Direct Application of Rep-PCR on Type I Sourdough Matrix to Monitor the Dominance and Persistence of a Lactobacillus plantarum Starter Throughout Back-Slopping

Direct Application of Rep-PCR on Type I Sourdough Matrix to Monitor the Dominance and Persistence of a *Lactobacillus plantarum* Starter throughout Back-slopping.

Paola Dolci* and Luca Cocolin

Department of Agricultural, Forest and Food Sciences, University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy

*corresponding author:

paola.dolci@unito.it

telephone +39 011 6708691

fax +39 011 6708559

Short version of title: Direct Application of Rep-PCR on Sourdough

Abstract

This study describes the optimization and application of repetitive element-PCR (rep-PCR) technique directly on microbial DNA extracted from type I sourdoughs for fast monitoring of a *L. plantarum* starter strain (P1FMC) throughout daily back-slopping, in response to pratical needs of a bakery factory. Co-inoculum trials, in flour matrix, with *L. plantarum* P1FMC and *L. lactis* LC71 strains and, subsequently, type I sourdough back-slopping trials were performed. The rep-PCR amplification profiles obtained were clearly referable to that of *Lb. plantarum* P1FMC starter in both co-inoculum trials (also when it was present with one order of magnitude less with respect to *L. lactis* LC71) and back-slopping trials where it dominated the fermentation process with loads of 10^8 cfu g^{-1} and prevailed on the autochthonous microbiota. Thus, the approach proposed in this paper could be considered a methodological advancement, based on a culture-independent one-step rep-PCR, suitable for fast monitoring of starter performance.

Keywords

Culture-independent method; *Lactobacillus plantarum*; repetitive element-PCR; sourdough back-

slopping; starter dominance.

Practical Application

The technical approach proposed in this paper has been optimized in response to practical needs of a bakery factory, and it can be suitable for fast monitoring of starter performance and dominance in sourdough back-slopping. Actually, this method can be applied when a single starter strain is used as inoculum. Although this is not the most frequent case in sourdough fermentations, this methodological approach could be taken into account for other applications in food fermentations where one-strain starter cultures are used, or for monitoring microbial biomass production in a bioreactor.

Introduction

The role and importance of lactobacilli in sourdough are known and well documented (Gänzle 2014; Minervini and others 2014; Brandt 2015). They may provide technological, nutritional and organoleptic advantages besides producing antifungal compounds, which could reduce contamination with mycotoxins and improve shelf life of bakery products.

In sourdough type I, different authors reported the prevalence of lactobacilli species, including *Lactobacillus sanfrancisciensis*, *Lactobacillus pontis*, *Lactobacillus fermentum*, *Lactobacillus brevis*, *Lactobacillus plantarum, Lactobacillus paralimentarius*, throughout subsequent back-slopping steps, where the inoculation of the flour with a small quantity of previously performed fermentation was used to optimize the process and to allow the dominance of few autochthonous strains reducing the risk of fermentation failure (Ehrmann and Vogel 2005; Hammes and others 2005).

To a lesser extent, some authors considered the use of starter cultures for sourdough type I propagation (Corsetti and others 2007; Siragusa and others 2009; Vogelmann and others 2009; Minervini and others 2010), and selected starter strains were used as inoculum once a week, followed by daily back-slopping. In this case, it became fundamental to monitor the robustness of the starter in terms of adaptability to environmental conditions and dominance on the autochthonous

microbiota during back-slopping propagation (Minervini and others 2010).

The methodological approaches to monitor the performance of a starter strain in sourdoughs are fundamentally based on culture-dependent techniques for which time-consuming could be considered the main disadvantage. Cultivation on selective media and subsequent molecular characterization of the isolates, based on genomic profiling (RAPD-PCR, REA-PFGE, ARDRA), have been carried out by different authors (Catzeddu and others 2006; Valmorri and others 2006; Corsetti and others 2007; Siragusa and others 2009; Minervini and others 2010).

In this work, a culture-independent repetitive element PCR (rep-PCR) protocol was applied directly on microbial DNA extracted from sourdough matrices. The method was optimized in response to the request of a bakery factory which asked for a method relatively low-cost and fast which, in one step, could detect the persistence of a *Lb. plantarum* starter strain, in sourdough type I back-slopping, and its dominance over the rest of microbiota.

Materials and methods

Sampling and microbial analysis

Flour samples were taken from three different batches of a mill (provider of the bakery factory). Ten grams of each sample were homogenised in 90 ml of sterile Ringer solution (Oxoid, Milan, Italy) in a Stomacher (Interscience, Rockland, MA, USA) for 3 min. The resulting suspension was serially diluted and analyzed for the presence of aerobic mesophilic microbial population on PCA (Oxoid) at 30 °C for 48 h, presumptive lactococci on M17 agar added with 5 g/L glucose (Oxoid) at 37 °C for 48 h, presumptive lactobacilli on modified MRS agar (Oxoid) added with 10 g/L maltose and 50 g/L fresh yeast extract (Corsetti and others 2007) at 37 °C, anaerobically, for 48 h, yeasts and moulds (Oxoid) on Malt agar supplemented with tetracycline (1ug/mL), at 25 °C for 96 h.

DNA extraction optimization

The same flour samples were submitted to trials for nucleic acid extraction to obtain DNA of good quality in terms of yield and purity and, thus, performance in PCR. Firstly, it was carried out the method based on phenol-chloroform separation, according to the protocol described by Rantsiou and others (2008). Then, DNeasy and RNeasy plant mini kit (Qiagen, Milan, Italy) and GenElute™

Plant Genomic DNA Miniprep kit (Sigma, Milan, Italy) were also used following manufacturer's instructions. DNA extractions were performed starting from 10 g of flour suspended in 100 mL, 50 mL and 30 mL of Ringer solution (Oxoid), with and without the addition of 10^9 cfu/ml of *Lb. plantarum* P1FMC (previously isolated from Panettone, DISAFA collection) and, separately, of 10^9 cfu/ml *Lactococcus lactis* LC71 (previously isolated form wheat sourdough, DISAFA collection). *Lb. plantarum* was chosen for its functional features, as robustness and competitiveness (Minervini and others 2010); while *L. lactis* was chosen as representative of lactic acid cocci which can be found, even if with less frequency than lactobacilli, in sourdough matrices (Corsetti and others 2001). The analysis of the three different flour:Ringer ratios was carried out in order to reach the best conditions in terms of DNA quality. In parallel, overnight pure cultures of both *Lb. plantarum* P1FMC (MRS, 37 °C) and *L. lactis* LC71 (M17, 37 °C) were submitted to DNA extraction as previously described (Cocolin and others 2001). All DNA samples were amplified by rep-PCR using a single oligonucleotide primer $(GTG)_5$ (Versalovic and others 1994), according to the protocol described by Dal Bello and others (2010).

Co-inoculum and back-slopping trials

Once DNA extraction conditions were optimized, co-inoculum trials were performed. Precisely, the following *Lb. plantarum* P1FMC:*L. lactis* LC71 ratios were inoculated in flour: 10^7 cfu/g:10^6 cfu/g (r1); 10^7 cfu/g:10^7 cfu/g (r2); 10^6 cfu/g:10^7 cfu/g (r3); 10^5 cfu/g:10^7 cfu/g (r4). The samples were submitted to rep-PCR and analyzed for the presence of *Lb. plantarum* P1FMC.

Finally, two type I sourdough back-slopping trials were performed in the factory pilot plant in order to assess rep-PCR sensitivity, in terms of detection of *Lb. plantarum* P1FMC directly in dough samples. Precisely, a final concentration of 10^6 cfu/g of *Lb. plantarum* P1FMC, used as starter culture, was inoculated in 400 g of flour added of 400 mL of water and incubated at 28 °C for 8 h. After starter fermentation, sourdough was propagated according to daily back-slopping protocol: an inoculum rate of 10% (w/w) was used to ferment 4 kg of fresh flour every day, for 12 days; sourdoughs were fermented at 28 °C for 6 h and stored at 10 °C overnight, before the subsequent inoculum. The presence and dominance of *Lb. plantarum* P1FMC were monitored by both direct detection of the strain in sourdoughs (totally, 16 samples collected during the two back-slopping trials), as described

above, and culture-dependent method by rep-PCR characterization of the isolates obtained on MRS agar (37 °C for 48 h, anaerobically) (totally, 160 randomly selected colonies).

Results and Discussion

In order to highlight efficacy and robustness of the culture-independent one-step rep-PCR protocol proposed in this study, co-inoculum trials, in flour matrix, with *Lb. plantarum* P1FMC and *L. lactis* LC71 strains, were performed, and the results are reported in figure 1. The rep-PCR profiles were clearly ascribable to the starter *Lb. plantarum* P1FMC when it was inoculated in flour with counts of an order of magnitude greater than *L. lactis* LC71 (10^7 cfu/g vs 10^6 cfu/g) (r1), when the loads of the two microorganisms were comparable (10^7 cfu/g)(r2), and also when it was present with one order of magnitude less with respect to *L. lactis* LC71 (10^6 cfu/g vs 10^7 cfu/g)(r3). In this last case a *L. lactis* LC71 amplification band appeared without interfering with the interpretation of the results. On the contrary, *L. lactis* LC71 rep-PCR profile prevailed (r4) when the microorganism was inoculated with load of two orders of magnitude greater than *Lb. plantarum* P1FMC (10^7 cfu/g vs 10^5 cfu/g). Thus, the results obtained from co-inoculum trials showed that when the starter *Lb. plantarum* P1FMC was present, in flour, with remarkable loads, as expected in sourdough back-slopping, this method allowed to monitor its dominance in spite of other microbial populations, which was the main purpose of our study. Other culture-independent methods (e.g. PCR-DGGE) have been described in literature, however their main focus was the monitoring of microbiota, throughout fermentation processes, at species level, without giving any information about the strain dominating the process (Meroth and others 2003; Randazzo and others 2005; Iacumin and others 2009; Scheirlink and others 2009; Vogelmann and others 2009).

DNA extraction was a critical point in the optimization of rep-PCR performance. The best results, in terms of clearly and reproducibility of rep-PCR profiles, were obtained starting from 10 g of dough diluted in 30 mL Ringer, followed by extraction with Qiagen kit. In figure 1, rep-PCR profiles of both *Lb. plantarum* P1FMC (lp) and *L. lactis* LC71 (lc), inoculated in flour with load of about 10^7-10^8 cfu/g, appeared absolutely comparable with profiles obtained from pure cultures (data not shown). Thus, it can be affirmed that preliminary optimization of DNA extraction method was fundamental for the

good performance of rep-PCR directly from a tricky matrix as flour is. Other authors underlined the importance of the choice of DNA extraction protocol when a culture-independent approach was used for the study of sourdough matrices (De Vuyst and Vancanneyt 2007; Minervini and others 2010).

Finally, DNA extracted directly from flour, with no addition of *Lb. plantarum* P1FMC and *L. lactis* LC71, showed an almost imperceptible amplification profile (figure 1, f) produced from autochthonous microbial populations, confirming flour low microbial counts detected on PCA (2.35 $\pm$ 0.6 LOG_{10} cfu/g), Malt agar (1.35 $\pm$ 0.7 LOG_{10} cfu/g), M17 agar (1.02 $\pm$ 0.3 LOG_{10} cfu/g) and modified MRS agar ($<$ 10 cfu/g).

The good quality of the extracted DNA allowed to obtain clear rep-PCR results also from the analysis of sourdoughs sampled in back-slopping trials where the presence and dominance of *Lb. plantarum* P1FMC was monitored. The profiles obtained were referable to the starter *Lb. plantarum* P1FMC which prevailed on flour autochthonous microbiota, as expected in fermentation processes. In particular, in figure 2, rep-PCR results obtained from sourdough samples are reported, as example. The amplification profiles (lines 1-8) were clearly referable to that of the starter (line 9, pure culture), which dominated the fermentation process with loads of 10^8 cfu/g (as determined by plate counts, data not shown), and prevailed on the autochthonous microbiota. These results are corroborated by those obtained from the rep-PCR characterization of the 160 colonies, 156 of which were found to belong to the inoculated strain. The presence of other indigenous *Lb. plantarum* strains developing during back-slopping cannot be completely excluded but it would not affect the efficiency in starter monitoring which was the main purpose of the present study.

The approach proposed in this paper could be considered a methodological advancement, based on a culture-independent one-step rep-PCR, suitable for fast monitoring of starter performance. This method has been optimized in response to practical needs of a bakery factory. Of course, it can be applied only when a single starter strain is used as inoculum. Although this is not the most frequent case in sourdough fermentations, this methodological approach could be taken into account for other applications in food fermentations where one-strain starter cultures are used, or for monitoring microbial biomass production in bioreactor.

Conclusions

The use of selected starter cultures is quite widespread in food fermentation industry and their monitoring, in relatively short time, in terms of persistence and dominance over the rest of microbiota, represents an undoubted advantage for the industrial routine and the quality of the final product. In this paper, an application of rep-PCR technique was optimized and used according to a culture-independent approach, in order to monitor the performance of a *L. plantarum* strain in sourdough back-slopping. This method was a response to practical needs of a bakery factory, and turned out to be reliable and suitable for following a dominant selected strain in a microbiologically complex matrix as it is sourdough. Moreover, the same approach could be applied to other food fermentations where one-strain starter cultures are used.

Author Contributions

Paola Dolci collected test data, interpreted the results and drafted the manuscript; Luca Cocolin designed the study and drafted the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Brandt MJ. 2015. Quality improvement and fermentation control in dough fermentations. In: Holzapfel W, editor. Advances in fermented foods and beverages. Cambridge, UK: Elsevier, pp 391-394.

Catzeddu P, Mura E, Parente E, Sanna M, Farris GA. 2006. Molecular characterization of lactic acid bacteria from sourdoughs breads produced in Sardinia (Italy) and multivariate statistical analysis of the results. Syst Appl Microbiol 29:138-144.

Cocolin L, Manzano M, Cantoni C, Comi G. 2001. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. Appl Environ Microbiol 67:5113-5121.

Corsetti A, Lavermicocca P, Morea M, Baruzzi F, Tosti N, Gobbetti M. 2001. Phenotypic and molecular identification and clustering of lactic acid bacteria and yeasts from wheat (species *Triticum durum* and *Triticum aestivum*) sourdoughs of Southern Italy. Int J Food Microbiol 64:95–104.

Corsetti A, Settanni L, Valmorri S, Mastrangelo M, Suzzi G. 2007. Identification of subdominant sourdough lactic acid bacteria and their evolution during laboratori-scale fermentations. Food Microbiol 24:592-600.

Dal Bello B, Rantsiou K, Bellio A, Zeppa G, Ambrosoli R, Civera T, Cocolin L. 2010. Microbial ecology of artisanal products from North West of Italy and antimicrobial activity of the autochthonous populations. LWT – Food Sci Technol 43:1151–1159.

De Vuyst L and Vancanneyt M. 2007. Biodiversity and identification of sourdough lactic acid bacteria. Food Microbiol 24:120–127.

Ehrmann MA and Vogel RF. 2005. Molecular taxonomy and genetics of sourdough lactic acid bacteria. Trends Food Sci Tech 16:31-42.

Ganzle MG. 2014. Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiol 37:2-10.

Hammes PP, Brandt MJ, Francis KL, Rosenheim J, Seitter MFH, Vogelmann A. 2005. Microbial ecology of cereal fermentations. Trends Food Sci Tech 16:4-11.

Iacumin L, Cecchini F, Manzano M, Osualdini M, Boscolo D, Orlic S, Comi G. 2009. Description of the microflora of sourdoughs by culture-dependent and culture-independent methods. Food Microbiol 26:128-135.

Meroth CB, Walter J, Hertel C, Brandt MJ, Hammes WP. 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR denaturing gradient gel electrophoresis. Appl Environ Microbiol 69:475-482.

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, De Angeli M, Di Cagno R, Pinto D, Siragusa S, Rizzello CG, Gobetti M. 2010. Robustness of *Lactobacillus plantarum* starters during daily propagation of wheat sourdough

type I. Food Microbiol 27:897-908.

Randazzo CL, Heilig H, Restuccia C, Giudici P, Caggia C. 2005. Bacterial population in traditional sourdough evaluated by molecola methods. J Appl Microbiol 99:251-258.

Rantsiou K, Urso R, Dolci P, Comi G, Cocolin L. 2008. Microflora of Feta cheese from four Greek manufacturers. Int J Food Microbiol 126:36-42.

Scheirlink I, Van derMeulen R, De Vuyst L, Vandamme P, Huys G. 2009. Molecular source tracking of predominant lactic acid bacteria in traditional Belgian sourdoughs and their production environments. J Appl Microbiol 106:1081-1092.

Siragusa S, Di Cagno R, Ercolini D, Minervini F, Gobetti M, De Angelis M. 2009. Taxonomic structure and monitoring of the dominant population of lactic acid bacteria during wheat flour sourdough type I propagation using *Lactobacillus sanfranciscensis* starters. Appl Environ Microbiol 75:1099-1109.

Valmorri S, Settanni L, Suzzi G, Gardini F, Vernocchi P, Corsetti A. 2006. Application of a novel polyphasic approach to study the lactobacilli composition of sourdoughs from the Abruzzo region (central Italy). Lett Appl Microbiol 43:343-349.

Versalovic J, Schneider M, De Bruijn F, Lupski J. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5:25–40.

Vogelmann SA, Seitter M, Singer U, Brandt MJ, Hertel C. 2009. Adaptability of lactic acid bacteria and yeasts to sourdoughs prepared from cereals, pseudocereals and cassava and use of competitive strains as starters. Int J Food Microbiol 130:205–212.

Figure 1. Rep-PCR profiles of flour samples inoculated with *Lb. plantarum* P1FMC and *L. lactis* LC71.

M: marker 1kb (Sigma); f: flour sample; lc: flour sample inoculated with 10^8 cfu/g *L. lactis* LC71; r1-r4: see materials and methods; lp: flour sample inoculated with 10^8 cfu/g *Lb. plantarum* P1FMC.

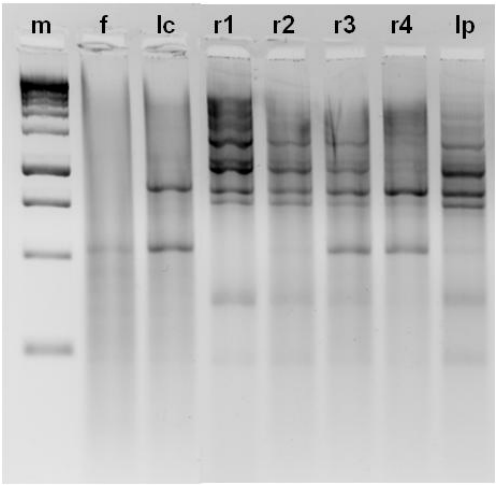

Figure 2. Rep-PCR profiles of sourdough samples inoculated with the starter *Lb. plantarum* P1FMC in back-slopping trials.

Lines 1-3-5-7: sourdoughs fermented for 6h at 28 °C after 1, 4, 8, 12 day back-slopping, respectively.

Lines 2-4-6-8: sourdoughs rested overnight at 10 °C after 1, 4, 8, 12 day back-slopping, respectively.

Line 9: *Lb. plantarum* P1FMC grown on MRS agar plate. Line 10: marker 1 kb (Sigma).