	–	6.15	2.10
0′	7.1 ± 0.1	–	–	6.12	1.55	7.1 ± 0.1	–	–	6.18	2.20	7.1 ± 0.2	–	–	6.15	2.10
8	8.4 ± 0.1	–	–	4.83	4.65	8.9 ± 0.1	–	–	5.58	2.80	8.8 ± 0.1	–	–	5.44	3.00
12	9.2 ± 0.1	–	–	4.05	7.90	9.7 ± 0.1	–	–	4.27	6.55	9.5 ± 0.1	–	–	4.23	6.60
24	9.2 ± 0.1	–	–	3.71	12.10	9.9 ± 0.1	–	–	3.76	11.50	9.8 ± 0.1	–	–	3.75	11.80
48	9.3 ± 0.1	–	–	3.61	16.90	9.5 ± 0.1	–	–	3.63	16.80	9.6 ± 0.1	–	–	3.63	16.05
72	9.1 ± 0.1	–	–	3.50	18.60	9.3 ± 0.1	–	–	3.56	18.00	9.3 ± 0.1	–	–	3.58	18.20
X_{max}	9.2	–	–	–	–	9.6	–	–	–	–	9.6	–	–	–	–

Time (h)	LC200-Ctl1					LC200-Ctl2					LC200-Ctl3				
	LAB	Yeast	AAB	pH	TTA (mL)	LAB	Yeast	AAB	pH	TTA (mL)	LAB	Yeast	AAB	pH	TTA (mL)
0	–	–	–	6.02	1.10	–	–	–	6.19	1.30	–	–	–	6.17	1.40
0′	7.4 ± 0.1	–	–	6.02	1.10	7.1 ± 0.1	–	–	6.19	1.30	7.3 ± 0.1	–	–	6.17	1.40
8	8.5 ± 0.1	–	–	4.60	4.20	8.7 ± 0.1	–	–	4.65	3.20	9.0 ± 0.1	–	–	4.41	3.80
12	9.3 ± 0.1	–	–	3.90	4.90	9.4 ± 0.1	–	–	4.01	4.20	9.2 ± 0.1	–	–	4.10	5.50
24	9.3 ± 0.1	–	–	3.60	10.20	9.6 ± 0.1	–	–	3.65	9.80	9.5 ± 0.1	–	–	3.66	11.35
48	9.7 ± 0.2	–	–	3.50	14.20	9.7 ± 0.1	2.8 ± 0.2	–	3.59	14.40	9.6 ± 0.1	–	–	3.58	14.30
72	9.2 ± 0.1	3.5 ± 0.2	–	3.44	15.55	9.7 ± 0.1	3.7 ± 0.1	–	3.55	15.20	9.5 ± 0.1	3.0 ± 0.1	–	3.52	16.20
X_{max}	9.4	–	–	–	–	9.7	–	–	–	–	9.5	–	–	–	–

pH of 8.5.

2.1.6. Microbiological analysis of sourdoughs

2.1.6.1. Culture-dependent microbial community dynamics and identifications. To determine the viable counts of presumptive LAB, AAB, and yeasts in the sourdough samples, appropriate decimal dilutions in sterile saline were made in triplicate and 100 µL of each dilution was plated on mMRS-5 agar medium (Harth et al., 2016) containing 100 ppm of cycloheximide (Sigma-Aldrich) and 5 ppm of amphotericin B (Sigma-Aldrich) to inhibit fungi, modified deoxycholate-mannitol-sorbitol (mDMS) agar medium (Papalexandratou et al., 2013) supplemented with 100 ppm of cycloheximide (Sigma-Aldrich) and 5 ppm of amphotericin B (Sigma-Aldrich), and yeast extract-peptone-dextrose (YPD) agar medium [5 g/L of yeast extract (Merck), 10 g/L of bacteriological peptone (Oxoid), and 20 g/L of glucose (Merck); Spitaels et al., 2014] containing 100 ppm of chloramphenicol (Sigma-Aldrich) to inhibit bacteria, respectively. The plates were incubated at 30 °C for 72 h. Average viable counts are expressed as CFU per mL or g of sourdough. A maximum number of ten colonies of LAB, AAB, and yeasts were randomly picked up from appropriate dilutions on the mMRS-5, mDMS, and YPD agar media, corresponding with 24, 48, and 72 h of fermentation. These colonies



(caption on next page)

Fig. 1. Culture-independent bacterial community dynamics at 0 (unfermented wheat flour-water mixture), 0′ (after inoculation), and after 8, 12, 24, 48, and 72 h of fermentation during wheat sourdough productions with *Lactobacillus crustorum* LMG 23699 as starter culture strain. This was done in the presence (Cit) and absence (Ctl) of citrate for both liquid (dough yield of 400, LC400) and firm (dough yield of 200, LC200) wheat sourdough productions. Sourdough fermentation samples are indicated as SD and plate wash samples as PW. The ladder was constructed using pure cultures of *Lactobacillus plantarum* IMDO 130201 (A), *Lactobacillus fermentum* IMDO 130101 (B), *Lactobacillus sakei* IMDO CG1 (C), *Lactobacillus amylovorus* DCE 471 (D), *Lb. crustorum* LMG 23699 (E), *Lactobacillus namurensis* LMG 23584 (F), and *Lactobacillus sanfranciscensis* IMDO 150101 (G). The closest relatives to the fragments sequenced are given: the red boxes refer to *Lactobacillus crustorum* (100% identity; accession no. NR_042533.1); other species identified are indicated with numbers: 1, *Staphylococcus xylosus* (100% identity; accession no. NR_113350.1); 2, *Pedinomonas tuberculata* (98% identity; accession no. KM462867.1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were grown in 10 mL tubes containing mMRS-5, mannitol-yeast extract-peptone (MYP; Moens et al., 2014), and YPD media, respectively, at 30 °C for 24 h. These cultures (2.0 mL) were stored in cryovials supplemented with glycerol (final concentration of 25%, v/v) at −80 °C, until further identification.

To identify the most abundant communities of bacteria and yeasts, $(GTG)_5$-PCR (bacteria) and M13-PCR (yeasts) fingerprinting analysis of genomic DNA from cultures of all isolates mentioned above was performed, as described previously (Harth et al., 2016). Briefly, DNA extraction was carried out as outlined in the protocol of the Nucleospin 96 tissue kit (Macherey-Nagel, Düren, Germany). PCR assays were carried out as described by Harth et al. (2016). Gel processing was carried out through numerical analysis using the BioNumerics version 5.10 software (Applied Maths, Sint-Martens-Latem, Belgium). Dendrogram construction was carried out by means of the unweighted pair group method with arithmetic averages (UPGMA) clustering algorithm, with correlation levels expressed as percentage values of the Pearson product-moment correlation coefficient. Final identification of representative isolates from each cluster was carried out by sequencing of the 16S rRNA gene (bacteria) or the internal transcribed spacer (ITS) regions (yeasts) based on PCR amplicons of genomic DNA (pA-pH primers and ITS1-ITS4 primers, respectively), followed by evaluation of the sequencing results through basic local alignment search tool (BLAST) analysis to determine the closest known relatives of the partial sequences obtained in the National Centre for Biological Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Similarities equal or higher to 98% were taken into account.

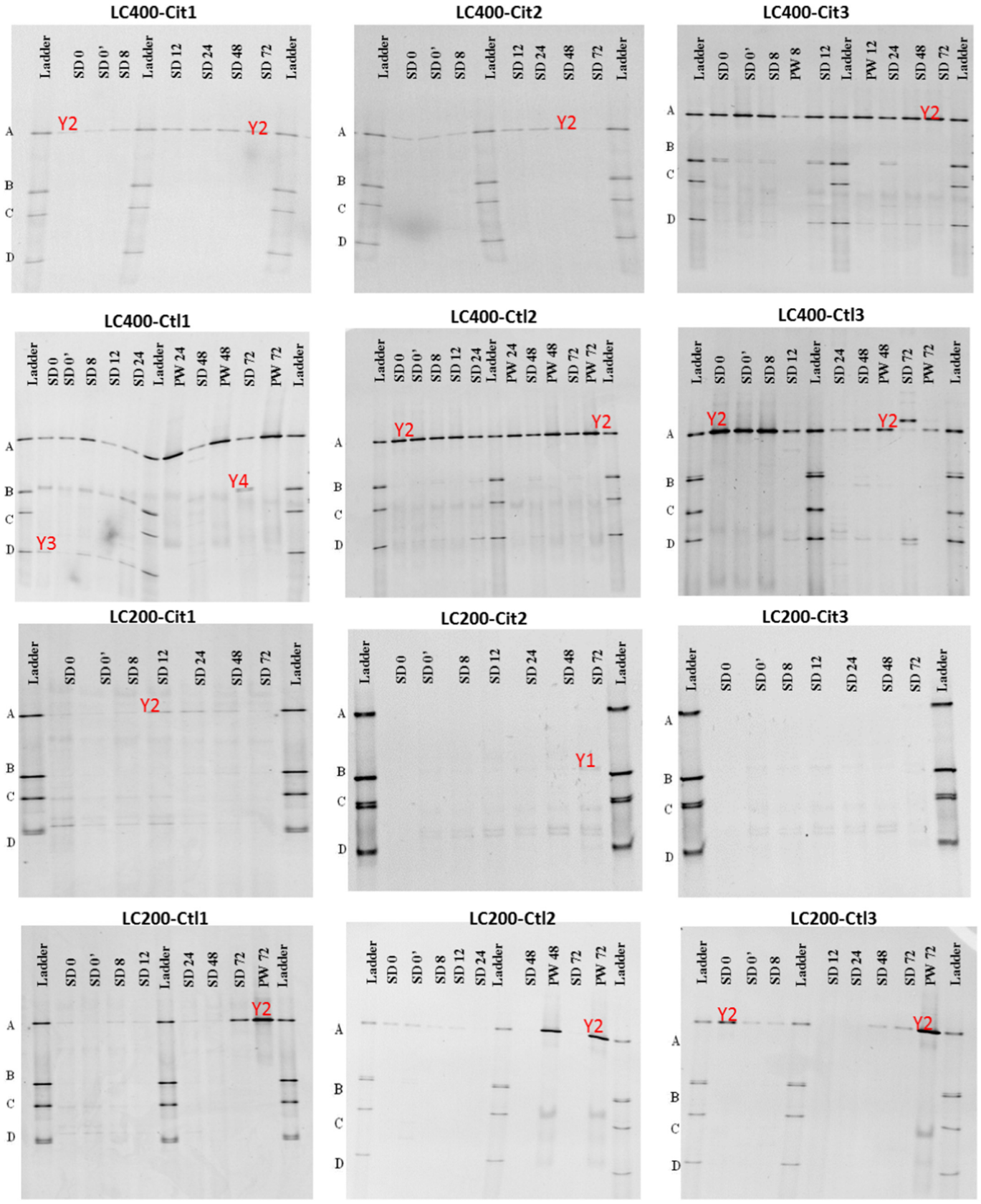
2.1.6.2. Culture-independent microbial community dynamics and identifications. To evaluate the prevalence of the starter culture during fermentation and to monitor its impact on the LAB, AAB, and yeast communities that were naturally present in the flour, DGGE of the PCR amplicons of a fragment of the 16S (bacteria) or 26S rRNA gene (yeasts) was performed (Harth et al., 2016). Therefore, the cell pellets from the sourdough samples were used (see Section 2.1.4). Also, plate washes from mMRS5, YPD, and mDMS agar media (see Section 2.1.6.1) were examined by collecting all cells from these agar media (corresponding with the 100 times dilutions) in 10 mL of peptone-physiological solution. These mixtures were centrifuged (4600 × *g* for 20 min at 4 °C), the supernatants were discarded, and the cell pellets were stored at −20 °C.

To extract bacterial and fungal DNA from the cell pellets obtained, a protocol described before was used (Van Kerrebroeck et al., 2016). To amplify bacterial DNA from the V3 region of the 16S rRNA gene, the universal primers F357-GC and 518R were used. To amplify fungal DNA from the D1 region of the 26S rRNA gene, the eukaryotic universal primers NL1-GC and LS2 were used. GC refers to a GC clamp. PCR amplifications were performed using a DNA T3 thermocycler (Biometra, Westburg, The Netherlands) in a final volume of 50 µL, containing 6 µL of 10× PCR buffer (Sigma-Aldrich), 2.5 µL of a solution of 0.1 mg/mL of bovine serum albumin (BSA; Acros Organics, Geel, Belgium), 0.5 µL of deoxy nucleotide triphosphates (dNTPs, 10 mM each; Sigma-Aldrich), 2 µL of each primer (5 µM; Integrated DNA Technologies, Leuven, Belgium), 0.25 µL of *Taq* DNA polymerase (5 U/mL; Sigma-Aldrich), 35.75 µL of ultrapure water, and 1 µL of DNA template (adjusted to 50 ng/µL). For the bacterial primers, the following PCR program was used: initial denaturation at 95 °C for 5 min; 34 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min, followed by cooling to 4 °C. For the yeast primers, the following PCR program was used: initial denaturation at 95 °C for 4 min; 29 cycles of denaturation at 95 °C for 1 s, annealing at 52 °C for 45 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 6 min, followed by cooling at 4 °C.

The PCR amplicon products were analysed by means of DGGE, as described previously (Camu et al., 2007). In short, separation of the different DNA fragments was obtained by means of a 35–60% denaturation gradient of formamide (14–24%; Sigma-Aldrich) and urea (2.4–4.2 M; National Diagnostics, Atlanta, GA, USA). The gels were normalized by using ladders of known bacterial and fungal DNA in at least three lanes per gel. Therefore, a mixture of PCR products originating from genomic DNA of pure cultures of *Lactobacillus amylovorus* DCE 471, *Lb. crustorum* LMG 23699, *Lb. fermentum* IMDO 130101, *Lactobacillus namurensis* LMG 23584, *Lactobacillus plantarum* IMDO 130201, *Lactobacillus sakei* IMDO CG1, and *Lb. sanfranciscensis* IMDO 150101 (laboratory collection of the research group IMDO) was used to analyse the LAB community profiles. To analyse the fungal community profiles, a ladder was constructed based on PCR products from genomic DNA of pure cultures of *S. cerevisiae* DIV/07-125X, *Candida glabrata* DIV/07-076BZ, *Wickerhamomyces anomalus* DIV/07-076BY, and *Kazachstania unispora* DIV/07-125CR (laboratory collection of the research group IMDO). DNA bands of interest were excised from the gels, resuspended in 30 µL of ultrapure water, and incubated at 4 °C for 48 h for DNA elution. Five microliter of this DNA solution was used to reamplify the PCR products. The primers F357/518R and NL1/LS2 were used for the bacterial and fungal DNA, respectively. All primers described above were designed with a specific clamp to increase the quality of the sequences of short PCR products. The amplicons were purified with a Wizard® SV Gel and PCR Clean up system (Promega, Madison, WI, USA) and sequenced in a commercial facility by means of capillary sequencing technology (VIB Genetic Service Facility, Antwerp, Belgium). A BLAST analysis was performed as described above. Similarities equal or higher to 98% were taken into account.

2.1.7. Metabolite target analysis to map substrate consumption and metabolite production

Glucose, fructose, sucrose, and maltose concentrations in the sourdough supernatants were determined by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), using an ICS 3000 chromatograph equipped with an AS-autosampler and a CarboPac PA10 column (Dionex, Sunnyvale, CA, USA), as described previously (Moens et al., 2014), with some modifications. Briefly, the column temperature was set to 30 °C and the flow rate of the mobile phase, consisting of ultrapure water (eluent A), 167 mM NaOH (eluent B) and 500 mM NaOH (eluent C), was 1.0 mL/min. The following gradient was applied: 0 min, 87% A, 13% B, and 0% C; 20 min, 87% A, 13% B, and 0% C; 25 min, 0% A, 0% B, and 100% C; 30 min, 0% A, 0% B, and 100% C; 31 min, 87% A, 13% B, and 0% C; and 35 min, 87% A, 13% B, and 0% C. Due to matrix interference, quantification was carried out with standard addition. Four standard solutions with the following compositions were prepared (g/L): ultrapure water (solution A); 1.0 glucose, 0.5 fructose, 0.5 sucrose, and 5.0 maltose



(caption on next page)

Fig. 2. Culture-independent yeast community dynamics at 0 (unfermented wheat flour-water mixture), 0′ (after inoculation), and after 8, 12, 24, 48 and 72 h of fermentation during wheat sourdough productions with *Lactobacillus crustorum* LMG 23699 as starter culture strain. This was done in the presence (Cit) and absence (Ctl) of citrate for both liquid (dough yield of 400, LC400) and firm (dough yield of 200, LC200) wheat sourdough productions. Sourdough fermentation samples are indicated as SD and plate wash samples as PW. The ladder was constructed using pure cultures of *Wickerhamomyces anomalus* DIV/07-076BY (A), *Kazachstania unispora* DIV/07-125CR (B), *Candida glabrata* DIV/07-076BZ (C), and *Saccharomyces cerevisiae* DIV/07-125X (D). The closest relatives to the fragments sequenced are given: Y1, *Sporobolomyces salmoneus/marcillae/Sporidiobolus metaroseus* (99% identity; accession no. KY109767.1/EU003479.1/KY109696.1); Y2, *Wickerhamomyces anomalus* (100% identity; accession no. EU057562.1); Y3, *Saccharomyces cariocanus* (99% identity; accession no. KY109235.1); and Y4, *Sporobolomyces patagonicus* (100% identity; accession no. AB279626.1).

(solution B); 2.0 glucose, 1.0 fructose, 1.0 sucrose, and 10.0 maltose (solution C); and 4.0 glucose, 2.0 fructose, 2.0 sucrose, and 20.0 maltose (solution D). Subsequently, 300 µL of the supernatants was mixed with 600 µL of acetonitrile (Sigma-Aldrich) and 300 µL of the solutions A, B, C, or D. Afterwards, all samples were vortexed, microcentrifuged (14,000 rpm, 20 min), diluted 10 times with ultrapure water, filtered using regenerated cellulose filters (0.2 µm Whatman filters; GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK), and injected (10 µL) into the column. The concentrations in the original samples were calculated as the intercepts with the X-axis of the linear regression of the peak heights (y-values) against the added concentrations (x-values) (Vrancken et al., 2008).

Concentrations of glycerol, erythritol, sorbitol, and mannitol were determined by HPAEC-PAD, using the same equipment as described above but with a CarboPac™ MA1 column (Dionex) (Wouters et al., 2013). The column temperature was set to 30 °C and the flow rate of the mobile phase, consisting of ultrapure water (eluent A) and 500 mM NaOH (eluent B), was 0.4 mL/min. The following gradient was applied: 0.0 min, 50% A and 50% B; 15.0 min, 50% A and 50% B; 25.0 min, 0% A and 100% B; 45.0 min, 0% A and 100% B; 45.5 min, 50% A and 50% B; and 55.0 min, 50% A and 50% B. Quantification was carried out with standard addition. Four standard solutions with the following compositions were prepared (g/L): ultrapure water (solution A); 0.15 glycerol, 0.20 erythritol, 0.45 sorbitol, and 0.70 mannitol (solution B); 0.30 glycerol, 0.40 erythritol, 0.90 sorbitol, and 1.40 mannitol (solution C); and 0.60 glycerol, 0.80 erythritol, 1.80 sorbitol, and 2.80 mannitol (solution D). Sample preparation was carried out as described above, using 0.2 µm Chromafil polyethersulfone filters (Macherey Nagel); 10 µL was injected into the column.

Concentrations of citric acid were determined by HPAEC with conductivity under ion suppression (CIS), using the same apparatus as described above, equipped with an AS-19 column (Dionex) (Camu et al., 2007). The column temperature was set to 30 °C and the flow rate of the mobile phase was 1.0 mL/min and consisted of ultrapure water (eluent A) and 100 mM KOH (eluent B). The following gradient was applied: 0 min, 98% A and 2% B; 8 min, 98% A and 2% B; 34 min, 60% A and 40% B; 36 min, 0% A and 100% B; 41 min, 0% A and 100% B; 42 min, 98% A and 2% B; and 44 min, 98% A and 2% B. Quantification was carried out with standard addition. Four standard solutions with the following compositions were prepared (g/L): ultrapure water (solution A); 1.0 citric acid (solution B); 2.0 citric acid (solution C); and 4.0 citric acid (solution D). Sample preparation was carried out as described above; 10 µL was injected into the column. Also the presence of succinate was checked qualitatively by the HPAEC-CIS method described above.

Concentrations of ethyl acetate, ethanol, acetic acid, diacetyl, and acetoin were determined through gas chromatography with flame ionization detection (GC-FID), using a Focus gas chromatograph (Interscience, Breda, The Netherlands), equipped with a Stabilwax-DA column (Restek, Bellefonte, PA, USA), a FID-80 detector (Interscience), and an AS 3000 autosampler (Interscience), as described previously (Moens et al., 2014). Briefly, the column temperature profile was as follows: 0 min, 40 °C; 10 min, 140 °C; 12 min, 230 °C; and 22 min, 230 °C. The injector and detector temperatures were 240 °C and 250 °C, respectively. One microliter was injected into the column, in split mode, with a split ratio of 40:1. External calibration was performed. The samples were analysed in triplicate.

The concentrations of D- and L-lactic acid were determined by high-performance liquid chromatography with ultraviolet detection (HPLC-UV), as described previously (Van Kerrebroeck et al., 2016). A Waters chromatograph (Waters, Milford, MA, USA) was used, equipped with a 600S controller, a 717Plus autosampler, a 486 UV detector (set at a wavelength of 253 nm), and a Shodex Orpak CRX-853 column (Showa Denko, Tokyo, Japan). The flow rate of the mobile phase was 0.8 mL/min and consisted of 0.5 mM $CuSO_4$ in water (eluent A) and acetonitrile (eluent B). The following gradient was applied: 0 min, 100% A and 0% B; 16 min, 100% A and 0% B; 17 min, 50% A and 50% B; 20 min, 50% A and 50% B; 21 min, 100% A and 0% B; and 35 min, 100% A and 0% B. External standards were used. Samples were prepared in triplicate by mixing 600 µL of supernatant and 600 µL of acetonitrile, followed by vortexing, centrifugation, filtering, and injection (30 µL) into the column, as described above.

Concentrations of volatile compounds of sourdough samples taken at 0, 24, 48, and 72 h were measured by headspace/solid-phase microextraction GC coupled to mass spectrometry (HS/SPME-GC–MS), as described previously (Harth et al., 2016). An Agilent 6890 gas chromatograph coupled to an Agilent 5973 N mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), a MPS2 Gerstel autosampler (Gerstel, Mülheim-an-der-Ruhr, Germany), and a DB-WAXetr capillary column (Agilent Technologies) were used. A 75-µm SPME divinylbenzene/carboxen/polydimethylsiloxane (DVS/CAR/PDMS) fiber (Agilent Technologies) was used. The injection port was operated in splitless mode and the oven temperature program was the following: 40 °C for 5 min; linear increase to 225 °C at 10 °C/min; and 225 °C for 5 min. Samples for analysis were prepared in triplicate in 20 mL headspace vials by adding 5.0 g of sourdough and 1.0 g of NaCl. Deconvolution and peak identification was performed using AMDIS version 2.71 (https://www.nist.gov; NIST, Gaithersburg, MD, USA) and NIST98 version 2.0 (https://www.nist.gov/; NIST) software, respectively. Peaks were taken into account when they were present in at least two of the three samples measured. Their surface areas were normalized and a heatmap analysis with Pearson correlation hierarchical clustering was performed using Multiple Experiment Viewer 4.9 software (http://mev.tm4.org) (Harth et al., 2016).

2.2. Bread production

Bread production and analysis was performed as described previously (Van Kerrebroeck et al., 2018).

2.2.1. Breadmaking

Dough of 0 h (unfermented wheat flour-water mixture) and sourdoughs of 24, 48, and 72 h of fermentation were used to produce leavened bread with sourdough as ingredient. The following recipe was applied: 342 g of wheat flour, 146 mL of ultrapure water, 3 g of dry commercial yeast (Instant Yeast Blue; Algist Bruggeman, Ghent, Belgium), 5 g of salt, and 72 g of sourdough. A bread machine (Home Bread Uno, Moulinex, Écully, France) was used with the following settings: preparation of the dough for 136 min and baking time of 63 min (program 3, weight of 1 kg, browning scale 3).

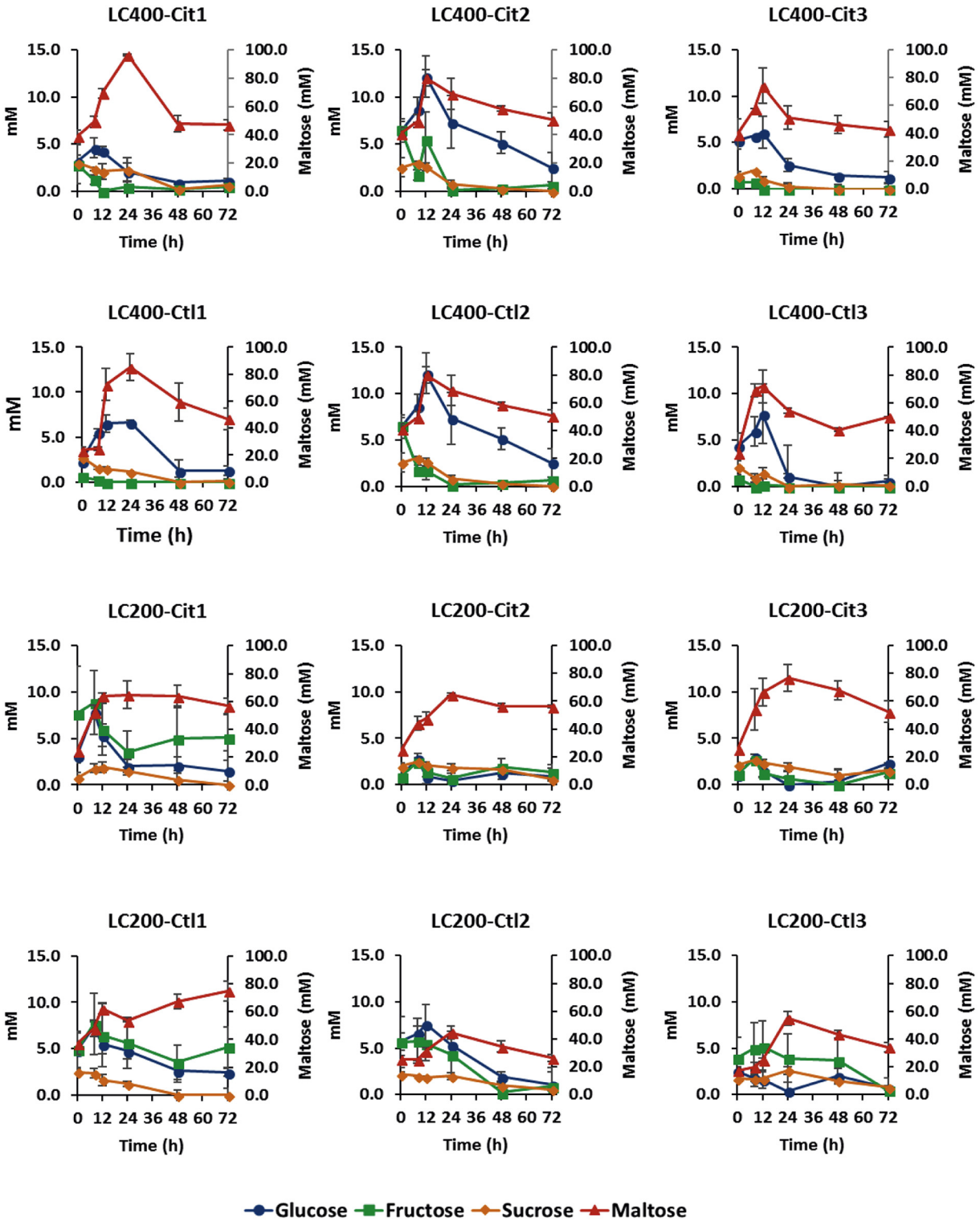


Fig. 3. Residual concentrations of glucose, fructose, and sucrose (left Y-axis), and maltose (right Y-axis) during wheat sourdough productions with *Lactobacillus crustorum* LMG 23699 as starter culture strain. This was done in the presence (Cit) and absence (Ctl) of citrate for both liquid (dough yield of 400, LC400) and firm (dough yield of 200, LC200) wheat sourdough productions.

2.2.2. Bread analysis

The pH and TTA of the breads were measured by mixing 10 g of bread crumb with 90 mL of water using the same apparatus and parameters described above. To analyse the volatile compounds of the crust and crumb of the breads, HS/SPME-GC–MS was performed after 1 h of cooling of the bread, as described above. Therefore, the crust was carefully separated from the crumb by means of a conventional knife and 3 g of crumb or crust were placed into 20 mL headspace vials. The measurements were done in triplicate.

2.3. Statistical analysis

Student's *t*-tests were used to check significant differences between sourdough pH and TTA, maximum viable LAB counts (X_{max}, obtained

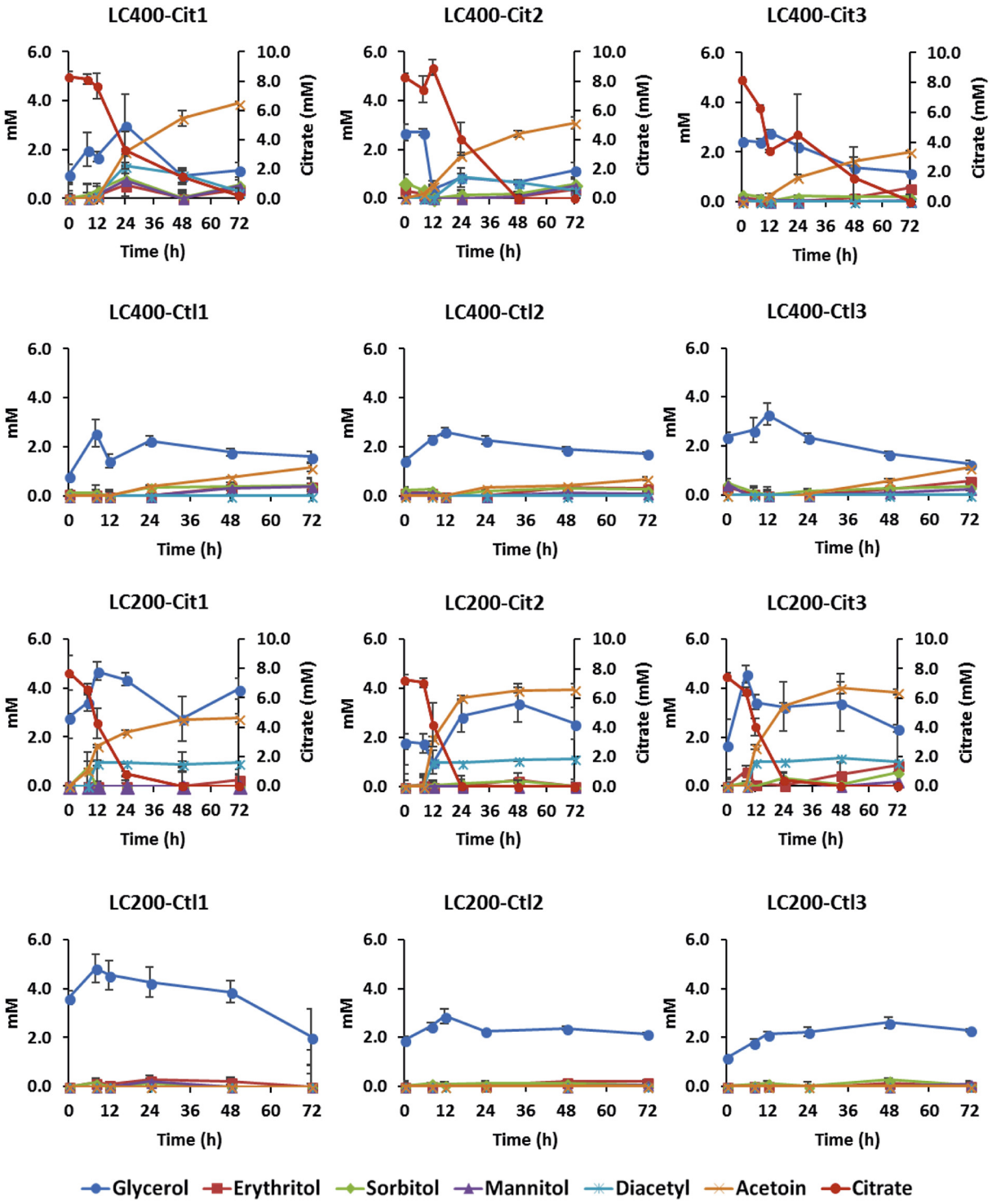


Fig. 4. Concentrations of glycerol, erythritol, sorbitol, mannitol, diacetyl, and acetoin (left Y-axis), and citrate (right Y-axis) during wheat sourdough productions with *Lactobacillus crustorum* LMG 23699 as starter culture strain. This was done in the presence (Cit) and absence (Ctl) of citrate for both liquid (dough yield of 400, LC400) and firm (dough yield of 200, LC200) wheat sourdough productions.

through fitting of the experimental data with the Baranyi and Roberts model using the DMFit package of ComBase software; https://www.combase.cc), consumption of citrate, production of metabolites, and bread pH and TTA. They were performed using SPSS software version 23 for Windows (IBM, Armonk, NY, USA). Cohen *d*-values were used and calculated, as described before (de Winter, 2013). Student's *t*-test is considered to be a valid approach for comparisons with low numbers of samples when the sample effects are large (Cohen *d*-values above 0.8). Differences were considered significant when the *p*-values of the *t*-tests and Cohen *d*-values were ≤ 0.05 and ≥ 0.8, respectively.

3. Results

3.1. Genome mining

Although the wheat sourdough strains *Lb. crustorum* LMG 23699 and

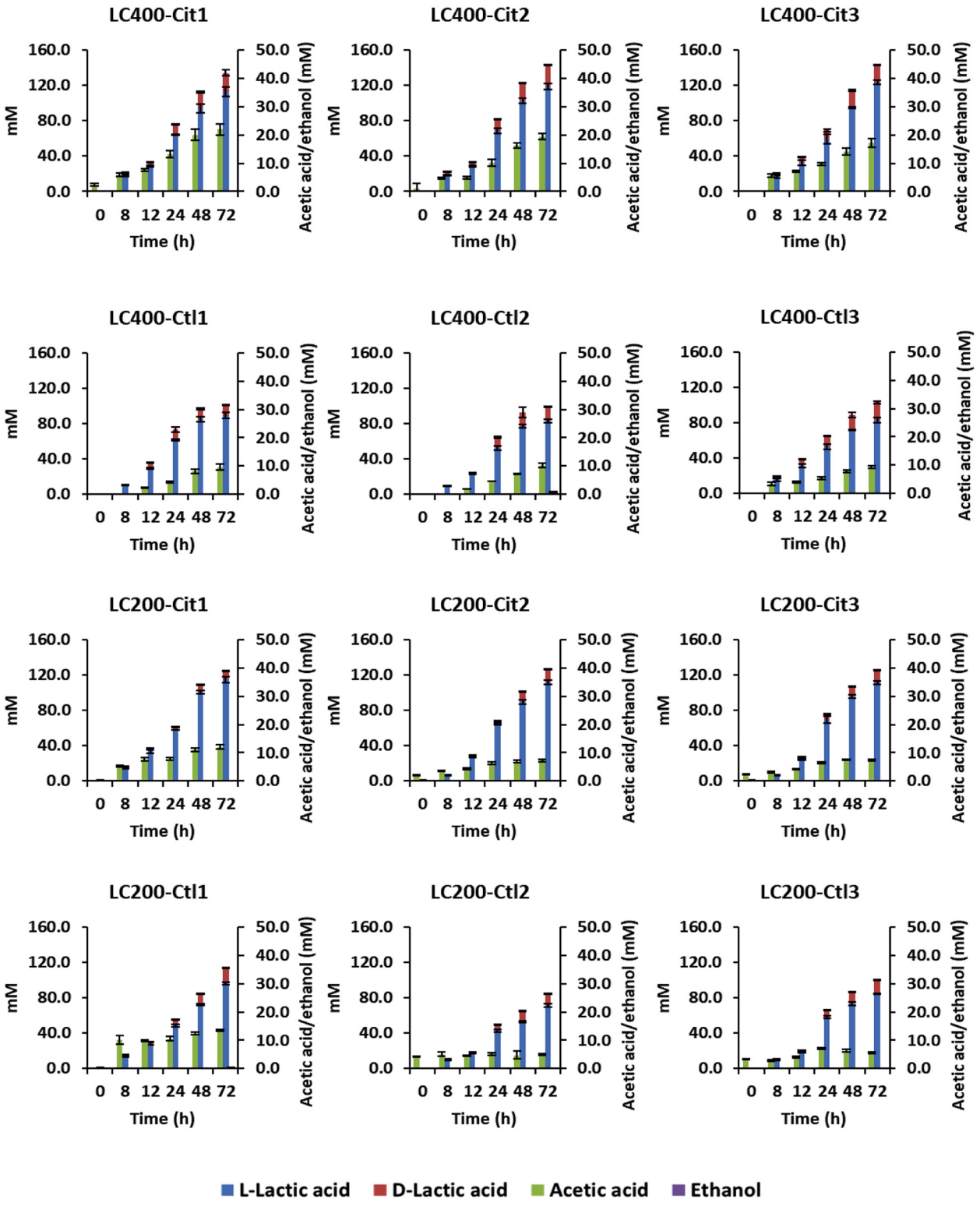
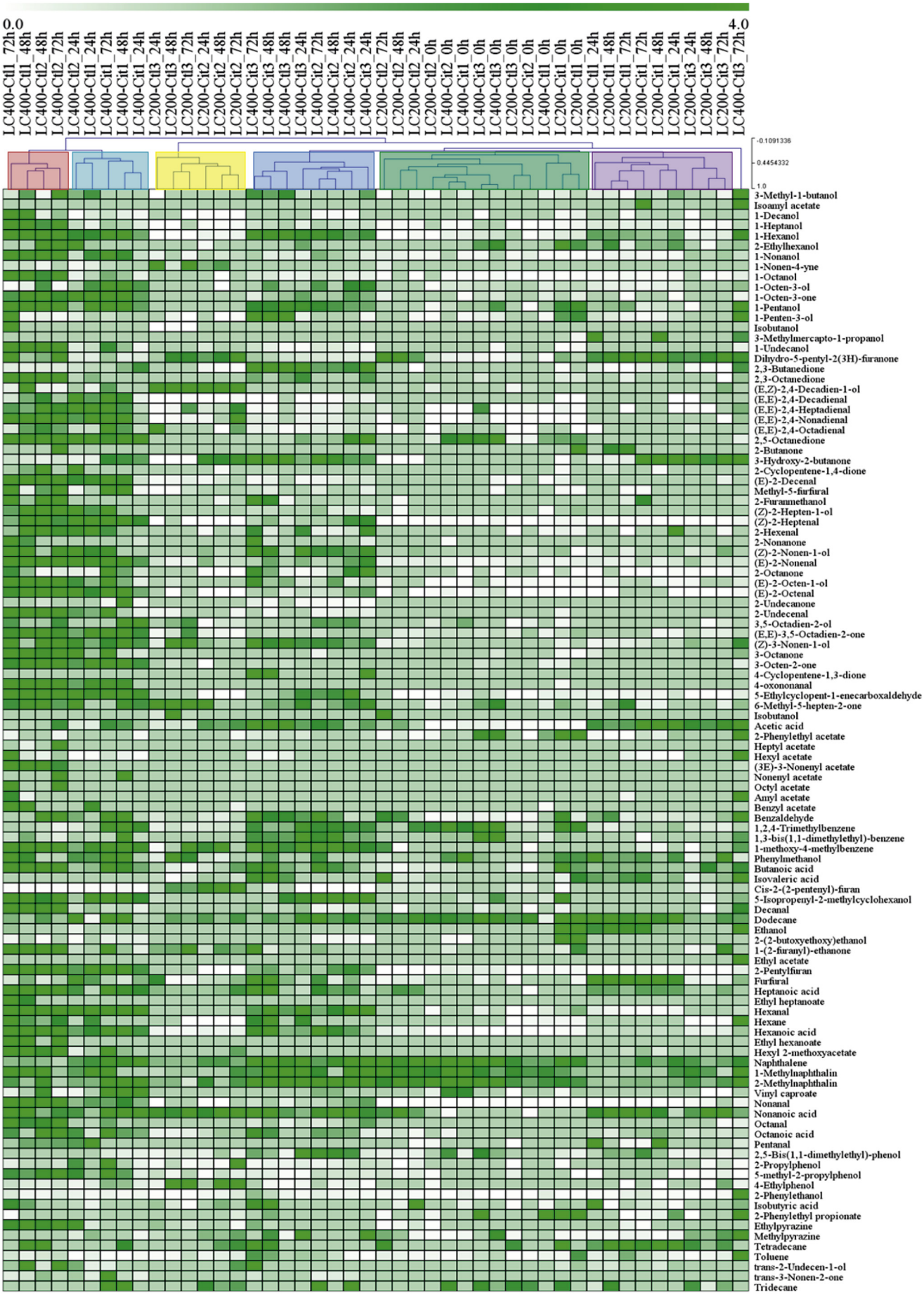


Fig. 5. Concentrations of D and L-lactic acid (left Y-axis), and acetic acid and ethanol (right Y-axis) during wheat sourdough productions with *Lactobacillus crustorum* LMG 23699 as starter culture strain. This was done in the presence (Cit) and absence (Ctl) of citrate for both liquid (dough yield of 400, LC400) and firm (dough yield of 200, LC200) wheat sourdough productions.

Lb. crustorum JCM 15951 actually represented the same strain (stored in two different culture collections), *Lb. crustorum* MN047 was isolated from koumiss. On protein level, all these *Lb. crustorum* strains possessed all the enzymes necessary to produce acetic acid, lactic acid, succinate, acetoin, diacetyl, and 2,3-butanediol from citrate (Table 1). In general, the identity and similarity of the respective enzymes of *Lb. crustorum* LMG 23699 were slightly higher when compared with those of *Lb. fermentum* 222 than with those of *Lc. lactis* IL1403. Only the acetoin reductase of *Lb. crustorum* MN047 displayed a slightly higher identity and similarity compared with the other *Lb. crustorum* strains (data not shown). Among the four lactate dehydrogenases present in the *Lb. crustorum* strains, three were L-lactate dehydrogenases. There were also



(caption on next page)

Fig. 6. Heat map of the volatile compounds, as detected by GC–MS, present in sourdoughs fermented with *Lactobacillus crustorum* LMG 23699 as starter culture strain. This was done in the presence (Cit) and absence (Ctl) of citrate for both liquid (dough yield of 400, LC400) and firm (dough yield of 200, LC200) wheat sourdough productions. The surface areas of the peaks obtained for the different compounds were normalized. Pearson correlation hierarchical clustering of the sourdough samples corresponding with different fermentation time points was applied. Clusters are highlighted with color boxes. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article.)

Table 3
Evolution of the pH and total titratable acidity (TTA) of the crumb of breads produced with liquid and firm wheat sourdoughs obtained through productions with *Lactobacillus crustorum* LMG 23699 as starter culture strain. This was done in the presence (Cit) and absence (Ctl) of citrate for both liquid (dough yield of 400, LC400) and firm (dough yield of 200, LC200) wheat sourdough productions. A bar (−) indicates that no samples were taken.

Time (h)	Cit1		Cit2		Cit3		Ctl1		Ctl2		Ctl3	
	pH	TTA (mL)	pH	TTA (mL)	pH	TTA (mL)	pH	TTA (mL)	pH	TTA (mL)	pH	TTA (mL)
DY400												
0	5.82	2.35	5.80	2.40	5.87	2.35	5.92	2.00	–	–	5.91	2.10
24	5.45	3.30	5.50	3.25	5.70	3.40	5.60	2.85	5.75	2.75	5.65	2.80
48	5.33	3.80	5.35	3.70	5.42	3.75	5.30	3.40	5.42	3.00	5.32	3.45
72	5.20	4.10	5.12	4.00	5.21	3.85	5.10	3.75	4.93	3.65	5.15	3.70
DY200												
0	–	–	5.79	2.60	–	–	–	–	5.60	2.50	–	–
24	4.85	4.20	4.89	4.00	4.95	4.10	4.88	4.20	5.15	3.75	4.90	3.80
48	4.75	4.60	4.72	4.35	4.66	4.50	4.72	4.45	4.95	3.90	4.82	4.00
72	4.69	4.90	4.66	4.35	4.70	4.60	4.62	4.50	4.85	4.00	4.75	4.30

three acetate kinases present in these strains.

3.2. Sourdough pH and total titratable acidity

When citrate was added, liquid wheat sourdoughs with a higher final pH ($p < 0.001$, $d = 27.8$), on average 3.61, were obtained compared to liquid wheat sourdoughs produced without citrate, pH on average 3.50 (Table 2). The TTA values at 72 h were higher in wheat sourdoughs [liquid sourdoughs ($p < 0.001$, $d = 10.6$); firm sourdoughs ($p = 0.002$, $d = 6.3$)] produced with the addition of citrate (on average 15.03 mL for liquid and 18.27 mL for firm sourdoughs) compared with those produced without citrate (on average 13.00 mL for liquid and 15.65 mL for firm sourdoughs). Moreover, the TTA values were higher for the firm wheat sourdoughs produced with the addition of citrate ($p < 0.001$, $d = 11.5$) or without the addition of citrate ($p = 0.002$, $d = 7.3$) compared with the liquid ones.

3.3. Culture-dependent microbial community dynamics and identifications

The viable LAB, AAB, and yeast counts were low (under the detection limit) at the start of all wheat sourdough productions (uninoculated wheat flour-water mixtures) (Table 2). After inoculation of the starter culture strain *Lb. crustorum* LMG 23699, viable LAB counts of on average log 7.0 CFU per mL (liquid wheat sourdough productions) or g (firm wheat sourdough productions) were found. No significant differences in X_{max} were found for the liquid and firm wheat sourdough productions in the presence or absence of citrate, indicating that citrate did not stimulate growth.

The mMRS-5 isolates from all time points examined during all sourdough productions performed were identified as *Lb. crustorum* (99% identity; accession no. NR_042533.1), throughout the liquid and firm wheat sourdough productions with and without citrate, indicating prevalence of the starter culture strain added. AAB were only present in the liquid wheat sourdough production LC400-Ctl3 and the mDMS isolates picked up were identified as *Acetobacter indonesiensis* (99% identity; accession no. NR_113847.1).

Yeasts were almost never found in the liquid and firm wheat sourdough productions in the presence of citrate, presumably because of viable yeast counts below the detection limit due to citrate's inhibitory activity (Table 2). Without citrate supplementation, low viable yeast counts [on average log (3.4 CFU/g)] were found after 48–72 h of fermentation during firm wheat sourdough productions; they were present at higher viable counts ($p = 0.002$, $d = 5.6$) during liquid wheat sourdough productions after 72 h of fermentation [on average log (5.5 CFU/mL)]. The YPD isolates picked up throughout all wheat sourdough productions were identified as *W. anomalus* (99% identity; accession no. NR_111210.1), except for one isolate that was identified as *Saccharomyces cerevisiae* (99% identity; accession no. KC881067.1) and picked up after 72 h of fermentation during liquid wheat sourdough production LC400-Ctl1.

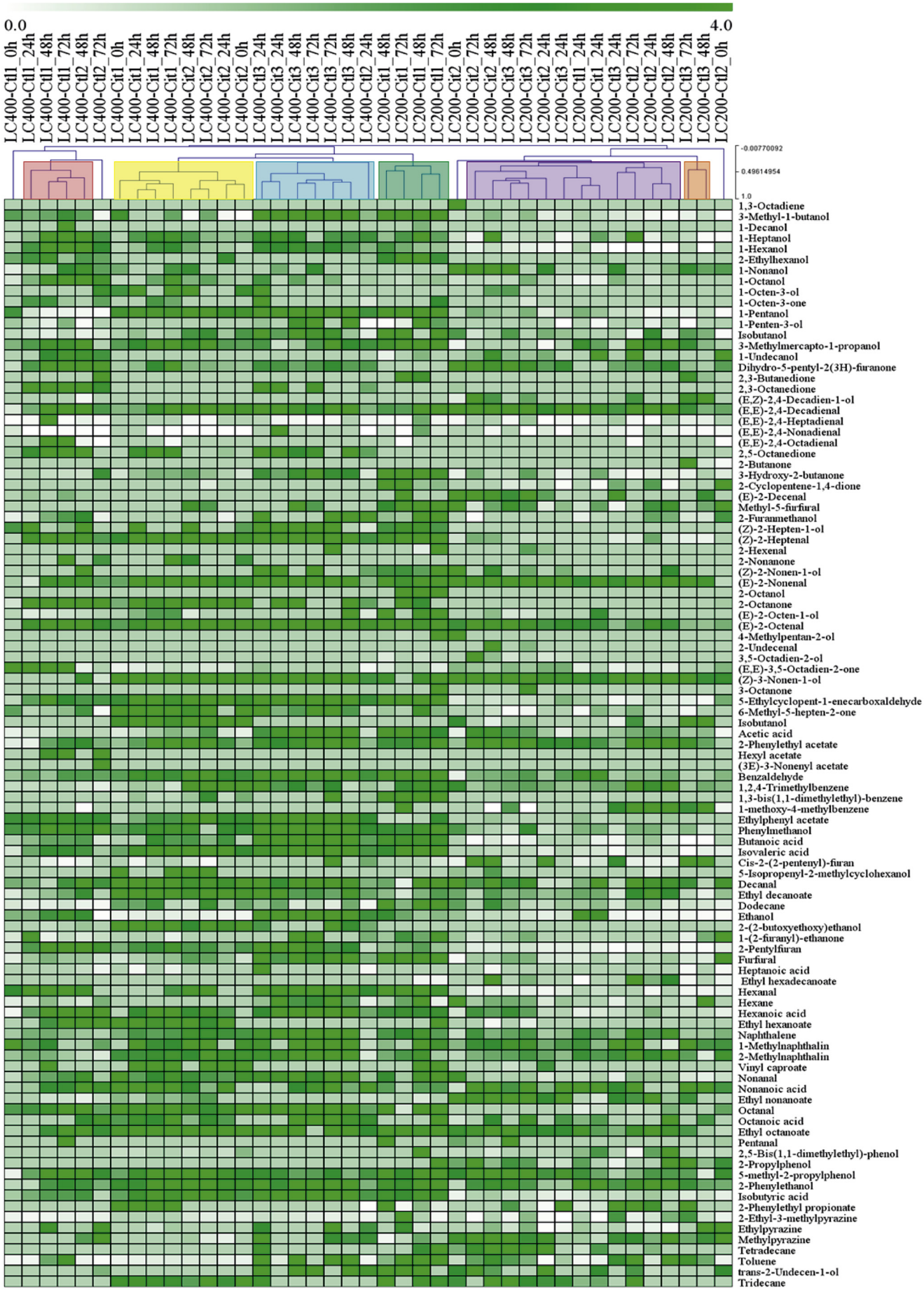
3.4. Culture-independent microbial community dynamics and identifications

The 16S rRNA-PCR-DGGE bacterial community profiles showed that the species *Lb. crustorum* was present throughout all wheat sourdough productions performed, both in fermentation samples and plate washes (Fig. 1). Presumably, due to the high concentration of the inoculated strain used, the *Lb. crustorum* bands in the gels corresponded with this strain, as were the isolates identified culture-dependently. One band corresponding with a sample of the wheat flour-water mixture of the firm wheat sourdough production LC200-Cit2 (plate wash) was attributed to *Staphylococcus xylosus*.

The 26S rRNA-PCR-DGGE yeast community profiles showed that *W. anomalus* was the most prevalent yeast species found throughout all wheat sourdough productions performed (Fig. 2). This was in agreement with the culture-dependent yeast identification data. Other yeast species were found sporadically. However, yeasts could hardly be detected in the firm wheat sourdough productions with citrate addition, confirming their low viable counts mentioned above.

3.5. Substrate consumption and metabolite production dynamics

Maltose was the main carbohydrate present at the beginning of all wheat sourdough productions performed, increasing rapidly during the first 12–24 h of fermentation and being metabolized thereafter (Fig. 3). Glucose and fructose concentrations slightly increased during the first hours of fermentation, due to sucrose conversion, and they were metabolized thereafter as well, whereby always higher residual maltose concentrations remained. Although glycerol concentrations did not



(caption on next page)

Fig. 7. Heat map of the volatile compounds, as detected by GC–MS, present in the crumb of breads produced from wheat sourdoughs fermented with *Lactobacillus crustorum* LMG 23699 as starter culture strain. This was done in the presence (Cit) and absence (Ctl) of citrate for both liquid (dough yield of 400, LC400) and firm (dough yield of 200, LC200) wheat sourdough productions. The surface areas of the peaks obtained for the different compounds were normalized. Pearson correlation hierarchical clustering of the bread crumb samples corresponding with different sourdough fermentation time points was applied. Clusters are highlighted with color boxes. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article.)

change much during all fermentations, sorbitol, mannitol and erythritol were present in very low concentrations (Fig. 4).

Citrate was consumed within a shorter time frame during the firm wheat sourdough productions (24 h) than during the liquid ones (48–72 h) ($p = 0.001$, $d = 6.91$) (Fig. 4). It was converted into acetoin, which was found in maximal concentrations between 3.90 ± 0.06 mM and 3.96 ± 0.23 mM in the liquid and firm wheat sourdough productions, respectively, toward the end of the fermentations. Acetoin was also found in concentrations below 1 mM at the end of the liquid wheat sourdough productions in the absence of citrate. Diacetyl was found too during the wheat sourdough productions in the presence of citrate, except for liquid wheat sourdough production LC400-Cit3. During the liquid wheat sourdough productions, its maximum concentration was reached after 24 h of fermentation (average of 0.73 mM), followed by a decrease, whereas during the firm wheat sourdough productions, the diacetyl concentrations increased during the first 12 h of fermentation and then remained stable (average of 0.97 mM). Succinate was never produced.

In the presence of citrate, more lactic acid was produced after 72 h of fermentation compared to its absence [liquid wheat sourdoughs ($p < 0.001$, $d = 9.53$); firm wheat sourdoughs ($p = 0.038$, $d = 2.49$)], which was more pronounced in the liquid wheat sourdough productions supplemented with citrate than in the firm ones supplemented with citrate ($p = 0.01$, $d = 31.88$) (Fig. 5). In the presence of citrate, more acetic acid was found, but its concentration was only significantly different in the liquid wheat sourdoughs ($p = 0.002$, $d = 5.67$). During all sourdough productions, more L-lactic acid than D-lactic acid was found, corresponding with 83–88% of the total lactic acid produced. Ethanol concentrations were below the detection limit, which was in agreement with the low abundance of yeasts.

A cumulative total of 109 different volatile compounds was detected in all wheat sourdoughs produced and heat mapping of the normalized data allowed the distinction of several clusters (Fig. 6). One main cluster corresponded with the unfermented wheat flour-water mixtures. Other clusters corresponded with differences in DY applied for the production of the wheat sourdoughs examined. Co-fermentation of citrate did not give a separate cluster. Independent of the addition of citrate or the DY applied, more organic acids (butanoic acid, isovaleric acid, heptanoic acid, hexanoic acid, nonanoic acid, and octanoic acid) were found at the end of the wheat sourdough productions. Based on the peak areas of the chromatograms, higher concentrations of aldehydes and alcohols were formed during the liquid wheat sourdough productions. Esters were prevalent during the liquid wheat sourdough productions without citrate, possibly reflecting yeast activity. In the presence of citrate, more diacetyl [only a significant difference in the case of the firm wheat sourdoughs ($p < 0.001$, $d = 13.76$)] and acetoin [liquid wheat sourdoughs ($p = 0.024$, $d = 2.91$); firm sourdoughs ($p = 0.001$, $d = 7.23$)] were produced during wheat sourdough productions than during sourdough productions without citrate, confirming the measurements described above.

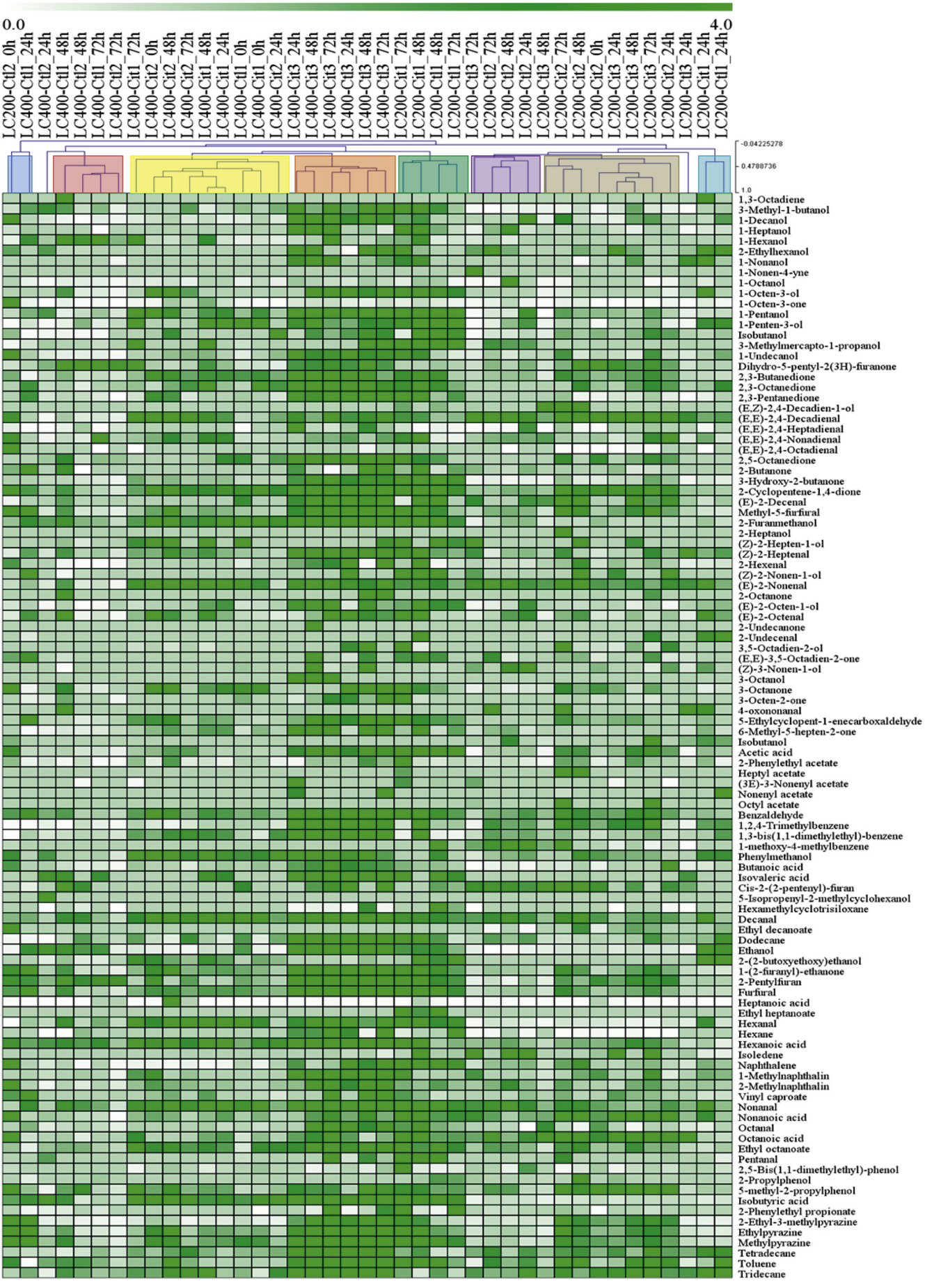
3.6. Bread production

The crumb of breads produced with all the 72-h fermented liquid wheat sourdoughs possessed higher pH values ($p \leq 0.001$–0.03, $d = 2.8$–13.0) and lower TTA values ($p = 0.02$, $d = 3.0$–3.1) than the firm wheat sourdoughs (average pH of 5.18 for liquid and 4.68 for firm wheat sourdoughs in the presence of citrate, and of 5.06 for liquid and 4.74 for firm wheat sourdoughs in its absence; average TTA values of 3.98 mL for liquid and 4.62 mL for firm wheat sourdoughs in the presence of citrate, and of 3.70 mL for liquid and 4.27 mL for firm wheat sourdoughs in its absence) (Table 3). Moreover, the TTA values of the crumb of breads produced with 72-h liquid wheat sourdoughs containing citrate were slightly higher ($p = 0.02$, $d = 3.0$) than those without (on average 3.98 mL and 3.70 mL, respectively).

A cumulative total of 102 and 105 volatile compounds were detected in the crumbs and crusts of the breads made with the wheat sourdoughs produced, respectively (Figs. 7 and 8). Compounds such as 2-phenylethanol, 3-methyl-1-butanol, 2-methylpropanoic acid, and 3-methylbutanoic acid were more present in the bread crumbs and crusts than in the respective sourdoughs. Clustering of the normalized data through heatmapping allowed to distinguish breads made with liquid and firm wheat sourdoughs and could show the impact of citrate addition. However, no cluster corresponded with breads produced with doughs containing unfermented wheat flour-water mixtures. Pyrazines and furans were predominantly present in the crusts of all breads produced, in particular in those made with firm wheat sourdoughs. Diacetyl and acetoin were especially present in breads produced with sourdoughs obtained in the presence of citrate, albeit that this was less pronounced in the bread crumbs and crusts compared with the corresponding original liquid and firm wheat sourdoughs.

4. Discussion

The present study showed the impact of the addition of citrate on the wheat sourdough fermentation dynamics with *Lb. crustorum* LMG 23699 as starter culture strain and on the aroma profile of the liquid and firm wheat sourdoughs obtained and breads produced thereof. It is well known that nonconventional ingredients used for sourdough production may be a source of microorganisms. The use of fruits and milk is an example (De Vuyst et al., 2017; Palomba et al., 2011; Zhang et al., 2011). Moreover, this study showed that chemical compounds of such ingredients may also function as precursor molecules for further co-fermentation or bioconversion and hence aroma formation, in particular when using a dedicated starter culture strain. For instance, citrate that is naturally present in milk (in a concentration of *circa* 10 mM) plays an important role as supplier of extra pyruvate for LAB. Furthermore, LAB can convert this extra pyruvate into buttery flavour compounds under acidic conditions through their C4 flavour pathway, which characterises some fermented dairy products such as certain cheeses and sour butter (Hugenholtz, 1993; Stefanovic et al., 2017). These LAB strains can be naturally present in the food matrix or are part of the starter culture used. However, citrate does not occur in wheat flour or at least not in concentrations requested for co-fermentation by LAB (Khakimov et al., 2014). Hence, it will not select for and enrich the potential occurrence of citrate-positive LAB during spontaneous sourdough fermentation processes. Yet, as shown during the present study, the supplementation of citrate led to butter flavour compounds in both sourdoughs and breads produced thereof in the presence of a citrate-positive starter culture strain, *in casu* the citrate-positive homofermentative *Lb. crustorum* LMG 23699. This strain was inoculated into the flour-water mixture and prevailed (shown both culture-dependently and culture-independently) during the liquid and firm wheat sourdough productions performed. It has been shown before that strains of *Lb. crustorum* initiate and sustain Type II wheat and rye sourdough fermentation processes, which are liquid sourdough productions carried out above room temperature (Ravyts and De Vuyst, 2011; Ripari et al., 2016a). Further, the present study showed that citrate addition did not



(caption on next page)

Fig. 8. Heat map of the volatile compounds, as detected by GC–MS, present in the crust of breads produced from wheat sourdoughs fermented with *Lactobacillus crustorum* LMG 23699 as starter culture strain. This was done in the presence (Cit) and absence (Ctl) of citrate for both liquid (dough yield of 400, LC400) and firm (dough yield of 200, LC200) wheat sourdough productions. The surface areas of the peaks obtained for the different compounds were normalized. Pearson correlation hierarchical clustering of the bread crust samples corresponding with different sourdough fermentation time points was applied. Clusters are highlighted with color boxes. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article.)

result in growth stimulation of this strain. This was in contrast with an increased growth rate and/or cell yield upon co-metabolism of glucose and citrate in the case of the sourdough-characteristic *Lb. sanfranciscensis* CB1 strain during wheat sourdough fermentations (Gobbetti and Corsetti, 1996), other sourdough-characteristic LAB strains (*e.g.*, *Lactobacillus panis* PM1; Kang et al., 2013), or LAB strains from other sources (*e.g.*, strains of *Lactococcus lactis* and *Leuconostoc* spp. during milk fermentations) (Gänzle, 2015; Hugenholtz, 1993; Vaningelgem et al., 2006). However, growth stimulation is not necessary for aroma enhancement. This has been shown for enterococci converting citrate into acetate, formate, ethanol, and acetoin during fermentations in milk-based media (De Vuyst et al., 2011).

Although maltose was not completely depleted, more lactic acid was produced in the presence of citrate, underlining co-fermentation of the energy source and the co-substrate by the *Lb. crustorum* LMG 23699 strain, and channelling pyruvate breakdown toward lactic acid, diacetyl and acetoin production, which was independent of the dough yield. Although genome mining could confirm this flux through the C4 flavour pathway, the genes of the reductive branch of the tricarboxylic acid cycle were present as well, but apparently not expressed, as succinate was not produced. In contrast, conversion of citrate into succinate is a common route for obligately heterofermentative lactobacilli (Damiani et al., 1996; Gänzle, 2015). The production of mainly L-lactate (Scheirlinck et al., 2007) was reflected in the presence of three L-lactate dehydrogenase genes in the genome of this homofermentative LAB strain. Also, it has been shown before that the strain *Lb. crustorum* LMG 23699 is able to produce diacetyl and/or acetoin (Ravyts and De Vuyst, 2011; Ripari et al., 2016a). The presence of low concentrations of mannitol possibly reflected activities of background heterofermentative LAB. Similarly, some acetoin can be produced by yeasts, possibly explaining the presence of low concentrations of acetoin in the liquid wheat sourdough productions without citrate (Pétel et al., 2017).

The prevailing yeasts, *W. anomalus* (shown both culture-dependently and culture-independently) and *S. cerevisiae* (shown culture-dependently only), are common members of the sourdough microbiota (De Vuyst et al., 2016; Van Kerrebroeck et al., 2017). Opportunistic contaminants such as *Acetobacter indonesiensis* (detected culture-dependently) and *Staphylococcus xylosus* (detected culture-independently) were not unique findings. *Acetobacter* species often occur in the sourdough matrix (Lhomme et al., 2015; Li et al., 2016; Minervini et al., 2012; Ripari et al., 2016a; Zhang and He, 2013) and Staphylococcaceae are common inhabitants of flour (Minervini et al., 2015; Richter et al., 1993). However, these microorganisms never prevail during spontaneous sourdough fermentations (De Vuyst and Neysens, 2005; De Vuyst et al., 2017; Minervini et al., 2015).

Unfermented wheat flour-water mixtures could be clearly distinguished from the wheat sourdoughs based on aroma compounds such as aldehydes, alcohols, ketones, and organic acids. These compounds are formed during wheat sourdough fermentation by microbial activities, in particular by the added starter culture strain as well as the background LAB and yeasts, and their formation can go further during dough preparation and baking due to bakers' yeast activity and/or chemical reactions (Birch et al., 2014; Cho and Peterson, 2010; De Vuyst et al., 2017; Hansen and Schieberle, 2005; Pico et al., 2015). For instance, organic acid formation in the wheat sourdoughs was stimulated in the presence of *Lb. crustorum* LMG 23699 but ester formation was not. All this had an impact on the aroma profile of the breads produced from these wheat sourdoughs. For instance, ester production during sourdough fermentation is due to yeast activity, but yeasts were not always present. Moreover, the ester notes could have been masked by the LAB strain activities. Indeed, citrate supplementation enhanced buttery aroma compound formation during both wheat sourdough fermentations and bread productions. Further, the DY influenced aroma production of compounds such as pyrazines and furans during the baking process. Increased pyrazine concentrations could be the result of the presence of higher concentrations of diacetyl that is converted into pyrazines through Maillard reactions during the baking process under acidic conditions (Guerra and Yaylayan, 2012).

In conclusion, this study showed the competitiveness and hence prevalence of *Lb. crustorum* LMG 23699 as starter culture strain during both liquid and firm wheat sourdough productions, with and without the addition of citrate. Production of *Lb. crustorum* LMG 23699-initiated wheat sourdoughs with citrate increased the concentrations of lactic acid, acetoin, and diacetyl, which had an impact on the sourdough and bread aroma profiles. Hence, citrate supplementation could be of interest to produce baked goods with enhanced buttery aroma compounds and notes.

Acknowledgements

The authors gratefully acknowledge the technical advice of ing. Wim Borremans. This work was supported by the Research Council of the Vrije Universiteit Brussel (SRP7 and IOF342 projects), the Hercules Foundation (project UABR09004), and Flanders' FOOD (project Innocereal II). Andrea Comasio and Henning Harth were recipients of PhD fellowships granted by the Vrije Universiteit Brussel.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Aplevicz, K.S., Mazo, J.Z., dos Santos Neto, N.K., Nalevaiko, F.S., Sant, E.S., 2014. Evaluation of sourdoughs for the production of bread using spontaneous fermentation technique. Acta Sci-Technol. 36, 713–719.
Birch, A.N., Petersen, M.A., Arneborg, N., Hansen, Å.S., 2013a. Influence of commercial baker's yeasts on bread aroma profiles. Food Res. Int. 52, 160–166.
Birch, A.N., Petersen, M.A., Hansen, Å.S., 2013b. The aroma profile of wheat bread crumb influenced by yeast concentration and fermentation temperature. Food Sci. Technol. Res. 50, 480–488.
Birch, A.N., Petersen, M.A., Hansen, Å.S., 2014. Aroma of wheat bread crumb. Cereal Chem. 91, 105–114.
Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., Ehrlich, S.D., Sorokin, A., 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Res. 11, 731–753.
Camu, N., De Winter, T., Verbrugghe, K., Cleenwerck, I., Vandamme, P., Takrama, J.S., Vancanneyt, M., De Vuyst, L., 2007. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. Appl. Environ. Microbiol. 73, 1809–1824.
Cho, I.H., Peterson, D.G., 2010. Chemistry of bread aroma: a review. Food Sci. Biotechnol. 19, 575–582.
Damiani, P., Gobbetti, M., Cossignani, L., Corsetti, A., Simonetti, M.S., Rossi, J., 1996. The sourdough microflora. Characterization of hetero- and homofermentative lactic acid bacteria, yeasts and their interactions on the basis of the volatile compounds produced. Z. Lebensm. Wiss. Technol. 29, 63–70.
De Vuyst, L., Neysens, P., 2005. The sourdough microflora: biodiversity and metabolic interactions. Trends Food Sci. Technol. 16, 43–56.
De Vuyst, L., Vaningelgem, F., Ghijsels, V., Tsakalidou, E., Leroy, F., 2011. New insights into the citrate metabolism of *Enterococcus faecium* and its possible impact on the production of fermented dairy products. Int. Dairy J. 21, 580–585.
De Vuyst, L., Van Kerrebroeck, S., Harth, H., Huys, G., Daniel, H.M., Weckx, S., 2014. Microbial ecology of sourdough fermentations: diverse or uniform? Food Microbiol. 37, 11–29.
De Vuyst, L., Harth, H., Van Kerrebroeck, S., Leroy, F., 2016. Yeast diversity of sourdoughs and associated metabolic properties and functionalities. Int. J. Food Microbiol. 239, 26–34.
De Vuyst, L., Van Kerrebroeck, S., Leroy, F., 2017. Microbial ecology and process technology of sourdough fermentation. Adv. Appl. Microbiol. 100, 49–160.

Gänzle, M.G., 2014. Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiol. 37, 2–10.
Gänzle, M.G., 2015. Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. Curr. Opin. Food Sci. 2, 106–117.
Gänzle, M.G., Ripari, V., 2016. Composition and function of sourdough microbiota: from ecological theory to bread quality. Int. J. Food Microbiol. 239, 19–25.
Gänzle, M.G., Vermeulen, N., Vogel, R.F., 2007. Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiol. 24, 128–138.
Gänzle, M.G., Loponen, J., Gobbetti, M., 2008. Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality. Trends Food Sci. Technol. 19, 513–521.
Gänzle, M.G., Zhang, C., Monang, B.S., Lee, V., Schwab, C., 2009. Novel metabolites from cereal-associated lactobacilli - novel functionalities for cereal products? Food Microbiol. 26, 712–719.
Gobbetti, M., Corsetti, A., 1996. Co-metabolism of citrate and maltose by *Lactobacillus brevis* subsp. *lindneri* CB1 citrate-negative strain: effect on growth, end-products and sourdough fermentation. Z. Lebensm. Unters. Forsch. 203, 82–87.
Gobbetti, M., Corsetti, A., Rossi, J., 1995. Maltose-fructose co-fermentation by *Lactobacillus brevis* subsp. *lindneri* CB1 fructose-negative strain. Appl. Microbiol. Biotechnol. 42, 939–944.
Gobbetti, M., Rizzello, C.G., Di Cagno, R., De Angelis, M., 2014. How the sourdough may affect the functional features of leavened baked goods. Food Microbiol. 37, 30–40.
Gobbetti, M., Minervini, F., Pontonio, E., Di Cagno, R., De Angelis, M., 2016. Drivers for the establishment and composition of the sourdough lactic acid bacteria biota. Int. J. Food Microbiol. 239, 3–18.
Gordún, E., Del Valle, L.J., Ginovart, M., Carbó, R., 2015. Comparison of the microbial dynamics and biochemistry of laboratory sourdoughs prepared with grape, apple and yogurt. Food Sci. Technol. Int. 21, 428–439.
Guerra, P.V., Yaylayan, V.A., 2012. Double Schiff base adducts of 2,3-butanedione with glycine: formation of pyrazine rings with the participation of amino acid carbon atoms. J. Agric. Food Chem. 60, 11440–11445.
Hansen, A., Hansen, B., 1996. Flavour of sourdough wheat bread crumb. Z. Lebensm. Unters. Forsch. 202, 244–249.
Hansen, A., Schieberle, P., 2005. Generation of aroma compounds during sourdough fermentation: applied and fundamental aspects. Trends Food Sci. Technol. 16, 85–94.
Hansen, A., Lund, B., Lewis, M.J., 1989. Flavour of sourdough rye bread crumb. Lebensm. Wiss. Technol. 22, 141–144.
Harth, H., Van Kerrebroeck, S., De Vuyst, L., 2016. Community dynamics and metabolite target analysis of spontaneous, backslopped barley sourdough fermentations under laboratory and bakery conditions. Int. J. Food Microbiol. 228, 22–32.
Hugenholtz, J., 1993. Citrate metabolism in lactic acid bacteria. FEMS Microbiol. Rev. 12, 165–178.
Illeghems, K., De Vuyst, L., Weckx, S., 2015. Comparative genome analysis of the candidate functional starter culture strains *Lactobacillus fermentum* 222 and *Lactobacillus plantarum* 80 for controlled cocoa bean fermentation processes. BMC Genomics 16, 766.
Kang, T.S., Korber, D.R., Tanaka, T., 2013. Contributions of citrate in redox potential maintenance and ATP production: metabolic pathways and their regulation in *Lactobacillus panis* PM1. Appl. Microbiol. Biotechnol. 97, 8693–8703.
Khakimov, B., Jespersen, B.M., Engelsen, S.B., 2014. Comprehensive and comparative metabolomic profiling of wheat, barley, oat and rye using gas chromatography-mass spectrometry and advanced chemometrics. Foods 3, 569–585.
Laëtitia, G., Pascal, D., Yann, D., 2014. The citrate metabolism in homo- and heterofermentative LAB: a selective means of becoming dominant over other microorganisms in complex ecosystems. Food Nutr. Sci. 5, 953–969.
Lhomme, E., Lattanzi, A., Dousset, X., Minervini, F., De Angelis, M., Lacaze, G., Onno, B., Gobbetti, M., 2015. Lactic acid bacterium and yeast microbiotas of sixteen French traditional sourdoughs. Int. J. Food Microbiol. 215, 161–170.
Li, Z., Li, H., Bian, K., 2016. Microbiological characterization of traditional dough fermentation starter (jiaozi) for steamed bread making by culture-dependent and culture-independent methods. Int. J. Food Microbiol. 234, 9–14.
Minervini, F., Lattanzi, A., De Angelis, M., Di Cagno, R., Gobbetti, M., 2012. Influence of artisan bakery- or laboratory-propagated sourdoughs on the diversity of lactic acid bacterium and yeast microbiotas. Appl. Environ. Microbiol. 78, 5328–5340.
Minervini, F., De Angelis, M., Di Cagno, R., Gobbetti, M., 2014. Ecological parameters influencing microbial diversity and stability of traditional sourdough. Int. J. Food Microbiol. 171, 136–146.
Minervini, F., Lattanzi, A., De Angelis, M., Celano, G., Gobbetti, M., 2015. House microbiotas as sources of lactic acid bacteria and yeasts in traditional Italian sourdoughs. Food Microbiol. 52, 66–76.
Minervini, F., Celano, G., Lattanzi, A., De Angelis, M., Gobbetti, M., 2016. Added ingredients affect the microbiota and biochemical characteristics of durum wheat type-I sourdough. Food Microbiol. 60, 112–123.
Moens, F., Lefeber, T., De Vuyst, L., 2014. Oxidation of metabolites highlights the microbial interactions and role of *Acetobacter pasteurianus* during cocoa bean fermentation. Appl. Environ. Microbiol. 80, 1848–1857.
Nutter, J., Fritz, R., Saiz, A.I., Iurlina, M.O., 2017. Effect of honey supplementation on sourdough: lactic acid bacterial performance and gluten microstructure. Food Sci. Technol. 77, 119–125.
Palomba, S., Blaiotta, G., Ventorino, V., Saccone, A., Pepe, O., 2011. Microbial characterization of sourdough for sweet baked products in the Campania region (southern Italy) by a polyphasic approach. Ann. Microbiol. 61, 307–314.
Papalexandratou, Z., Lefeber, T., Bahrim, B., Lee, O., Daniel, H.M., De Vuyst, L., 2013. *Hanseniaspora opuntiae*, *Saccharomyces cerevisiae*, *Lactobacillus fermentum*, and *Acetobacter pasteurianus* predominate during well-performed Malaysian cocoa bean box fermentations, underlining the importance of these microbial species for a successful cocoa bean fermentation process. Food Microbiol. 35, 73–85.
Pétel, C., Onno, B., Prost, C., 2017. Sourdough volatile compounds and their contribution to bread: a review. Trends Food Sci. Technol. 59, 105–123.
Pico, J., Bernal, J., Gomez, M., 2015. Wheat bread aroma compounds in crumb and crust - a review. Food Res. Int. 75, 200–215.
Ravyts, F., De Vuyst, L., 2011. Prevalence and impact of single-strain starter cultures of lactic acid bacteria on metabolite formation in sourdough. Food Microbiol. 28, 1129–1139.
Richter, K.S., Dorneanu, E., Eskridge, K.M., Rao, C.S., 1993. Microbiological quality of flours. Cereal Foods World. 38, 367–369.
Ripari, V., Cecchi, T., Berardi, E., 2016a. Microbiological characterisation and volatiles profile of model, ex-novo, and traditional Italian white wheat sourdoughs. Food Chem. 205, 297–307.
Ripari, V., Gänzle, M.G., Berardi, E., 2016b. Evolution of sourdough microbiota in spontaneous sourdoughs started with different plant materials. Int. J. Food Microbiol. 232, 35–42.
Scheirlinck, I., Van der Meulen, R., Van Schoor, A., Huys, G., Vandamme, P., De Vuyst, L., Vancanneyt, M., 2007. *Lactobacillus crustorum* sp. nov., isolated from two traditional Belgian wheat sourdoughs. Int. J. Syst. Evol. Microbiol. 57, 1461–1467.
Spitaels, F., Wieme, A.D., Janssens, M., Aerts, M., Daniel, H.M., Van Landschoot, A., De Vuyst, L., Vandamme, P., 2014. The microbial diversity of traditional spontaneously fermented lambic beer. PLoS One 9, e95384.
Stefanovic, E., Fitzgerald, G., McAuliffe, O., 2017. Advances in the genomics and metabolomics of dairy lactobacilli: a review. Food Microbiol. 61, 33–49.
Stolz, P., Böcker, G., Hammes, W.P., Vogel, R.F., 1995a. Utilization of electron acceptors by lactobacilli isolated from sourdough. I. *Lactobacillus sanfrancisco*. Z. Lebensm. Unters. Forsch. 201, 91–96.
Stolz, P., Vogel, R.F., Hammes, W.P., 1995b. Utilization of electron acceptors by lactobacilli isolated from sourdough. II. *Lactobacillus pontis*, *L. reuteri*, *L. amylovorus* and *L. fermentum*. Z. Lebensm. Unters. Forsch. 201, 402–410.
Sun, Z., Harris, H.M.B., McCann, A., Guo, C., Argimon, S., Zhang, W., Yang, X., Jeffery, I.B., Cooney, J.C., Kagawa, T.F., Liu, W., Song, Y., Salvetti, E., Wrobel, A., Rasinkangas, P., Parkhill, J., Rea, M.C., O'Sullivan, O., Ritari, J., Douillard, F.P., Paul Ross, R., Yang, R., Briner, A.E., Felis, G.E., de Vos, W.M., Barrangou, R., Klaenhammer, T.R., Caufield, P.W., Cui, Y., Zhang, H., O'Toole, P.W., 2015. Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. Nat. Commun. 6, 8322.
Thiele, C., Gänzle, M.G., Vogel, R.F., 2002. Contribution of sourdough lactobacilli, yeast, and cereal enzymes to the generation of amino acids in dough relevant for bread flavor. Cereal Chem. 79, 45–51.
Van Kerrebroeck, S., Bastos, F.C., Harth, H., De Vuyst, L., 2016. A low pH does not determine the community dynamics of spontaneously developed backslopped liquid wheat sourdoughs but does influence their metabolite kinetics. Int. J. Food Microbiol. 239, 54–64.
Van Kerrebroeck, S., Maes, D., De Vuyst, L., 2017. Sourdoughs as a function of their species diversity and process conditions, a meta-analysis. Trends Food Sci. Technol. 68, 152–159.
Van Kerrebroeck, S., Comasio, A., Harth, H., De Vuyst, L., 2018. Impact of starter culture, ingredients, and flour type on sourdough bread volatiles as monitored by selected ion flow tube-mass spectrometry. Food Res. Int. 106, 254–262.
Vaningelgem, F., Ghijsels, V., Tsakalidou, E., De Vuyst, L., 2006. Cometabolism of citrate and glucose by *Enterococcus faecium* FAIR-E 198 in the absence of cellular growth. Appl. Environ. Microbiol. 72, 326–329.
Vrancken, G., Rimaux, T., De Vuyst, L., Leroy, F., 2008. Kinetic analysis of growth and sugar consumption by *Lactobacillus fermentum* IMDO 130101 reveals adaptation to the acidic sourdough ecosystem. Int. J. Food Microbiol. 128, 58–66.
de Winter, J.C.F., 2013. Using the Student's *t*-test with extremely small sample sizes. Pract. Assess. Res. Eval. 18, 1–12.
Wouters, D., Bernaert, N., Conjaerts, W., Van Droogenbroeck, B., De Loose, M., De Vuyst, L., 2013. Species diversity, community dynamics, and metabolite kinetics of spontaneous leek fermentations. Food Microbiol. 33, 185–196.
Yi, L., Guo, X., Liu, L., Shao, C., Lü, X., 2017. First report on the complete genome sequence of *Lactobacillus crustorum* MN047, a potent probiotic strain isolated from koumiss in China. Genome Announc. 5, e00048-17.
Yu, Y., Wang, L., Qian, H., Zhang, H., Qi, X., 2018. Contribution of spontaneously-fermented sourdoughs with pear and navel orange for the bread-making. Food Sci. Technol. 89, 336–343.
Zaunmüller, T., Eichert, M., Richter, H., Unden, G., 2006. Variations in the energy metabolism of biotechnologically relevant heterofermentative lactic acid bacteria during growth on sugars and organic acids. Appl. Microbiol. Biotechnol. 72, 421–429.
Zhang, G., He, G., 2013. Predominant bacteria diversity in Chinese traditional sourdough. J. Food Sci. 78, 1218–1223.
Zhang, J., Liu, W., Sun, Z., Bao, Q., Wang, F., Yu, J., Chen, W., Zhang, H., 2011. Diversity of lactic acid bacteria and yeasts in traditional sourdoughs collected from western region in Inner Mongolia of China. Food Control 22, 767–774.
Zhao, C.J., Kinner, M., Wismer, W., Gänzle, M.G., 2015. Effect of glutamate accumulation during sourdough fermentation with *Lactobacillus reuteri* on the taste of bread and sodium-reduced bread. Cereal Chem. 92, 224–230.