Performance of *Leuconostoc citreum* FDR241 during wheat flour sourdough type I propagation and transcriptional analysis of exopolysaccharides biosynthesis genes

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ABSTRACT

This study focused on the performance of the dextran producer *Leuconostoc citreum* as starter culture during 30 days of wheat flour type I sourdough propagation (back-slopping). As confirmed by RAPD-PCR analysis, the strain dominated throughout the propagation procedure, consisting of daily fermentations at 20 °C. The sourdoughs were characterized by consistent lactic acid bacteria cell density and acidification parameters, reaching pH values of 4.0 and mild titratable acidity. Carbohydrates consumption remained consistent during the propagation procedure, leading to formation of mannitol and almost equimolar amount of lactic and acetic acid. The addition of sucrose enabled the formation of dextran, inducing an increase in viscosity of the sourdough of 2–2.6 fold, as well as oligosaccharides. The transcriptional analysis based on glucosyltransferases genes (GH70) showed the existence in *L. citreum* FDR241 of at least five different dextransucrases. Among these, only one gene, previously identified as forming only α-(1–6) glycosidic bonds, was significantly upregulated in sourdough fermentation conditions, and the main responsible of dextran formation. A successful application of a starter culture during long sourdough back-slopping procedure will depend on the strain robustness and fermentation conditions. Transcriptional regulation of EPS-synthetizing genes might contribute to increase the efficiency of industrial processes.

1. Introduction

The positive influence of sourdough on bread quality has been the subject of many studies, focusing on the complex reactions occurring during microbial metabolism of lactic acid bacteria and yeasts, and their interactions with the endogenous enzymatic activity of the raw material (De Vuyst and Neysens, 2005; Gänzle, 2014). In addition to improved aroma, storability and general enhancement of nutritional properties of bread, sourdough can also ameliorate baked goods structure, particularly when exopolysaccharides (EPS) synthetized by lactic acid bacteria are present. EPS are long-chain carbohydrates polymers widely employed in food industry, particularly in fermented dairy food, thanks to their positive effects on texture and sensory attributes on the final product (Ruas-Madiedo et al., 2002). In most recent years, the use of EPS has been explored also for baked goods manufacturing with the aim of enhancing the quality of the final products. Essentially, due to their hydrocolloid activity, EPS deeply affect dough textural properties, influencing several attributes of baked goods, including improved structure and volume, increased softness and delayed staling (Lynch et al., 2017). In this respect, the synthesis of EPS *in situ* during sourdough fermentation can substitute additives of plant origin such as xanthan for bread manufacturing. Compared to commercial hydrocolloids which need to be declared on the product label and might not meet the consumers' request for additive-free food, EPS synthetized in the sourdough do not require labelling and constitute a clear advantage for food producers (Galle and Arendt, 2014;

Table 1
Sourdough production and propagation procedure.

Samples	Wheat flour (g)	Water (g)	Sucrose (g)	Sourdough (g)	Total weight (g)	Dough yield	Temperature	Time
Sourdough fermentation (T1)	127.4	198.4	14.2 (10% on dry weight)	–	340	240	20 °C	24 h
Sourdough propagation (T2-T30)[a]	70	105	5 (5% on dry weight)	60	240	240	20 °C	8 h

[a] Propagation procedure: 25% w/w of sourdough previously fermented, was added to the ingredients reported, keeping a constant dough yield (dough weight/ flour + sucrose weight x100) of 240, and fermented in the above conditions.

Waldherr and Vogel, 2009). This so-called "clean label" approach is an increasing trend among consumers and should be taken into account by food manufacturers when developing new products (Asioli et al., 2017). Many studies have reported potential health effects of EPS, based on different host interactions such as immunomodulation and cell adhesion, and prebiotic effect (as reviewed by Caggianiello et al., 2016). The latter is particularly relevant for baked goods as it might contribute to increase their health benefits. As recently reviewed by Lynch et al. (2017), in cereal and bakery application, the major part of EPS studied/ applied are homopolysaccharides of the dextran or levan type, consisting of repeated units of a single monosaccharide, i.e. D-glucose or D-fructose, respectively. The synthesis of the above microbial EPS takes place through the activity of α-transglycosylases, belonging to the family 70 of the glycoside hydrolases (GH70), using sucrose as glycosyl donor. Dextrans are α-glucans with a linear backbone made of α-(1–6)-linked D-glucopyranosyl units, in which the amount of α-(1–6) linkages can vary depending on environmental conditions and microbial strain (Jeanes et al., 1954; Kim et al., 2003).

Among the different artisan and industrial processes for sourdough preparation, type I sourdough, corresponding to traditional method, is one of the most used. Sourdough type I is achieved through daily propagation (back-slopping) to keep the microorganisms in a metabolically active state (Siragusa et al., 2009). The procedure consist of mixing the mother sponge from the preceding fermentation with flour and water and allowing it to ferment again at a certain temperature for a specific time (De Vuyst and Neysens, 2005). However, in industrial conditions the use of starter cultures to initiate the fermentation at periodic intervals, e.g. weekly or monthly, followed by daily propagation is considered a better solution to achieve consistent results (De Vuyst and Vancanneyt, 2007; Siragusa et al., 2009). The starter should possess high adaptation capacity to dominate during back-slopping process, and its metabolism must rapidly adapt to the sourdough environmental conditions to dominate the spontaneous microflora during type I propagation (Corsetti et al., 2007; Minervini et al., 2010; Siragusa et al., 2009; Vogelmann et al., 2009). Therefore, selection based on pro-technological properties and robustness of the starters is fundamental to guarantee their persistence throughout the propagation procedure (De Vuyst and Neysens, 2005; Minervini et al., 2010). The main aim of this study is to assess the performance of *Leuconostoc citreum* FDR241, a dextran producing strain isolated from bakery, as starter culture during 30 days of back-slopping procedure. *Leuconostoc* spp. are typical members of the microbial community of plants (Björkroth and Holzapfel, 2006) and have been frequently isolated from sourdough environment (Corsetti et al., 2001; Gabriel et al., 1999; Vrancken et al., 2011). The variety of glucansucrases and polymers produced by leuconostocs has been previously explored, showing a very high biodiversity (Amari et al., 2015; Bounaix et al., 2010). For instance, different strains of *L. citreum* originating from sourdough synthetized dextrans with different amount of α-(1–6), α-(1–3), and α-(1–2) linkages as well as mutan and fructans (Amari et al., 2015). In spite of the several reports available on the presence of *Leuconostoc* spp. in sourdough and on its glycosyltransferases properties, to the best of our knowledge, there is no report on the use of *L. citreum* as starter for propagation of wheat flour sourdough type I.

Studies on the dextransucrase activity of *Leuconostoc* spp. have been performed so far only in synthetic substrates in the presence of different carbon sources. In this study, the main characteristics of the sourdough in terms of acidification, carbohydrates metabolism and dextran synthesis were monitored throughout the propagation time, and the dominance of the starter was described. In addition, the transcription profiles of selected glycosyltransferases genes during sourdough fermentation and propagation was analyzed to identify the candidate enzymes responsible for the dextran production.

2. Materials and methods

2.1. Microorganism and growth conditions

Leuconostoc citreum FDR241 previously isolated from rye bran samples stored in a bakery environment (data not shown), and deposited in the VTT internal strain collection (under the code B8723), was used as starter for fermentation. The strain was routinely cultivated on MRS broth (de Man-Rogosa-Sharpe, Oxoid LTD, Basingstoke, UK) at 30 °C for 24 h. After the exponential phase of growth was reached, cells were recovered by centrifugation, washed with 50 mM phosphate buffer, pH 7.0 and re-suspended in an aliquot of the tap water used for sourdough preparation as described below.

2.2. Sourdough propagation

Common wheat flour (total fat, 2.1%; carbohydrates, 70%; sugars, 0.5%; protein, 12.5%), was provided by Helsingin Mylly Oy, Finland. Sourdough production and propagation was based on the protocol reported in Table 1. Wheat flour (127.4 g), tap water (198.4 g), sucrose (14.2 g) and cell suspension containing about 9.0 log cfu/ml of the above mentioned starter (final cell number in the dough of 6.0 log cfu/g per g), were used to prepare 340 g of dough (T0). The dough was fermented at 20 °C for 24 h (T1). Successively, sourdoughs were propagated according to a back-slopping protocol. In detail, the sourdough was used at inoculum rate of 25% w/w to ferment a mixture of flour (70 g) sucrose (5 g) and tap water (105 g) producing a dough with final weight of 240 g. Fermentation was carried out at 20 °C for 8 h. Sourdoughs were stored at 4 °C for about 16 h between back-slopping steps. Sourdough propagation was carried out for 30 days. All the sourdoughs were produced with the same dough yield (dough weight/flour + sucrose weight x100) of 240. Samples before fermentation (T0) after the first fermentation step (T1), and at intervals during back-slopping procedure (T2, T5, T10, T15, T20, T30) after 8 h of fermentation, were withdrawn for further analyses as described below. Sourdoughs were produced in triplicate.

2.3. Microbial counts and lactic acid bacteria isolation

Ten grams of sourdough were homogenized with 90 ml of sterile sodium chloride (0.9%, w/v) solution using a Stomacher. For the determination of presumptive lactic acid bacteria, serial dilutions were plated on MRS agar (LabM, United Kingdom) supplemented with 0.01% cycloheximide. After incubation at 30 °C for 48 h, 20–25 colonies were

randomly taken from plates containing the two highest sample dilutions from the first (T2) and intermediate (T5-T15) back-slopped sourdoughs, and final sourdough (T30). Gram-positive, catalase negative, non-motile isolates were cultivated in MRS broth at 30 °C for 24 h and re-streaked into MRS agar. Isolates able to acidify the culture medium after incubation at 30 °C for 24 h were considered for further analyses. Stock cultures were stored at −80 °C in 10% (v/v) glycerol. In addition, the last dilutions were plated on MRS supplemented with 2% sucrose to visually assess EPS production of the lactic acid bacteria. The plates were incubated for 48–72 h at 30 °C. For yeast counting, Yeast Agar medium (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l dextrose) supplemented with 0.01% chloramphenicol was used. The plates were incubated for 72 h at 25 °C. Isolation procedure and analyses were carried out on duplicate sourdoughs.

2.4. DNA extraction and genotypic characterization of lactic acid bacteria isolates

Genomic DNA was extracted using a DNeasy® Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's instructions, and with the addition of lysozyme (80 mg/ml, Sigma Aldrich, Canada) to the lysis buffer solution. The obtained pure genomic DNA of isolates was stored at −20 °C for RAPD-(Random Amplification of Polymorphic DNA)-PCR analyses and 16SrRNA gene sequencing. Three oligonucleotides, P1 50-GCGGCGTCGCTAATACATGC-30, P4 50-CCGCAGC GTT-30, and M13 50-GAGGGTGGCGGTTCT-30 were used for isolates biotyping. The reaction mixture and PCR conditions were as described by Coda et al. (2006). The molecular sizes of the amplified DNA fragments were estimated by comparison with the 1-kb DNA molecular size markers (Invitrogen Life Technologies). RAPD-PCR profiles were acquired by Gel Doc EQ System (Bio-Rad, Hercules, CA). The similarity of the electrophoretic profiles was evaluated by determining the Dice coefficients of similarity and by using the unweighted pair group method with average linkage (UPGMA). The presence or absence of fragments was recorded as 1 or 0, respectively. Only reproducible well-marked amplified fragments were scored, faint bands being ignored. The reproducibility of the RAPD fingerprints was assessed by comparing the PCR products obtained with primers P1, P4, and M13 and DNA prepared from separate cultures of the same strain. To confirm their identity, selected isolates were subjected to 16SrRNA gene fragment amplification, using the primers LacbF/LacbR as described in De Angelis et al. (2006). The identification queries were performed by a BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

2.5. RNA extraction and real time qPCR

Sourdough samples at selected time points (T0, T1, T2, T5, T30) were used for this analysis. Ten grams of each sourdough sample were mixed with 90 ml of potassium phosphate buffer (50 mM; pH 7.0), and homogenized for 5 min at room temperature. Afterwards, homogenates were centrifuged (1000 × g, 5 min, 4 °C) and the supernatants were recovered and centrifuged again (5000 × g, 15 min, 4 °C). Total RNA from collected cells was extracted using the RiboPure™ RNA Purification Kit, bacteria (Ambion RNA, Life Technologies Co., Carlsbad, CA, USA), according to the manufacturer's instructions. The quality of RNA was monitored through agarose gel electrophoresis. The RNA concentration was measured in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). The cDNA was obtained by the First Strand cDNA Synthesis Kit for qPCR (Roche) according to the manufacturer's instructions.

The genomic DNA and cDNA was analysed by qPCR in a LightCycler 480 Instrument II (Roche) and the analysis was performed with the accompanying software (Advance Relative Quantification tool). The qPCR primer pairs were designed with the PerlPrimer v1.1.21 software, based on the sequences retrieved from draft genomes of three *L. citreum* strains, LBAE E16, LBAE C10, and LBAE C11 (Amari et al., 2015;

Laguerre et al., 2012; Passerini et al., 2015). The primers used for the analysis are listed in Table 5. The qPCR analysis was performed in quadruplicates, and the signals were normalized for reference gene (*recA*) expression in each time point. The reactions were assembled with the LightCycler480 SYBR Green I Master (Roche) according to manufacturer's instructions. Sequence alignments of the partial sequences of dextransucrase genes A (*dsrA*), B, (*dsrB*), E (*dsrE*), M (*dsrM*), and branchingsucrase A (*brsA*) were obtained with the Clustal Omega tool at the EMBL-EBI website (https://www.ebi.ac.uk/Tools/msa/clustalo/) and visualized with the BoxShade tool at the ExPASy website (https://embnet.vital-it.ch/software/BOX_form.html). Alignments are reported in 2.5S and Figs. 2S–6S.

2.6. Determination of pH, titratable acidity and organic acids

The pH values were measured before and after fermentation, with a pH meter (Knick, Portamess 752, Germany). The titratable acidity (TTA) was measured on 10 g of dough diluted with 90 ml of Milli-Q water with a manual Titrator Mettler Toledo DL53 (Switzerland) with 0.1 mol/l NaOH until pH reached 8.5. TTA was expressed as the total NaOH amount (ml).

Organic acid analysis was performed using a Aminex HPX-87H column (ion exclusion, Biorad) as described in Zeppa et al. (2001). Results are expressed as % on flour basis.

2.7. Viscosity measurements

Viscosity of sourdoughs was measured immediately after fermentation at 20 °C. Before viscosity measurement, doughs were mixed thoroughly, and 60 g of each was taken. Viscosity as a function of shear rate was performed with a rotational rheometer (Rheolab QC, Anton Paar GmbH, Vienna, Austria) under different shear rates, from 2 to 100 1/s (up and down sweeps). In order to evaluate the specific viscosity changes of the doughs after fermentation, the viscosity values at the shear rate of 100 1/s were compared.

2.8. Sugars and mannitol analysis

Mono-, di-, and oligosaccharides and mannitol were analyzed at selected times to follow the carbohydrate metabolism during sourdough propagation. For this analysis, 100 mg of freeze-dried sourdough samples were mixed with 5.0 ml of Milli-Q water and vortexed for 5 min to allow the complete dissolution of free sugars and mannitol. Then, the suspensions were boiled for 5 min to inactivate enzymes and microbes. After cooling, samples (400 μl) were filtered using Amicon Ultra-0.5 centrifugal filter units (Millipore, Billerica, MA) at 12,000 × g for 10 min to remove polymeric molecules (above 10 kDa). Before further analysis, samples were diluted with Milli-Q water (Katina et al., 2009). Sucrose, glucose, fructose and maltose were analyzed by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) system using a CarboPac PA1 column (250 × 4 mm i. d., Dionex, Sunnyvale, CA), as previously described by Xu et al. (2017). Results are expressed as % on flour basis. Oligosaccharides were analyzed by HPAEC-PAD system, equipped with a CarboPac PA-100 column (250 × 4 mm, i. d., Dionex), a DECADE detector (Antec Leyden, The Netherlands), a Waters 717 autosampler, and two Waters 515 pumps, as described by Rantanen et al. (2007). Mannitol was quantified by the HPAEC-PAD system equipped with a CarboPac MA-1 analytical column (250 × 4 mm i. d., Dionex), a DECADE detector, a Waters 717 autosampler and two Waters 515 pumps according to Xu et al. (2017). Mannitol (Sigma-Aldrich) was used as the standard for quantification and the results are expressed as % on flour basis.

2.9. Dextran analysis

The amount of dextran was analyzed by an enzyme assisted method using a mixture of dextranase (Sigma-Aldrich) and α-glucosidase (Megazyme, Ireland) as previously described by Katina et al. (2009). After inactivation of the enzymes and centrifugation, glucose in the sourdough supernatants was analyzed by HPAEC-PAD as described above.

2.10. Statistical analysis

The numerical results of all the analyses in this study are averages of three different sourdoughs for each time point, when not otherwise stated. Data were analyzed by one-way analysis of variance (ANOVA) using Origin 8.6 (OriginLab Inc.). Means comparison was determined by Tukey's test ($P < 0.05$).

3. Results

3.1. Microbial counts and lactic acid bacteria profiling

Before fermentation (T0), the cell density of presumptive lactic acid bacteria was 6.04 $\pm$ 0.05 log cfu/g. After 24 h (T1), the highest cell number among the sourdoughs 9.16 $\pm$ 0.04 log cfu/g was reached. From this time onward, the cell density presented similar values, ranging from 8.8 $\pm$ 0.05 to log 9.07 $\pm$ 0.1 log cfu/g. Additionally, slime formation was observed for the dilution plated on MRS + sucrose. The yeast cell density was below 2.0 log cfu/g throughout all the back-slopping procedure. In order to confirm the presence of the starter, 170 isolates of presumptive lactic acid bacteria isolated from T2, T5, T15 and T30 were subjected to RAPD-PCR analysis using the single primers M13, P1 and P4. The use of primers M13 and P4 gave distinct profiles in the conditions here applied, while only one band was observed for P7 for all the isolates. All the isolates analyzed showed the same RAPD patterns of the starter *L. citreum* FDR241, with the exception of 6 isolates retrieved at T2 and T5, having identical RAPD pattern, and further identified as *Weissella cibaria/confusa*. The attribution of 10 representative isolates from the time points considered to be *L. citreum* FDR241 was further confirmed with 16SrRNA gene sequencing. A representative image of the RAPD patterns obtained is shown in Fig. 1S.

3.2. Acidification during sourdough propagation

The acidification process of the sourdoughs was consistent throughout propagation time (Fig. 1). At T0, the initial pH value was 6.1 and the highest drop of 2.2 units was observed after 24 h of fermentation, corresponding to final pH value of 3.9. From this time onward, the pH values showed none or very little variations, in the range of 3.9–4.0 units. Additionally, the daily pH drop after 8 h of fermentation was measured, corresponding to 0.8–0.9 pH units. In agreement with pH values, the lowest TTA was recorded for T0, 1.6 ml of NaOH 1M, while the highest value 9.2 ml of NaOH 1M was found for T1. With the exception of T2, for which a significantly different TTA value 8.20 ml of NaOH 1M was measured, from T5 until T30, TTA values remained stable in the range 7.3–7.6 ml of NaOH 1M, showing no significant differences. In agreement with the pH and TTA measurements, the concentration of lactic and acetic acid was the highest at T1, reaching values of 3.380 and 1.658%, respectively (Table 2). The amount of organic acids significantly decreased at T2 and showed similar values from T5 onward, in the range of 1.851–1928 and 0.898–0.910% for lactic and acetic acid, respectively. The fermentative quotient, the molar ratio between lactic acid and acetic acid, was approximately 1.2–1.7 throughout the propagation time.

3.3. Sugar consumption and mannitol formation

Sucrose, glucose, and maltose were found at T0, and sucrose concentration corresponded to the amount added before fermentation (Table 3). After the first 24 h at 20 °C, sucrose was not detected any more in any of the samples while fructose was retrieved as consequence of microbial utilization and endogenous enzymatic activity of the flours. At T1, glucose content did not show any significant difference from T0, while maltose accumulated up to 3.70%, reaching its highest concentration among the samples. After the first propagation step (T2), glucose content did not show any significant variation while a slight decrease in fructose and maltose content was observed. From T5 to T30 glucose, fructose and maltose content remained stable at approximately 0.3, 1.3 and 3.2%, respectively. Considering the sucrose addition (10% on flour basis), the consequent theoretical 5% of fructose liberation (corresponding to 278 mmol/kg) and its use as external electron acceptor, the amount of mannitol at T1, 3.88%, or 215 mmol/kg, was quite in line with the expected amount. The same trend was observed for all the time points, indicating a correlation between the amount of mannitol and the theoretical amount of fructose deriving from sucrose addition and back-slopping.

3.4. Viscosity measurements, dextran and oligosaccharides formation

Changes in dough texture were evaluated by viscosity analysis. In order to assess the specific modifications after fermentation and during propagation, viscosity values at the shear rate of 100 1/s were compared. All the sourdoughs had a typical shear thinning and thixotropic behavior. As expected, the addition of sucrose induced viscosity increase, which overall varied during the propagation time within the range 0.70–2.19 Pa s (Table 4). The lowest value was found at T0, and it increased approximately 3 folds after the first fermentation step (T1). After this, the viscosity values varied from 1.36 to 2.19 Pa s during the propagation step (T2-T30), showing the highest value in T2. The dextran content in the sourdough ranged from 0.35 to 0.71% and did not show any significant variation throughout the propagation time. Generally, the amount of dextran in the sourdoughs was significantly less than it could be theoretically produced from the addition of 10% sucrose, i.e. 5% after the first fermentation, or at the subsequent propagation steps when 5% sucrose was added and potentially 2.5% of dextran could be formed. Oligosaccharides formation from sucrose, potentially lowering the yield of dextran, was assessed and their profile is reported in Fig. 2. Before fermentation the high peak of sucrose and low peaks of glucose and maltose are visible. After the first 24 h of fermentation, sucrose is not detected anymore while oligosaccharides are visible. A similar profile was obtained for all the sourdoughs. However, due to a lack of pure standard compounds, no quantification of oligosaccharides was possible.

3.5. L. citreum FDR241 transcription analysis

To identify genes encoding dextransucrases in the strain *L. citreum* FDR241 used in this study, the presence of several candidate genes previously identified/annotated in genomes of *L. citreum* strains isolated from sourdough was verified (Table 5) (Laguerre et al., 2012). Primers were designed to amplify two separate regions of each candidate gene and, together with primers for the *recA* gene, used in qPCR reactions where the genomic DNA of *L. citreum* FDR241 served as a template (Table 5). Five putative dextransucrase genes were identified in the genome, providing ~1:1 qPCR signal ratio towards the *recA* (data not shown), and selected for transcription analysis. Overall, based on the similarity of the genes identified in *L. citreum* FDR241 a very closed relation with the previously identified sourdough isolates was observed (Figs. 2S–6S); however, depending on the gene/strain, different level of similarity were found. In addition, the presence of all the six *L. citreum* FDR241-specific gene transcripts, as determined by the
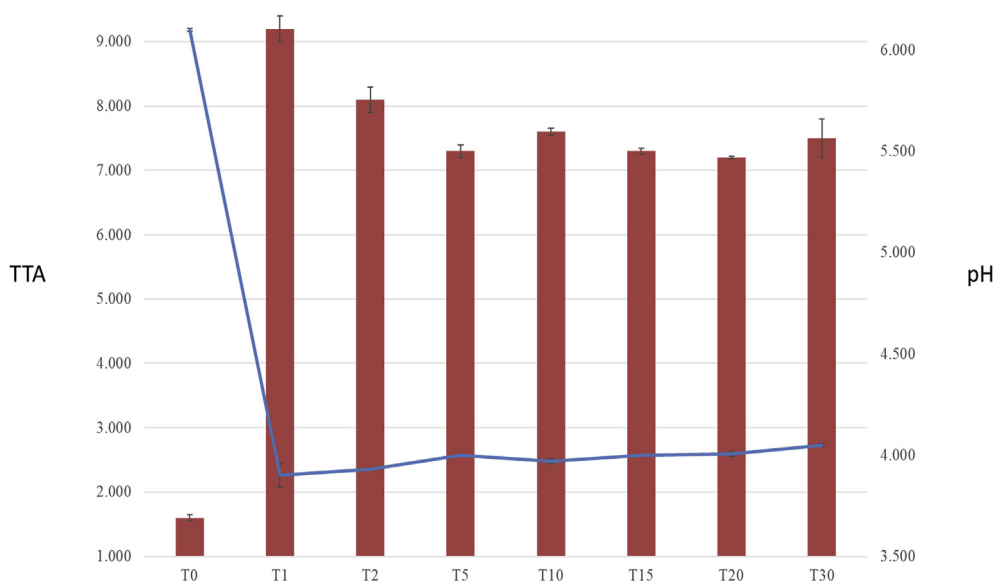
Fig. 1. Values of pH units (line) and TTA (bars) expressed as ml of NaOH 1M, in sourdoughs before fermentation (T0) and during propagation procedure (T1-T30).

Table 2
Lactic and acetic acid concentration (expressed as % on flour basis) in selected sourdough samples before fermentation (T0) and during propagation procedure (T1-T30).

Sample	Lactic acid (%)	Acetic acid (%)
T0	0.141 ± 0.008^a	0.078 ± 0.008^a
T1	3.380 ± 0.161^b	1.658 ± 0.104^b
T2	1.958 ± 0.128^c	1.060 ± 0.054^c
T5	1.851 ± 0.086^c	0.898 ± 0.059^d
T10	1.908 ± 0.079^c	0.910 ± 0.072^d
T15	1.928 ± 0.123^c	0.899 ± 0.043^d
T20	1.843 ± 0.074^b	0.905 ± 0.040^d
T30	1.863 ± 0.117^b	0.900 ± 0.048^d

[a-d] Values in the same column with different letters are significantly different ($p < 0.05$).

transcription analysis of the total mRNA samples, indicated consistently high abundance of the starter throughout the 30 days of propagation.

The transcription analysis (Fig. 3) revealed rather dissimilar expression pattern of the identified genes during the 30 days of backslopping. While the *dsrA* gene, encoding the dextransucrase responsible for synthesis of α-(1–6) and α-(1–3) linkages, seemed to retain low and constitutive expression over the whole course of the experiment, the *dsrE* gene appeared to be down-regulated in the initial phase of the cultivation, showing the highest expression in the MRS inoculum and significantly decreasing in sourdough fermentation conditions. Analogous pattern was detected for the *bsrA* gene, encoding an enzyme responsible for α-(1–2) branching. Its initial downregulation was,

Table 4
Viscosity values and dextran content of sourdough samples before fermentation (T0) and during propagation procedure (T1-T30).

Sample	Viscosity (Pa s) [A]	Dextran (%) [B]
T0	0.70 ± 0.15^a	–
T1	$1.92 \pm 0.15^{b, d, e}$	0.71 ± 0.09^a
T2	2.19 ± 0.12^b	0.63 ± 0.19^a
T5	$1.36 \pm 0.04^{c, d}$	0.46 ± 0.22^a
T10	$1.88 \pm 0.11^{b, d, e}$	0.56 ± 0.08^a
T15	$1.70 \pm 0.04^{d, e}$	0.39 ± 0.11^a
T20	1.72 ± 0.06^e	0.38 ± 0.11^a
T30	$1.60 \pm 0.01^{c, d, e}$	0.35 ± 0.11^a

[A] Viscosity values were taken at the shear rate of 100 1/s. [B] Dextran content was based on dry weight. [a-e] Values in the same column with different letters are significantly different ($p < 0.05$).

however, less pronounced than in the case of *dsrE*, and the expression somewhat increased in later time points. The highest induction was observed for the *dsrB* gene, showing the minimal expression level upon inoculation and reaching the maximum expression during the first 24 h of fermentation, after which the expression stabilized and remained constant for all the time points tested. A similar trend was observed for the *dsrM* gene, even though the expression level was below that of the *dsrB*. The expression pattern of the *dsrB* gene indicated that the dextransucrase encoded by this gene is likely to be responsible for majority of dextran produced in the conditions of this study, and the results allow to predict the production of dextran mainly characterized by α-
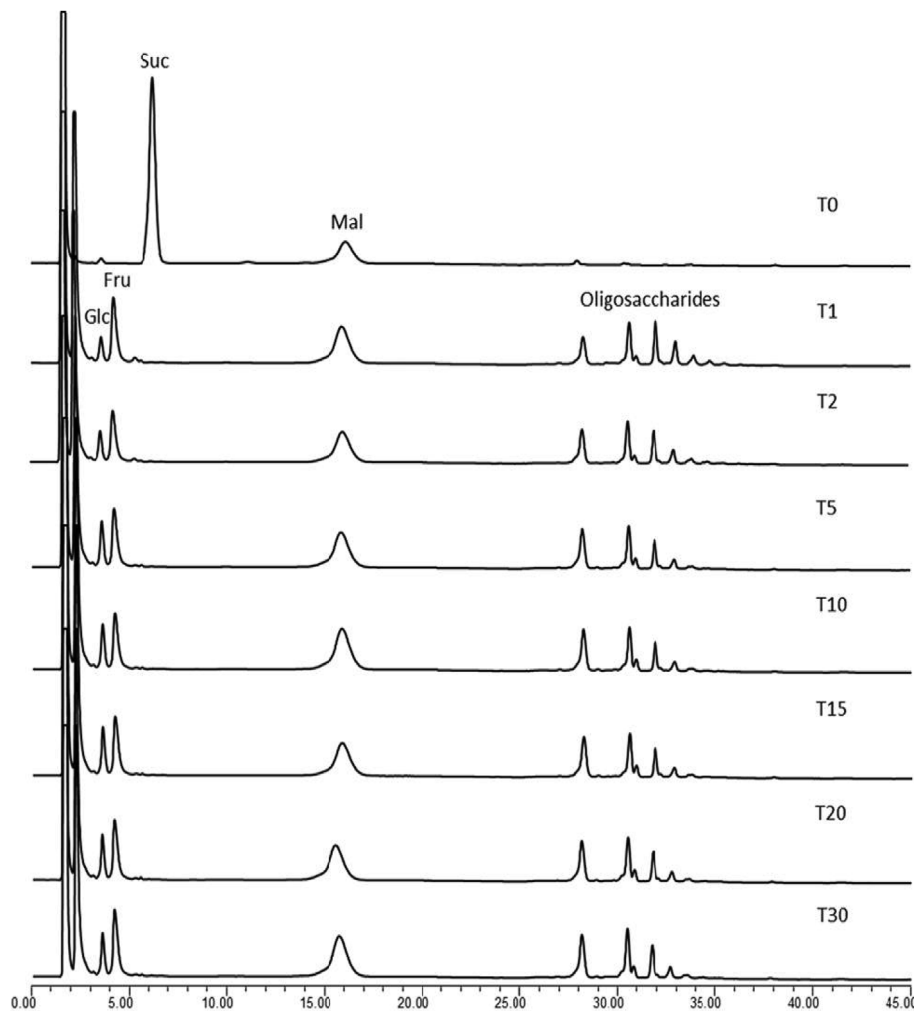
Table 3
Sucrose, glucose, fructose, maltose and mannitol concentration (expressed as % on flour basis) in sourdough samples before fermentation (T0) and during propagation procedure (T1-T30).

Sample	sucrose	glucose	fructose	maltose	mannitol
T0	10.58 ± 0.98	0.16 ± 0.02^a	nd	2.24 ± 0.26^a	–
T1	nd	$0.18 \pm 0.04^{a, c}$	1.73 ± 0.24^a	3.70 ± 0.46^b	3.88 ± 0.04^a
T2	nd	0.13 ± 0.02^a	1.16 ± 0.19^b	$2.79 \pm 0.61^{a, b}$	3.27 ± 0.12^b
T5	nd	$0.36 \pm 0.02^{b, c}$	$1.33 \pm 0.11^{a, b}$	$3.24 \pm 0.18^{a, b}$	2.58 ± 0.02^c
T10	nd	$0.35 \pm 0.03^{b, c}$	$1.24 \pm 0.11^{a, b}$	3.57 ± 0.32^b	2.64 ± 0.08^c
T15	nd	$0.35 \pm 0.01^{b, c}$	$1.39 \pm 0.01^{a, b}$	3.59 ± 0.41^b	2.57 ± 0.04^c
T20	nd	$0.30 \pm 0.06^{b, c}$	$1.24 \pm 0.29^{a, b}$	$2.92 \pm 0.43^{a, b}$	2.63 ± 0.04^c
T30	nd	0.27 ± 0.04^c	$1.28 \pm 0.21^{a, b}$	$3.17 \pm 0.40^{a, b}$	2.61 ± 0.12^c

[A] Sugars were all calculated on dry weight base. [a-c] Values in the same column with different letters are significantly different ($p < 0.05$).

Fig. 2. Mono- and oligo-saccharides profile of sourdoughs before fermentation (T0) and during propagation procedure (T1-T30). Glc, glucose; Fru, fructose; Suc, sucrose; Mal, maltose.

(1–6) linkages, with the presence of α-(1–2) branching.

4. Discussion

The effective implementation of sourdough in industrial conditions requires the use of efficient starter cultures able to dominate the environment and to perform consistently (Minervini et al., 2010). In this study, *L. citreum* robustness and consistent performance throughout 30 days of sourdough propagation were demonstrated. Previously, a few studies have focused on the dominance of specific lactic acid bacteria strains during 10 days of sourdough type I propagation, reporting that not all the selected strains of *Lactobacillus plantarum* and *Lactobacillus sanfranciscensis* used as starters were able to persist in all the cases (Minervini et al., 2010; Siragusa et al., 2009).

In the conditions applied in this study, since the first day of propagation, the lactic acid bacteria cell number reached values typical of mature sourdoughs, remaining consistent for all the 30 days (Gobbetti, 1998; Vrancken et al., 2011). No yeasts were found in any of the sourdoughs at any time, in analogy with what observed when using an EPS producing strain of *Weissella cibaria* as starter for sourdough (Di Cagno et al., 2006). The absence of yeasts might depend on several factors, including the fermentation temperature of 20 °C, significantly lower than the growth range of 25–27 °C reported as optimal for many yeast species; the relatively short fermentation time, unfavorable to yeasts growth, as well as possible competition for carbohydrates (De Vuyst et al., 2009, 2016). The results of our culture dependent approach

and RAPD-PCR analyses confirmed the high abundance of *L. citreum* FDR241 throughout the propagation time, and allowed the occasional retrieval of *Weissella cibaria/confusa*. However, the presence of other lactic acid bacteria species and strains cannot be ruled out, since flour is a recognized source of competitive lactic acid bacteria, and the use of different substrates might have aided the retrieval of higher biodiversity (Corsetti et al., 2001; De Vuyst and Vancanneyt, 2007).

Nonetheless, fermentation time and temperature can also strongly influence the microbial dynamics of sourdough (Vrancken et al., 2011). In spontaneous sourdough fermentation carried out at 23 °C, *L. citreum* was able to dominate the environment confirming the low-temperature adaptation of this specie (Vrancken et al., 2011; Yim et al., 2008). In addition to favoring the dominance of the starter, the relatively low temperature of 20 °C for sourdough fermentation was selected to avoid excessive acidification. The fermentation and propagation protocol here applied allowed to maintain a pH close to 4.0 during all the procedure. Sourdough with too high acidity is not generally recommended because of its repercussions on bread attributes and overall negative effect on baked goods quality, thus counteracting the positive influence of EPS (Kaditzky and Vogel, 2008; Katina et al., 2009). Indeed, despite the low pH value of the sourdough, and with the exception of the first fermentation, the TTA was of about 7.2–7.9 from the second until the last back-slopping. These data, indicating a mild acidification, are similar or lower than other wheat flour sourdoughs containing EPS fermented by *W. confusa* (Katina et al., 2009), *W. cibaria* (Wolter et al., 2014), or *Leuconostoc* spp. (Di Monaco et al., 2015) strains. With the exception of

Table 5

The qPCR primer pairs used for dextransucrase genes identification and transcription analysis (in bold) in *L. citreum* FDR421 strain.

Name	Sequence[a]	Template gene[b]	Present in FDR241[c]
recA_qPCR_F	**TTGTTGATTCAGTCGCAGCC**	*recA L. citreum* LBAE E16	Yes
recA_qPCR_R	**TGTCTCAGGATTACCAAACATCAC**		
dsrA_qPCR_F1	ATGCCTAATGATAAACGGAGTG	*dsrA*-homolog in *L. citreum* LBAE E16	Yes
dsrA_qPCR_R1	CTGTTGAATCTAACCCTGTTGAG		
dsrA_qPCR_F2	**AAGGCAGACATGGTATATGGT**		
dsrA_qPCR_R2	**TCGGGTGTTGTTGGAAAGTC**		
dsrB_qPCR_F1 dsrB_qPCR_R1	AAACCTTCCACTCAAATGCTG GAATCTAAGAAACTGGTATCGGTG	*dsrB*-homolog in *L. citreum* LBAE E16	Yes
dsrB_qPCR_F2	**GGTCACTTCTGGCTTCACTG CCATCATTACCCAAATAGAACCAC**		
dsrB_qPCR_R2			
dsrE_qPCR_F1	**GTAGCGGGTGACTATCAGGA**	*dsrE*-homolog in *L. citreum* LBAE E16	Yes
dsrE_qPCR_R1	**GTATTCACCATTGCCTTTATCGTC**		
dsrE_qPCR_F2	GCAAGACAATACACAAATTCGTGG		
dsrE_qPCR_R2	GGCTGGAAGTTAGAGAAACCT		
GSE16_4_qPRC_F1	GATACAAGTCAAAGGTCAGGCA	*GSE16-4* in *L. citreum* LBAE E16	No
GSE16_4_qPRC_R1	AAGAAACCATCCACAAGATTCC		
GSE16_4_qPRC_F2	GTTGCTGGTGGTCAAATAATGG		
GSE16_4_qPRC_R2	CCGCTACTATTGGATACGATGAC		
GSE16_5_qPRC_F1	ATGCCCATCAAGTTTATCGG	*GSE16-5* (*dsrM*-homolog) in *L. citreum* LBAE E16	No
GSE16_5_qPRC_R1	TACCCATACAGCTAAGTAACCA		
GSE16_5_qPRC_F2	TTAACACAGACTACACTGCCT		
GSE16_5_qPRC_R2	GATTAAGTTCCAGCTCACACC		
GSC_11_3_qPRC_F1	AGAATCAACTTTACCGTGCC	*GSC11-3* (*asr*-homolog) in *L. citreum* LBAE C11	No
GSC_11_3_qPRC_R1	ACCCAGACACCTAAATAACCC		
GSC_11_3_qPRC_F2	CAGACTACACTGCCTACATCAC		
GSC_11_3_qPRC_R2	TTCCAACTCACACCCTTACTG		
brsA_qPRC_F1	**TTTGATACCATTACGGCCCT**	*brsA*-homolog in *L. citreum* LBAE C10	Yes
brsA_qPRC_R1	**GTCGTTTGCTATAACAACACC**		
brsA_qPRC_F2	AGTAGGTGGAGGTAAGTATCAG		
brsA_qPRC_R2	TAGTATCCGTATCTAATTCCGCTC		
brsM_qPCR_F1	**ATGCTCAAACTGGATACGCT**	*dsrM*-homolog in *L. citreum* LBAE C10	Yes
brsM_qPCR_R1	**AATTTCTTCTACGATGTCCCTG**		
dsrM_qPCR_F2	TTAGCATCATTAACGAGACCC		
dsrM_qPCR_R2	GCCAGTAAAGCCTTATCAACAG		

[a] Sequences of the forward and reverse primer pairs are shown in 5′-to-3′ direction.

[b] Gene from a particular *L. citreum* strain used for the primer design. The names are based on previously published information (Amari et al., 2015; Passerini et al., 2015).

[c] Presence of the gene in the genome of *L. citreum* FDR421 strain based on qPCR, where both primer pairs gave a positive result.

the first fermentation and propagation, as expected, sugars content remained consistent during all the back-slopping procedure. *L. citreum* was able to utilize efficiently the added sucrose, converting it into glucose and fructose. Concomitantly, fructose was reduced to mannitol, leading to the production of acetic acid (Ganzle et al., 2007). In previous studies on other heterofermentative species the molar ratio of

mannitol to acetate was of 2:1 (Korakli et al., 2002). In our conditions, the molar ratio of mannitol to acetate is close to 1. Some possible explanations for this outcome include the use of other electron acceptors such as oxygen, which presence into the dough is facilitated by the back-slopping procedure and daily mixing, as well as metabolic difference between the starters (Erten, 1998; Gobbetti, 1998).
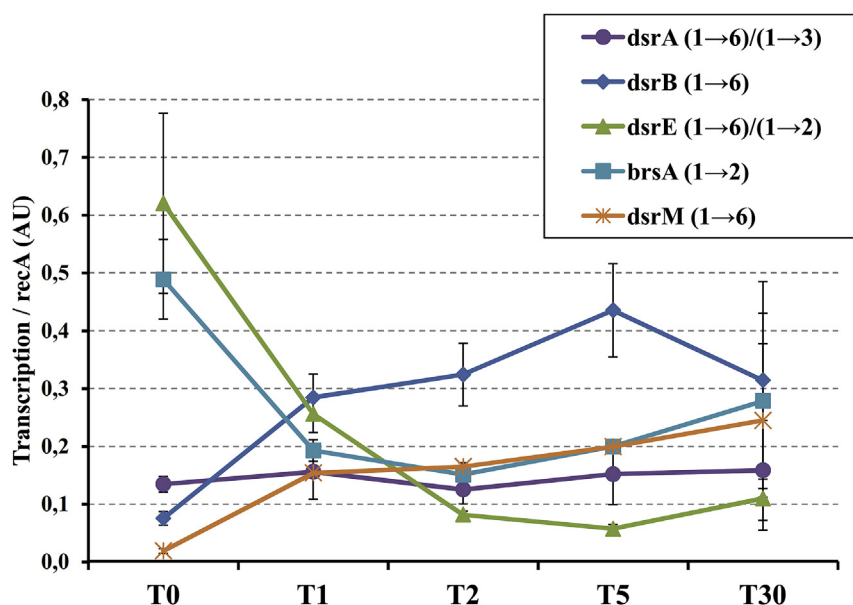

Fig. 3. Transcription analysis of dextransucrase encoding genes as reported in Table 5, of representative time points before fermentation (T0) and during propagation procedure (T1-T30). The genes and their coding are defined based on previously published information (Laguerre et al., 2012; Amari et al., 2015; Passerini et al., 2015).

The amount of fructose deriving from sucrose hydrolysis well correlated with the mannitol formed; fructose was not completely used during the fermentation and a slight accumulation of maltose was also found. Based on these results, we can hypothesize that maltose was also used by the starter. However, an accurate metabolite balance cannot be achieved in these conditions. In fact, the endogenous enzymatic activity of the flour contributed to the formation of maltose and fructose through the degradation of starch and fructosans, which are typically found in wheat flour at concentrations of 55 and 1%, respectively (Koehler and Wieser, 2013). Because of dextran synthesis, compared to the beginning of fermentation, a viscosity increase of approximately 2–2.6 fold characterized all the sourdoughs, with the sole exception of T2, showing a slightly higher value. An increase in viscosity induced by dextran presence has been observed before (Di Cagno et al., 2006; Katina et al., 2009; Xu et al., 2017) and it is due to the EPS water-binding capacity and ability to interact with proteins in doughs. However, due to the several physical changes occurring during sourdough fermentation, contributing to viscosity modification, an exact correlation between viscosity and dextran content is not possible (Katina et al., 2009; Xu et al., 2017). As expected, the addition of sucrose enabled dextran formation; nonetheless, the amount of dextran retrieved, 0.35–0.7% on flour basis, was lower than the theoretical amount, i.e. close to 5% for T1 and 2.5% for the other time points, corresponding to a yield of approximately 15–20%. The low dextran yield obtained can be a consequence of the presence of sucrose and maltose used by dextransucrase as acceptor molecule for the formation of maltooligosaccharides, as also indicated by the maltose found in the sourdoughs, released through the activity of endogenous α-amylase throughout the propagation time. The formation of maltooligosaccharies by dextransucrase activity in wheat substrate is a common phenomenon and can compete with the formation of polymeric dextran (Katina et al., 2009; Kaditzky and Vogel, 2008; Kajala et al., 2016; Robyt et al., 2008). Notwithstanding the lower amount achieved compared to the expectations, the dextran content of the sourdoughs is well in line with the amount of hydrocolloids required to act as dough improvers i.e. 0.1–1% of flour basis (Guarda et al., 2004; Rosell et al., 2001), therefore having technological relevance for baked goods manufacturing. The quantity of residual fructose and mannitol formed in this process, however, seem to exclude the formation of relevant amounts of fructans in these conditions. Strains of *L. citreum* originating from sourdough synthetized dextrans with different amount of α-(1–6), α-(1–3), and α-(1–2) linkages as well as mutan and fructans (Bounaix et al., 2009). The existence of bonds different than α-(1–6), representing the degree of branching of the molecule, is an important feature as it influences the water solubility and other rheological properties of the dextrans and, consequently, of the dough. Overall, only a few studies showed the effects of glucan structure and molecular weight in baking (Chen et al., 2016). Previously, it was shown that dextrans with a linear structure were more effective in increasing bread volume compared to dextrans of the same size but with more branching (Lacaze et al., 2007). Besides technological functionality, also nutritional functionality depends on EPS structure. Dextrans and oligosaccharides with α-(1–6) and α-(1–2) structure possess prebiotic properties and might have very relevant repercussion on health (Sarbini et al., 2011, 2014). In the conditions of this study, significant amount of dextran and maltooligosaccharides were formed. Notwithstanding the necessity to characterize further the structure and molecular weight of the EPS and oligosaccharides produced, based on the results of the genetic expression discussed below, we can hypothesize that the main structural feature of the dextrans produced in this study is mostly α-(1–6) with possible α-(1–2) linkages. Therefore, a positive influence of these polymers on both the technological and nutritional quality of derived baked goods can be assumed. Due to their relevance in several industrial applications, many dextransucrases have been characterized, but not enough information is yet available about the regulation of the expression.

RT(reverse transcription)-qPCR analysis was performed using total RNAs extracted from sourdough propagation from selected time points as previously described. The targeted genes were selected from dextransucrase genes previously identified in *L. citreum* strains isolated from wheat sourdough (Laguerre et al., 2012). *L. citreum* FDR241 possessed a combination of at least five GH70 enzyme-encoding genes previously detected in diverse *L. citreum* strains (Passerini et al., 2015).

In this strain, only the *dsrB* gene was significantly upregulated and the main responsible of dextran formation, while other analyzed genes were downregulated, low or not expressed. The *dsrB* gene was previously shown to synthesize dextrans made only of α-(1–6), as well as oligosaccharides composed of α-(1–6) linked glucosyl residues in addition to the maltosyl residue in the presence of acceptor maltose in *L. citreum* NRRL B-1299 (Monchois et al., 1998). The *brsA* gene, found for the first time in the above strain and acting as natural α-(1–2) branching sucrase (Passerini et al., 2015), was detected in *L. citreum* FDR241. However, it was downregulated in sourdough conditions and its expression was lower compared to *dsrB*. The *dsrA* gene previously associated with branched dextran composed of α-(1–6) and α-(1–3) linkages (Monchois et al., 1996) showed a very low level of expression in the conditions of this study.

Notwithstanding the possibility of other dextransucrases involved, in this investigation, at least one showed its highest activity during the first 24 h of sourdough fermentation, remaining consistent until the end of the process. Transcriptional analysis of *L. mesenteroides* NRRL B-512F dextransucrase has shown that sucrose act as atypical activator, detecting dextransucrase activity only after several hours of contact with high concentration of sucrose (Quirasco et al., 1999). In this study, *dsrE* and *brsA*, typically associated to α-(1–2) branching, where highly expressed during cultivation condition in MRS, as reflected on the initial sourdough inoculum (T0), but their expression diminished afterwards, in sourdough fermentation conditions. Analogous result was observed for genes loosely related to *dsrE* in other *L. citreum* strains isolated from sourdoughs after cultivation in MRS (Amari et al., 2015). This might be due to multiple factors, including CCpA-mediated regulation as response to a growth environment characterized by high amount of sucrose (Vastano et al., 2016). However, more in depth understanding requires further study. Similarly, the amount of EPS formed depends also on environmental factors, which might play a role in the regulation of genes not related to EPS synthesis, responsible of overall carbohydrate metabolism of lactic acid bacteria, such as CcpA or other sugar regulators (Ravcheev et al., 2013; Vastano et al., 2016).

It was previously suggested that, in spontaneous sourdough back-slopping, the selection of specific environmental parameters, such as temperature, and propagation time could affect the final microbiota composition (Vrancken et al., 2011). The fermentation parameters used in this study, such as temperature of 20 °C and consistent supply of high sucrose concentration (146–292 mmol/kg), allowed to establish an environment in which *L. citreum* FDR241 dominated and showed a consistence performance. Understanding of transcriptional regulation of EPS-synthetizing genes in different sourdough conditions may have high technological relevance in industrial processes.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2018.05.003.

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