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Antifungal sourdough lactic acid bacteria as biopreservation tool in quinoa and rice bread

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ABSTRACT

The use of sourdough fermented with specific strains of antifungal lactic acid bacteria can reduce chemical preservatives in bakery products. The main objective of this study was to investigate the production of antifungal carboxylic acids after sourdough fermentation of quinoa and rice flour using the antifungal strains *Lactobacillus reuteri* R29 and *Lactobacillus brevis* R2Δ as bioprotective cultures and the non-antifungal *L. brevis* L1105 as a negative control strain. The impact of the fermentation substrate was evaluated in terms of metabolic activity, acidification pattern and quantity of antifungal carboxylic acids. These *in situ* produced compounds ($n = 20$) were extracted from the sourdough using a QuEChERS method and detected by a new UHPLC-MS/MS chromatography. Furthermore, the sourdough was applied *in situ* using durability tests against environmental moulds to investigate the biopreservative potential to prolong the shelf life of bread. Organic acid production and TTA values were lowest in rice sourdough. The sourdough fermentation of the different flour substrates generated a complex and significantly different profile of carboxylic acids. Extracted quinoa sourdough detected the greatest number of carboxylic acids ($n = 11$) at a much higher concentration than what was detected from rice sourdough ($n = 9$). Comparing the lactic acid bacteria strains, *L. reuteri* R29 fermented sourdoughs contained generally higher concentrations of acetic and lactic acid but also the carboxylic acids. Among them, 3-phenyllactic acid and 2-hydroxyisocaproic acid were present at a significant concentration. This was correlated with the superior protein content of quinoa flour and its high protease activity. With the addition of *L. reuteri* R29 inoculated sourdough, the shelf life was extended by 2 days for quinoa (+100%) and rice bread (+67%) when compared to the non-acidified controls. The *L. brevis* R2Δ fermented sourdough bread reached a shelf life of 4 days for quinoa (+100%) and rice (+33%). However, the shelf life was similar to the chemically acidified control indicating that the preservation effect of the carboxylic acids seems to have a minor contribution effect on the antifungal activity in gluten-free breads.

1. Introduction

Highly processed food containing artificial preservatives are increasingly rejected by the consumers who increasingly prefer natural and wholesome food products. Nevertheless, microbial safety is required to be preserved within the product's shelf life besides appearance, taste and texture quality aspects. This challenge has led the food industry to reconsider fermentations and biopreservation techniques. Among bakery products the most economically and ecologically friendly method is the use of lactic acid bacteria (LAB) applied as starter culture for sourdough (Zannini et al., 2012).

Bread spoilage is caused predominantly by fungal spores. LAB are known to produce a range of active compounds inhibiting fungal growth. This activity is believed to result from a synergistic effect between pH decrease due to the production of organic acid (*i.e.*, lactic acid, acetic acid) and other antifungal metabolites produced by LAB (Cortés-Zavaleta et al., 2014; Ndagano et al., 2011). These include low molecular mass compounds such as carboxylic acids; phenyl and substituted phenyl derivates (3-phenyllactic, 4-hydroxyphenyllactic or benzoic acid), cyclic dipeptides, hydroxy fatty acids or antifungal peptides (Black et al., 2013; Broberg et al., 2007; Coda et al., 2008; Lavermicocca et al., 2003; Niku-Paavola et al., 1999; Ryan et al., 2011; Ström et al., 2002). The production of antifungal compounds depends on the microbial growth and metabolic activity of each strain which are further influenced by endogenous factors such as flour carbohydrates, enzymes and microbial interaction (Axel et al., 2015b; Meroth et al., 2003; Van Der Meulen et al., 2007). Accordingly, the evaluation is highly complex.

In the last decades, considerable effort has been directed to screen the antifungal activity of LAB in order to find suitable antifungal bioprotective cultures. This content was recently reviewed by a number of studies (Crowley et al., 2013; Dalié et al., 2010; Oliveira et al., 2014) and specifically for bread biopreservation (Axel et al., 2016).

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However, the application of antifungal starter strains for sourdough fermentation is still limited and more strains should be investigated *in situ*.

Little attention has been directed to determine the concentration of antifungal compounds formed by LAB during different sourdough fermentations. A few studies report the extraction of antifungal carboxylic acids from the sourdough matrix (Axel et al., 2015b; Dallagnol et al., 2011, 2015; Ryan et al., 2009). Disadvantages encountered were either long extraction procedures, usage of large amounts of solvents or high matrix effects and partially low recoveries. With the method described herein a multi-compound (20) UHPLC-MS/MS method is described offering shorter analysis time and good recoveries for the determination of *in situ* formed compounds from sourdough.

In a recent study by this group the antifungal activities of three different *Lactobacillus* species and their production of antifungal carboxylic acids in wheat sourdough was investigated (Axel et al., 2015a). It is noteworthy that, *in situ* tests with the artificial application of the carboxylic acids revealed the contribution of these compounds to synergistic antifungal activity in the wheat system.

The main focus of this study addresses the investigation of further substrates for sourdough fermentation with antifungal potential to prolong the shelf life of bread. Therefore, quinoa and rice flour were selected as fermentation substrate. *Lactobacillus reuteri* R29 and *Lactobacillus brevis* R2Δ were chosen as the bioprotective cultures due to their antifungal activity *in vitro* and *in situ* (Axel et al., 2015a). The non-antifungal strain L. *brevis* L1105 was included as the negative control strain. The impact of the substrate on the quantity of antifungal carboxylic acids produced after LAB sourdough fermentation was evaluated. Finally, the biopreservatives were applied in sourdough breads and the shelf life was evaluated using a mould environmental durability test, which was compared to acidified and non-acidified controls.

2. Material and methods

2.1. Materials

The flours used in this study were quinoa flour (Irish Independent Health Food Ltd., Ballyvourney, Ireland); 10.6% moisture, total starch 57.5% of dry matter (DM) and protein (N × 6.25) 15.9% of DM, fat 6.8% of DM, ash 3.2% of DM and white rice flour (Doves Farm Foods Ltd., Berkshire, UK), 13.9% moisture, total starch 87.3 of DM, protein (N × 5.95) 7.4% of DM, fat 0.8% of DM and ash 0.5% of DM. Dry yeast was supplied by Puratos, Groot-Bijgaarden, Belgium, sugar by Siucra, Dublin, Ireland and salt by Glacia British Salt Limited, Cheshire, UK.

Chemicals and analytical standards and base ingredients for media were mainly purchased from Sigma Aldrich (Dublin, Ireland), unless otherwise stated. The antifungal compound 3-phenyllactic acid was acquired from BaChem (Weil am Rhein, Germany). All analytical standards had a purity of ≥95%. The dispersive SPE Kit Cat #: 5982-4956 (15 mL; 150 mg C18, 900 mg $MgSO_4$) were received from Agilent (Dublin, Ireland).

2.2. Enzymatic activities of quinoa and rice flours

The endogenous activity of α- and β-amylase as well as amyloglucosidase were analysed enzymatically (K-CERA, K-BETA3, *R*-AMGR3, Megazyme, Bray, Ireland). The activity of *endo*-proteinase was analysed spectrophotometrically using haemoglobin as substrate (Brijs et al., 1999).

2.3. Sourdough type II preparation

L. reuteri R29, *L. brevis* L1105 and *L. brevis* R2Δ were obtained from the culture collection of UCC (School of Food and Nutritional Science, University College Cork, Cork, Ireland). The strains were stored in a 35% glycerol stock at −80 °C and routinely maintained on deMan-Rogosa (MRS) agar (48 h at 37 °C). For the preparation of working cultures, single colonies were picked from the agar plates, cultured in MRS broth (10 mL) at 37 °C for 24 h, and sub-cultured (1%) for 16 h. Cells were harvested by centrifugation (2410 ×*g*, 10 min), washed, re-suspended in sterile water and added to the sourdough to an initial cell count of approximately 10^7 cfu/g dough. Sourdoughs were prepared with an equal weight of flour and water; dough yield (DY) of 200. The doughs were fermented at 37 and 30 °C (growth optimum for *L. reuteri* and *L. brevis*, respectively) for 48 h. A fermentation time of 48 h was chosen based on previous literature for the production of functional sourdough displaying antifungal activity (Axel et al., 2015a; Belz et al., 2012; Dal Bello et al., 2007; Garofalo et al., 2012). LAB cell counts were determined at 0 h and 48 h of fermentation for determining the cell growth. Chemically acidified (CA) sourdough was acidified with a mixture of lactic and acetic acid (4:1) according to Ryan et al. (2008) to reach a concentration of 11.25 and 2.75 g/kg, respectively.

2.4. General sourdough analysis

Total titratable acidity (TTA) and pH values of the fermented sourdoughs were measured using a standard procedure (Arbeitsgemeinschaft Getreideforschung e.V., 1994). An Agilent 1260 high performance liquid chromatography system equipped a Hi-Plex H column (300 × 7.7 mm, 8 μm; Agilent, Cork, Ireland) and an appropriate guard column (50 × 7.7 mm, 8 μm; Agilent, Cork, Ireland) was used to quantify sugars (0.125–2.5 mM) and organic acids (2–32 mM) from extracted freeze dried sourdough. Samples preparation was done according to Wolter et al. (2014). Concentrations of fructose, glucose, maltose, raffinose and sucrose in flour and sourdough samples were analysed using a refractive index detector. Lactate and acetate acid were detected using a diode array detector (λ = 210 nm). Samples for sugar and acid determination were eluted with water or 0.004 M sulphuric acid at a flow rate of 0.6 ml/min and 25 °C or 65 °C, respectively.

2.5. Quantification of antifungal carboxylic acids using the QuEChERS method and UHPLC-MS/MS analysis

Stock solutions (2 mg/mL) of carboxylic acids (azelaic acid, benzoic acid, caffeic acid, catechol, *p*-coumaric acid, ferulic acid, hydrocaffeic acid, hydrocinnamic acid, hydroferulic acid, 4-hydroxybenzoic acid, 2-hydroxydodecanoic acid, β-hydroxylauric acid, 2-hydroxyisocaproic acid, hydroxymyristic acid, 4-hydroxyphenyllactic acid, methylcinnamic acid, 3-phenyllactic acid, phloretic acid, salicylic acid, vanillic acid) in water or acetonitrile were individually prepared. 100 μL of each was combined (2 mL; 20 × 100 μL) in a vial to make a 100 μg/mL standard mix solution.

2.5.1. Sample preparation

Freeze-dried sourdough sample (2 g ± 0.01 g) with 10 mL of water were vortexed for 30 s in a polypropylene tubes (50 mL), hydrocinnamic acid D9 and salicylic acid D6 internal standard solution (100 μL; 50 μg/mL) was added. After 15 min, 10 mL of ethyl acetate containing 0.1% formic acid was dispensed into the samples which were then vortexed for 30 s. NaCl (1 g) and MgSO4 (4 g) were added and shaken immediately upon addition for 1 min. After centrifugation (10 min, 2842 ×*g*) the upper organic phase was transferred to a 15 mL Agilent dSPE tube, vortexed for 30 s and centrifuged (10 min, 2842 ×*g*). The supernatant (5 mL which is equivalent to 1/2 of the original samples; 1.0 g) was transferred to a 15 mL polypropylene tube with 500 μL of DMSO and evaporated under nitrogen at 50 °C on a Turbovap LV system. Filtered extracts (0.2 μm PTFE 13 mm, Millipore) were injected onto the UHPLC-MS/MS system (5 μL).