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Application of *Lactobacillus amylovorus* DSM19280 in gluten-free sourdough bread to improve the microbial shelf life

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ABSTRACT

The present study investigated the antifungal activity of *Lactobacillus amylovorus* DSM19280 as a starter culture for gluten-free quinoa sourdough bread under pilot-plant conditions to extend the microbial shelf life. Challenge tests against environmental moulds were conducted and a negative control with non-antifungal strain, *L. amylovorus* DSM20531^T, as well as a chemically acidified and a non-acidified control were included. Organic acid production, antifungal metabolites, carbohydrates changes during fermentation and bread quality were compared to wheat counterparts. The application of quinoa sourdough fermented with the antifungal *L. amylovorus* DSM19280 extended the mould free shelf life by 4 days compared to the non-acidified control. No significant difference in lactic acid production was found between the lactobacilli strains. HPLC-UV/DAD was used to quantify antifungal compounds. The concentration of 4-hydroxyphenyllactic acid, phloretic acid, 3-phenyllactic acid and hydroferulic acid were significantly higher ($P < 0.01$) in the quinoa sourdough fermented with the antifungal *L. amylovorus* DSM19280 when compared to the non-antifungal strain, thus indicating their contribution to the antifungal activity. Evaluation of bread characteristics such as specific volume or crumb hardness, revealed that the addition of *L. amylovorus* fermented sourdough also improved bread quality. In conclusion, the combination of quinoa flour fermented with the antifungal *L. amylovorus* DSM19280 serves a great potential biopreservative ingredient to produce gluten-free breads with an improved nutritional value, better bread quality and higher safety due to an extended shelf life, and therefore meeting consumer needs for good quality and preservatives-free food products.

Chemical compounds studied in this article

4-Hydroxyphenyllactic acid (PubChem CID: 9378); Phloretic acid (PubChem CID: 10394); 3-Phenyllactic acid (PubChem CID: 3848); Hydroferulic acid (PubChem CID:14340); 4-Hydroxybenzoic acid (PubChem CID: 135); Vanillic acid (PubChem CID: 8468); Lactic acid (PubChem CID: 612).

1. Introduction

Interest in the application of alternative gluten free (GF) crops for the production of cereal-based foods is growing constantly, mainly due to the rising incidence of coeliac disease. As a result, the trend for ethnic and ancient grains has increasingly attracted bakery industries as well as consumers worldwide. Due to its high nutritional value, the pseudo-cereal quinoa as such holds exceptional promise for utilisation in food and beverages (Arendt and Zannini, 2013). It is rich in proteins and essential amino acids. The lipid fraction contains high amounts of polyunsaturated fatty acids and in comparison to other grains, quinoa is found to be rich in the vitamins A, E and folate. During the last years, novel products appeared on the market labelled with proposed healthier and more natural features compared to their classic wheat counterparts. However, GF bread production results in major challenges for bakers and cereal technologists. Complex recipe formulations,

Abbreviations: CA, chemically acidified; DAD, diode array detector; DY, dough yield; GF, gluten free; HPLC, high performance liquid chromatography; LAB, lactic acid bacteria; RID, refractive index detector; SPE, solid phase extraction; TPA, texture profile analysis; TTA, total titratable acidity; UV, ultra violet.

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using different starches, proteins, gums and hydrocolloids, are necessary to mimic the structure-building and water binding properties of gluten (Zannini et al., 2012). Although proceedings in GF bread formulations have been achieved so far, the inclusion of the above mentioned ingredients/additives presents several disadvantages such as excessive prices (Moroni et al., 2009), other allergic reactions (Ortolani and Pastorello, 2006) and predominantly, the use of additives does not match the actual consumers' requirements for natural products (Zannini et al., 2012). Furthermore, consumers' concerns about safety and additive contents in food has received much attention over the last years. In turn, such minimal processed food without chemical preservatives should be still of high-quality and have an extended shelf life (Pawlowska et al., 2012). The shelf life of bread is determined by both staling behaviour and microbial deterioration. The latter one is a serious and expensive problem implicated with an estimated loss of the world's bread production of 5–10 % (Pitt and Hocking, 2009). Apart from the unpleasant sight of visible mould growth, fungi are also responsible for off-flavour formation, the production of mycotoxins as well as other allergenic compounds, which might be produced even before fungal outgrowth is visible. Furthermore, due to higher water contents and also higher water availability, GF breads are more susceptible for fungal spoilage (Hager et al., 2012a).

The “free-from” trend leads the bakery industry to reconsider traditional preservation methods and replace chemical preservatives with natural alternatives to guarantee a clean label. One of the most established food biopreservation is fermentation, a process based on the growth of microorganisms in foods. Among bakery products the microorganisms most widely used are lactic acid bacteria (LAB) applied as starter cultures for sourdough. A further benefit from sourdough LAB, in particular those belonging to the genus *Lactobacillus*, is that many species have been referred to the European Food Safety Authority (EFSA) for safety assessment without raising safety concerns. As a result, they have been included in the QPS (Qualified Presumption of Safety) list for authorised use in the food and feed chain within the European Union (EFSA, 2012). This promotes many lactobacilli starter cultures to be categorised as biological agents excluded for labelling by the EU regulation on food additives (EEC, 2008). The same applies to the US, where they enjoy the generally regarded as safe (GRAS) status regulated by the U.S. Food and Drug Administration.

Formerly, it was believed that the organic acids produced by LAB, particularly lactic and acetic acid, were the main agents responsible for antifungal activity due to the pH decrease. Besides organic acids, a range of other secondary metabolites produced by LAB has been identified additionally as the source of the antifungal activity. A comprehensive overview about individual substances, their possible antifungal mechanism and some applications of antifungal acting LAB was recently reviewed by Crowley et al. (2013). Antifungal compounds also include fatty acids, acids with phenyl groups, proteinaceous compounds and a variety of other low-molecular weight compounds. In recent years, remarkable effort has been directed to research the antifungal activity of LAB in order to reduce fungal spoilage of foods and extend shelf life. However, many of them were studied under laboratory conditions. Various applications in food *in situ* systems exist, but particular research in GF sourdough bread systems is still limited (Moore et al., 2008). In fact, neither the suitability of antifungal strains as starter cultures for the fermentation of GF flour nor the quality of the final breads have been completely evaluated to date.

The aim of the present study was to apply antifungal LAB fermented sourdough as natural preservative in GF bread to inhibit spoilage and consequently improve safety and quality. Therefore, the antifungal activity of *Lactobacillus amylovorus* DSM19280 was investigated in a simple GF bread system, particularly in quinoa sourdough bread, under pilot-plant conditions. Bread characteristics such as specific volume and textural and visual crumb properties were evaluated and compared to their wheat counterpart. In addition, the flours used were fully characterised for their chemical composition. Sourdough was further analysed for pH value, total titratable acidity (TTA) as well as metabolite profile.

2. Material and methods

2.1. Materials

The flours used in this study were quinoa flour (Irish Independent Health Food Ltd, moisture 10.7 %) and wheat flour (baker's flour, Odlums, Ireland, moisture 13.5 %). Dry yeast was supplied by Puratos, Belgium; sugar from Siucra, Ireland and salt from Glacia British Salt Limited, UK.

Chemicals and analytical standards were mainly purchased from Sigma Aldrich (Dublin, Ireland). The antifungal compound 3-phenyllactic acid was acquired from BaChem (Weil am Rhein, Germany). All analytical standards had a purity of ≥95 %.

2.2. Cultures, media and growth conditions

The patented antifungal strain *L. amylovorus* DSM19280 (Arendt et al., 2009) was obtained from the culture collection of UCC (School of Food and Nutritional Science, University College Cork, Cork, Ireland) and *L. amylovorus* DSM20531^T, provided from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was preliminary evaluated for its *in vitro* antifungal activity (data not shown) and then chosen as a negative control.

The lactobacilli cultures stored in 35 % glycerol stock solution at −80 °C were routinely refreshed on deMan-Rogosa-Sharpe agar (MRS) 5 agar (Meroth et al., 2003). For optimal growth condition after the dormant state, sugars (maltose, glucose and fructose) were autoclaved separately to avoid Maillard reaction and a sterile filtered vitamin mixture (cobalamin, folic acid, nicotinic acid amide, pantothenic acid, pyridoxal phosphate, and thiamine, 0.2 g/L each) was added to the agar after sterilisation. For sourdough cell counts commercial MRS agar was used (CM0361, Oxoid, Basingstoke, Hampshire, England). Both MRS5 and MRS agar were dyed with 0.05 g/L bromocresol green (Sigma–Aldrich, Steinheim, Germany). Lactobacilli were grown at 30 °C for 48 h under anaerobic conditions.

2.3. Starch hydrolysis test

To test the amylolytic activity of the strains, starch agar was prepared containing 3 g/L meat extract, 10 g/L soluble starch as well as 12 g/L agar. After 48 h incubation time using 10 μL of a 16 h culture, the plate surface was flooded with Gram Iodine solution to make starch hydrolysis visible by a colourless zone surrounding colonies.

2.4. Flour characterisation

For compositional analysis, crude fat, protein, moisture and ash content of the flours were determined according to the AACC (American Association of Cereal Chemists) methods 30-10, 46-12, 44-15A and 08-01, respectively. Protein content was calculated with a protein factor of 6.25 for quinoa and 5.83 for wheat. Analysis of total amino acids was carried out according to the EU regulation Nr. 152/09 after hydrolysis with 6 M hydrochloric acid for 23 h (EEC, 2009). The amino acids were separated by ion exchange chromatography and determined by reaction with ninhydrin using