Exploiting synergies of sourdough and antifungal organic acids to delay fungal spoilage of bread

Mattia Quattrini [a,b,⁋], Nuanyi Liang [a,⁋], Maria Grazia Fortina [b], Sheng Xiang [a], Jonathan Curtis, [a], Michael Gänzle [a,c*]

[a] *Dept. of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Canada*

[b] *Dept. of Food, Environmental and Nutritional Sciences, University of Milan, Milan, Italy*

[c] *College of Bioengineering and Food Science, Hubei University of Technology, Wuhan China*

⁋ Both authors contributed equally to the manuscript.

* Corresponding author.
Michael Gänzle
Dept. of Agricultural, Food and Nutritional Science
University of Alberta
4-10 Agriculture/Forestry Centre
Tel: + 1 780.492.0774
Edmonton, Alberta, Canada T6G 2P5

E-mail address: mgaenzle@ualberta.ca

Highlights

- Sourdough baking can delay fungal spoilage
- Acetic acid is the most relevant antifungal metabolites of lactobacilli
- Sourdough enhances the antifungal activity of antifungal organic acids in bread

ABSTRACT

Fungal spoilage of bread remains an unsolved issue in bread making. This work aimed to identify alternative strategies to conventional preservatives in order to prevent or delay fungal spoilage of bread. The minimum inhibitory concentration (MIC) of bacterial metabolites and chemical preservatives was evaluated *in vitro*, and compared to their *in situ* activity in baking trials. Calcium propionate, sorbic acid, 3-phenyllactic acid, ricinoleic acid, and acetic acid were tested both individually and in combination at their MIC values against *Aspergillus niger* and *Penicillium roqueforti*. The combination of acetic acid with propionate and sorbate displayed additive effects against the two fungi. For these reasons, we introduced sourdough fermentation with specific strains of lactobacilli, using wheat or flaxseed, in order to generate acetate in bread. A combination of *Lactobacillus hammesii* and propionate reduced propionate concentration required for shelf life extension of wheat bread 7 fold. Flaxseed sourdough bread fermented with *L. hammesii*, excluding any preservative, showed a shelf life 2 days longer than the control bread. The organic acid quantification indicated a higher production of acetic acid (33.8 ± 4.4 mM) when compared to other sourdough breads. Addition of 4% of sucrose to sourdough fermentation with *L. brevis* increased the mould free shelf-life of bread challenged with *A. niger* by 6 days. The combination of *L. hammesii* sourdough and the addition of ricinoleic acid (0.15% or 0.08%) prolonged the mould free shelf-life by 7-8 days for breads produced with flaxseed or wheat sourdoughs. In conclusion, the *in vitro* MIC of bacterial metabolites and preservatives matched the *in situ* antifungal effect. Of the different bacterial metabolites evaluated, acetic acid had the most prominent and consistent antifungal activity. The use of sourdough fermentation with selected strains able to produce acetic acid allowed reducing the use of chemical preservatives.

Keywords: Bread, fungal spoilage, propionic acid, acetic acid, lactobacillus; flaxseed; ricinoleic acid.

1. Introduction

Fungal spoilage is a key limiting factor for the shelf life of bread and causes considerable economic losses. Bakery products are easily colonized by fungal conidiospores from diverse genera including *Aspergillus*, *Cladosporium*, *Endomyces*, *Penicillium*, and *Rhizopus* (Dal Bello et al., 2007). Conidiospores of filamentous fungi are ubiquitous in the biosphere and are dispersed by air unless contamination is controlled by clean room technology (Denyer and Baird, 2006). The water activity and pH of bread support growth of mycelial fungi on bread that is stored at ambient temperature (Belz et al., 2012; Zhang et al., 2010). Refrigeration delays fungal growth but also accelerates starch retrogradation and bread staling (Gray and Bemiller, 2003).

UV light and pulsed light technology reduce spore contamination of bread but find only limited commercial application (Smith et al., 2004). Chemical preservatives are more commonly used to extend the shelf life of bread. Ethanol vapors delay germination of fungal spores (Salminen et al., 1996), however, calcium propionate and sorbic acid are more widely used as preservatives in pre-packed and sliced white bread (Smith et al., 2004). However, the use of preservatives conflicts the aim to develop "clean label" products that avoid the use of additional chemicals. (Anonymous, 2018a and 2018b).

Lactic acid bacteria are used in baking applications as leavening agents, to achieve dough acidification, or to improve specific quality attributes of bread (Gobbetti et al., 2014; Hammes and Gänzle, 1998). Lactic acid bacteria also produce metabolites with antifungal activity; however, most of their antifungal metabolites are uncharacterized, unproven in food, or negatively impact bread flavour (Axel et al., 2017; Black et al., 2013; Quattrini et al., 2018). Acetic acid is produced in primary carbohydrate metabolism; acetic acid has antifungal activity but also impacts flavour and texture of bread (Gerez et al., 2009; Magnusson and Schnürer, 2001; Kaditzky et al., 2008). The levels of acetic acid produced in sourdough fermentations is readily adjusted by addition of pentoses, or by addition of sucrose as electron acceptor in heterofermentative metabolism (Gänzle, 2015). Propionic acid was

generated in co-fermentation of *L. diolivorans* and *L. buchneri*; however, propionic acid also impacts bread flavour when added at concentrations that are effective against fungi (Zhang et al., 2010). 3-Phenyllactic acid and cyclic dipeptides have antifungal activity *in vitro* but their contribution to the inhibition of fungal growth on bread remains unproven (Axel et al., 2017; Ryan et al., 2009; Vermeulen et al., 2006). The antifungal effect of hydroxylated unsaturated fatty acids has been proven in bread but their accumulation to active concentrations in sourdough remains to be demonstrated (Liang et al., 2017; Black et al., 2013). *In situ* preservative effects of lactic acid bacteria have often been attributed to uncharacterized compounds (Axel et al., 2017; Mandel et al., 2013).

The use of alternative flours in baking can provide plant-derived antifungal compounds to support the activity of bacterial metabolites. Compounds with antifungal activity were isolated from legume flours (*Pisum sativum*, *Phaseolus vulgaris*) and were successfully employed to extend the mould-free shelf life of what bread (Rizzello et al., 2015 and 2017). Flaxseeds have a high oil content with a high proportion of linoleic acid, a substrate for enzymatic or microbial conversion to antifungals fatty acids (Black et al., 2013). The microbial and enzymatic conversion products, 10-hydroxy-12-octadecenoic acid and coriolic acid, respectively, have similar antifungal activity acid (Black et al., 2013; Liang et al., 2017).

The use of multiple antifungal metabolites to exploit synergies may improve the antifungal effect of sourdough while minimizing the impact of organic acid on bread flavour (Ryan et al., 2008; Zhang et al., 2010). Assessment of the synergistic effects of different antifungals metabolites is greatly facilitated by establishment of the correlation of *in vitro* MIC and *in situ* preservative effects. This study therefore aimed to compare the minimum inhibitory concentration of antifungal compounds to their antifungal effect in bread. Antifungal compounds were assessed bread produced with straight dough process, and in sourdough bread. Wheat sourdoughs were compared to flaxseed sourdoughs.

2. Materials and methods

2.1 Strains and growth conditions

Lactobacillus hammesii DSM16381 from French sourdough (Valcheva et al., 2006) and *Lactobacillus plantarum* C264 and *Lactobacillus brevis* C186 from maize bran (Decimo et al., 2017) were cultivated on modified MRS (mMRS) medium (Black et al., 2013) 30 °C. *Aspergillus niger* FUA5001 and *Penicillium roqueforti* FUA5005 were used as target strains for the antifungal assay, as representative of common fungal spoilage in bread (Zhang et al., 2010). Fungal strains were cultivated on malt extract agar medium at 25 °C for 72 h, and spores were collected by adding physiological solution (0.85% NaCl, 0.01 % Tween80). After filtration with Whatman N.1 filter paper, the suspensions were stored at -20° C until further use. Spore suspensions were diluted to proper spore density (10^2 or 10^4 spores/mL) counted with a hemocytometer (Fein-Optik, Jena, Germany).

2.2 Antifungal activity assay

Minimum inhibitory concentrations (MIC) were determined with serial 2-fold dilutions of ricinoleic acid, 3-phenyllactic acid, acetic acid, calcium propionate and sorbic acid (Merck, Darmstadt, Germany) in 96-well microtiter plates (Magnusson and Schnürer, 2001). In the MIC assays, the pH was controlled at pH 4.5 by adjustment of the pH of the medium and the stock solutions of antifungal compounds. Microtiter plates were inoculated with mMRS broth containing 10^4 spores/ mL of *A. niger* or *P. roqueforti* and incubated at 25 °C for 5 days. The MIC was determined as the lowest concentration of compound inhibiting the mould growth. Ethanol, which was used as solvent for ricinoleic acid, was removed by evaporation under a laminar flow hood before the addition of the fungal spores.

A checkerboard procedure (Gänzle et al., 1999) was carried out to determine the combined inhibitory activity of two compounds. The plates were inoculated and incubated at 25 °C for 5 days. The MIC was determined as the lowest concentration of the two compounds inhibiting the mould growth. Experiments were performed in triplicate.

2.3 Sourdough fermentation and bread preparation

118 *L. hammesii*, *L. plantarum* and *L. brevis* were used to prepare sourdough bread. Cells from an
119 overnight culture in mMRS medium were washed twice and suspended in sterile tap water to a
120 concentration of 10^8 CFU/mL. Sourdough was prepared by mixing white wheat flour or flaxseed flour,
121 sterile tap water, and culture in a ratio of 2:1:1 (wt/wt/wt). The dough was fermented at 30 °C for 24 h.
122 Samples were taken at time 0 and after 24 hours for determination of cell counts and pH values, and for
123 quantification of organic acids. Colony morphology and uniformity were used to verify the identity of
124 fermentation microbiota with the inoculum. Cell counts for the three strains reached 10^9-10^{10} CFU/g after
125 24 h.

126 Bread formulations shown in Tables 1 and 2. Sourdough bread was prepared with 10% addition of
127 sourdough. Bread with chemical preservatives was prepared with different concentrations according to
128 MIC results. Bread making procedure was described by Black et al. (2013). After baking, the breads
129 were cooled to 20°C on racks for 120 min, and samples were taken for challenge test, pH determination,
130 and quantification of organic acids.

131 The same protocol was used in the bread experiments to investigate the antifungal effect of the
132 combination of *L. hammesii* sourdough and ricinoleic acid, with minor modifications. Sourdough was
133 fermented for 2 days and 50 g-flour breads (i.e. all the ingredients were used in the same proportion
134 shown in Table 1 and 2, but half of the amount) were made for these experiments. Bread was hand-
135 kneaded for extra 3 min after mechanical mixing. The second proofing was 85min. Bread experiment
136 groups include control without addition of sourdough and ricinoleic acid ([control]); *L. hammesii*
137 fermented sourdough bread with addition 2% linoleic acid during sourdough fermentation,; *L. hammesii*
138 sourdough bread with addition of 0.03%, 0.08% and 0.15% ricinoleic acid added at the bread stage,
139 respectively.

140 *2.4 Bread challenge test against* P. roqueforti *and* A. niger

141 Mould challenge test was conducted as described by Black et al. (2013). Bread samples were sliced
142 in 25-mm thick slices and inoculated with a suspension containing 10^2 spores/mL. The spore suspensions
143 were sprayed on each corner of the slice and in the middle, delivering 90 μL of suspension or about 10
144 spores on each spot. The inoculated slices were placed into sterile plastic bags with filter tips ensure
145 aerobic conditions. Slices were incubated for 12 d at 20 °C and monitored every 12 h. The last day before
146 visible mycelial growth is reported as mould-free shelf life. The effect of chemical preservatives or
147 sourdough fermentation or the combination of the two was determined in triplicate independent
148 experiments (triplicate sourdough fermentation and baking). Statistical analysis was done with Tukey's
149 test with Graphpad Software or SPSSStatistics Software. Significant differences were reported at a
150 confidence level of *P* values of 0.05.

151 *2.5 Quantification of acetic acid with high performance liquid chromatography (HPLC).*

152 Acetic acid was determined by HPLC with an Aminex HPX-87 column (300 mm × 7.8 mm, Biorad,
153 USA) at a temperature of 80 °C and a flow rate of 0.4 mL/min with 5 mM H_2SO_4 as the eluent. The
154 injection volume was 10 μL. Refractive index detector and UV detector (210 nm) were used for detection.
155 For sample preparations, 2 g of bread was diluted with 10 mL of MilliQ water and incubated for 3 h at
156 80 °C. After centrifugation, 7% perchloric acid were added and the solution incubated at 4 °C overnight.
157 Precipitated protein was removed by centrifugation. The samples were filtered before injection in the
158 column.

159 **3. Results**

160 *3.1 MIC of preservatives and combination effects*

161 The individual MIC for each of the five compounds was tested *in vitro* against the two indicator strains
162 *A. niger* and *P. roqueforti* at pH of 4.5. Sorbic acid was the strongest inhibitor (0.4 ± 0.1 and 0.2 ± 0.0
163 mM for *A. niger* and *P. roquefortii*, respectively), followed by calcium propionate (1.3 ± 0.2 and 12.0 ±
164 0.0 mM), ricinoleic acid (1.7 ± 0.0 and 3.5 ± 0.0 mM) and acetic acid (8.2 ± 3.4 and 25.0 ± 5.5 mM).

165 3-Phenyllactic acid was the weakest inhibitor with MIC values of 30 ± 10 and 50 ± 0 mM against *A.*
166 *niger* and *P. roquefortii*. Synergistic activities of acetic acid with other inhibitors were determined with
167 checkerboard assays. Acetic acid exhibited additive activity with calcium propionate, sorbic acid and
168 ricinoleic acid (Figure 2). MIC values of calcium propionate and acetic acid combination were lower
169 than the individual MICs, respectively, with 0.6 + 6.2 mM against *A. niger* and 3.1 + 6.2 mM against *P.*
170 *roquefortii* (Fig. 2). The combination of sorbic acid and acetic acid was active at 0.2 + 3.1 mM against
171 *A. niger* and 0.2 + 6.2 mM against *P. roqueforti* (Fig. 2).

172 *3.2 Antifungal effect of organic acids addition to bread*

173 The organic acids were used in baking trials; compounds or combination of compounds were added
174 approximately at the level of their respective MIC. Bread was challenged by inoculation with *A. niger* or
175 *P. roqueforti* and stored until visible mycelial growth, or for 12 days. The results are shown in Table 3.
176 With the exception of ricinoleic acid, the results obtained *in vitro* are comparable with the data obtained
177 *in situ*. 3-Phenyllactic acid, the weakest inhibitor *in vitro*, showed no antifungal effect *in situ* when added
178 at a level corresponding to 20 mmol / kg bread (Table 3). Acetate, calcium propionate and sorbic acid
179 significantly extended the mould-free shelf life of bread; sorbic acid and acetic acid extended the shelf
180 life by 5-6 days. Acetic acid extended the shelf life of bread by three days ($p<0.05$) in combination with
181 propionic acid; acetic acid in combination with sorbic acid extended the shelf life only by two days
182 ($P<0.1$) relative to the control (Table 3).

183 To determine whether the antifungal effects relate to the pH, the pH of breads is also shown Table 3.
184 The pH of control bread was 5.5. Addition of acetic acid and phenyllactic acid reduced the pH to values
185 below 4.5 while other organic acids had no major effect on the pH.

186 *3.3. Antifungal effect of sourdough addition to bread*

187 The effect of sourdough alone or in combination with preservatives on the mould-free shelf life was also
188 assessed in challenge studies with *P. roqueforti* and *A. niger*. A first series of sourdoughs was prepared

189 with wheat flour, fermented with *L. plantarum*, or *L. brevis* or *L. hammesii*. Use of wheat sourdough
190 fermented with these three lactobacilli moderately but significantly extended the shelf life of bread
191 challenged with *A. niger* but was ineffective against *P. roqueforti* (Table 4). The acetic acid
192 concentrations in breads produced with *L. hammesii*, *L. plantarum* and *L. brevis* sourdoughs were 12.6
193 ± 3.4, 13.2 ± 4.7 and 16.2 ± 2.3 mmol/kg, respectively.
194 The use of flaxseed sourdough in baking reduced the shelf life of bread except for sourdoughs fermented
195 with *L. hammesii*. The acetate concentrations in bread produced with flaxseed sourdoughs fermented
196 with *L. hammesii*, *L. plantarum* and *L. brevis* were 33.8 ± 4.4, 17.8 ± 6.3 and 23.8 ± 3.8 mmol/kg of
197 bread, respectively, which was substantially higher than acetate concentrations obtained with wheat
198 sourdoughs.
199 Addition of calcium propionate (3.1 mM) to *L. hammesii* sourdough bread prolonged the shelf life of
200 wheat bread challenged with *P. roqueforti* and *A. niger*; the combination of *L. hammesii* sourdough with
201 addition of sorbic acid (0.2 mM) extended the shelf life of bread challenged with *A. niger* but not with
202 *P. roquefortii*.
203 To additionally evaluate the effect of acetic acid concentrations, wheat or flaxseed sourdoughs were
204 fermented with addition of 4% sucrose. Remarkably, the addition of sucrose to sourdough did not
205 increase the concentration of acetic acid in bread relative to the bread without sucrose addition (data not
206 shown). The mould-free shelf life of bread nevertheless increased, particularly for *L. brevis* sourdoughs,
207 which increased the shelf life to 8.5 and 9 days for bread challenged with *P. roquefortii* and *A. niger*,
208 respectively. A similar shelf-life was only obtained with the addition of chemical preservatives.
209 Ricinoleic acid inhibited fungal growth *in vitro* (Figure 1) but did not delay fungal growth when added
210 as sole preservative to bread (Table 3). To determine its activity in combination with *L. hammesii*
211 sourdough, 0.03% to 0.15% ricinoleic acid, corresponding to 1 to 5 mM, were added to bread produced
212 with *L. hammesii* wheat and flaxseed sourdoughs. Sourdough fermented with addition of 2% linoleic

213 acid, the substrate for formation of the antifungal 10-hydroxy-12-octadecenoic acid by *L. hammesii*, was
214 additionally evaluated. Addition of 0.08 or 0.15% ricinoleic acid increased the shelf life of wheat bread
215 challenged with *A. niger* or *P. roquefortii* to more than 12 days (Figure 2); addition of 0.03% ricinoleic
216 acid was effective only against *A. niger*. Addition of linoleic acid to sourdoughs fermented with *L.*
217 *hammesii* did not delay fungal growth (Fig. 2). An extension of the shelf life by sourdough in combination
218 with ricinoleic acid was not observed in wheat bread with flaxseed sourdough; the increase of the average
219 shelf life was less than experimental error (Figure 2).

220 **4. Discussion**

221 Bread is a perishable product and subject to rapid deterioration after baking**.** Fungal spoilage is one of
222 the main causes of bread spoilage**.** Moreover, formation of mycotoxins production by filamentous fungi
223 represents a health risk (Sirot et al., 2013). *P. roqueforti* is resistant to biological or chemical
224 preservation; this organism also often occurs as spoilage agent in bread (Axel et al., 2017). The present
225 study confirms that *P. roquefortii* is most difficult to control both with respect to the *in vitro* resistance
226 to preservatives and with respect to the mould-free shelf life of bread. In addition, the inoculum used in
227 the challenge studies, about 100 spores per slice of bread, is substantially higher than the environmental
228 contamination in industry practice. Environmental mould contamination is difficult to control and to
229 reproduce, however, studies on the mould-free shelf life of bread consistently demonstrate that spoilage
230 by environmental contaminants is substantially slower and more readily controlled by preservatives when
231 compared to bread challenged with *Penicillium* spp. (Axel et al., 2015; Belz et al., 2012; Black et al.,
232 2013). Challenge studies with *P. roqueforti* therefore represent a worst case scenario.

233 In this work, we compared the *in vitro* MIC of antifungal bacterial metabolites and chemical
234 preservatives. Phenyllactic acid has the weakest antifungal activity at pH 4.5. In keeping with prior
235 observations, inhibition of fungal growth was observed only at concentrations exceeding 30 mmol / L,
236 corresponding to 4 g / L (Axel et al., 2016; Ryan et al., 2011). During growth in sourdough, lactobacilli

produce phenyllactate from phenylalanine, however, the concentration of phenyllactate in sourdough remains below 0.2 mmol / kg or less than 1% of the MIC (Axel et al., 2016; Ryan et al., 2009; Vermeulen et al., 2006). The combination of different organic acids displays additive rather than synergistic activity when adjusting for the pH (this study); therefore, phenyllactate is not likely to make a contribution to inhibition of fungal growth in bread.

Calcium propionate, sorbic acid, ricinoleic acid and acetic acid displayed antifungal activity in the range of 1 – 24 mmol / L and the *in situ* activity matched the *in vitro* activity when assayed at the same pH. The pH plays a key role for the activity of weak organic acids (Lind et al., 2005). Undissociated acids penetrate the fungal membrane and acidify the cytoplasm, leading to cell death (Stratford and Eklund, 2003). The pKa of ricinoleic acid, acetic acid, sorbic acid, and propionic acid is 4.74, 4.75, 4.76, and 4.90, respectively, indicating that their activity in sourdough bread with pH < 5.0 is much higher than their activity in yeast-leavened bread with a pH of 5.5. Indeed, ricinoleic acid was ineffective in bread with a pH of 5.5 but displayed antifungal activity in sourdough bread. Sourdough fermentation thus has a double role in preservation as it accumulates antifungal organic acids and reduces the pH, thus increasing their antifungal activity.

Lactic acid bacteria produce multiple metabolites with *in vitro* activity against fungal spores, including organic acids, cyclic dipeptides, and long-chain hydroxyl fatty acids (Axel et al., 2017; Black et al., 2013; Gerez et al., 2009). The present study identified acetic acid as the most relevant antifungal compound produced by lactic acid bacteria, as it is readily accumulated to concentrations matching the MIC against fungal spores. Acetate formation by heterofermentative lactic acid bacteria can be adjusted by addition of sucrose, providing fructose to allow regeneration of co-factors and increased acetate formation in heterofermentative metabolism (Stolz et al., 1995; Gänzle, 2015). Addition of acetic acid to bread delayed fungal spoilage (Tables 3 and 4); however, high levels of acetic acid also result in an

unacceptable flavor (Hansen and Schieberle, 2005) and interfere with development of the gluten network in wheat baking, thus negatively affecting volume and texture (Kaditzky et al., 2008).

The combination of acetate with other antifungal compounds reduces or prevents the adverse impact of individual organic acids on bread flavour. Proof of concept was provided by prior studies using sourdough containing propionic and acetic acids (Zhang et al., 2010), or using sourdough in combination with propionate (Ryan et al., 2008). The present study confirms these observations, and demonstrated by addition of acetic and propionic acids that the effect is attributable to the additive antifungal activity of these two organic acids (Tables 3 and 4). In combination with acetic acid or sourdough, the propionate concentration required for shelf life extension of wheat sourdough bread was reduced 7 fold when compared to the amount required for preservation of straight dough bread. Moreover, we extended prior observations by demonstrating additive activity of sourdough or acetic acid with ricinoleic acid and sorbic acid.

The additive activity of *L. hammesii* sourdough and ricinoleic acid, an unsaturated hydroxy-fatty acid present in castor oil, was further explored by adding different levels of ricinoleic acid to bread produced with *L. hammesii* sourdough. The antifungal activity of ricinoleic acid is comparable to other unsaturated hydroxy fatty acids including coriolic acid and 10-hydroxy-12-octadecenoic acid, which are produced by enzymatic or microbial conversion of linoleic acid in sourdough (Black et al., 2013; Liang et al., 2017). The addition of 0.1% coriolic acid to bread also significantly increased the mould-free shelf life of bread (Black et al., 2013). Our study demonstrates that a combination of sourdough and ricinoleic acid displayed a similar antifungal performance at a ricinoleic acid concentration of 0.08%.

Of note, sucrose addition to sourdough did not substantially increase the acetate concentration in bread. The availability of sucrose and other substrates for co-factor regeneration in sourdough supports formation of 10 – 20 mM acetate in wheat sourdough; the acetate concentration can be increased by addition of sucrose (Korakli et al., 2001). With a sourdough addition of 10%, most of the acetic acid that

284 is present in bread, 10 – 20 mM, was produced after the final mixing in the bread dough where sucrose
285 levels were not different. Heterofermentative lactobacilli produce acetate rather than ethanol as long as
286 electron acceptors are available (Korakli et al., 2001; Stolz et al., 1995). The extended mould-free shelf
287 life of bread produced with *L. brevis* sourdoughs containing sucrose is thus attributable to unknown
288 factors and may relate to a moderate reduction of the water activity.

289 Replacement of wheat with other substrates for sourdough fermentation and / or baking significantly
290 impacts the mould-free shelf life of bread (Axel et al., 2015 and 2016). Different substrates support
291 formation of different levels of organic acids (Axel et al., 2015) and are a potential source of plant
292 bioactives with antifungal activity (Gänzle, 2014). We explored the use of flaxseed sourdough; flaxseed
293 is rich in linoleic acid (Dubois et al., 2007) and may support the enzymatic or microbial formation of
294 antifungal hydroxy fatty acids from linoleic acid. In addition, flaxseed offers health benefits in relation
295 to cardiovascular diseases that are derived from its high fibre content and the content of ω-3 fatty acids
296 (Caligiuri et al., 2014; Cunnane et al., 1995; Kajla et al., 2015). Fungal growth on bread produced with
297 flaxseed or flaxseed sourdoughs was equal or faster when compared to the wheat counterparts. Bread
298 produced with flaxseed sourdoughs contained higher levels of acetate than the corresponding wheat
299 breads; however, flaxseed also contains mucilage with high water binding capacity (Kaewmanee et al.,
300 2014). Hydrocolloids may increase the water activity of bread and hence accellerate fungal spoilage. Our
301 data suggest that linoleic acid bound in triglycerides does not support formation of the antifungal 10-
302 hydroxy-12-octadecaenoic acid by *L. hammesii* in flaxseed sourdoughs. Bacterial hydration of free
303 unsaturated fatty acids is a mechanisms of detoxification (Volkov et al., 2010) and past studies aiming
304 to convert plant oil to bioactive lipids by lactic acid bacteria employed lipase to achieve hydrolysis of
305 triglycerides (Ogawa et al., 2005).

306 In conclusion, we demonstrate that the *in vitro* MIC of bacterial metabolites and preservatives is
307 matches the *in situ* antifungal effect. We also demonstrated that the accumulation of antifungal

metabolites in sourdough is a difficult proposition – because sourdough is used at a dosage of only 10 – to 20%, antifungal metabolites are relevant only if they are produced in bread dough, or if the concentration of antifungal metabolites in sourdough need to exceed the MIC 5 – 10 fold. Acetic acid is the most significant antifungal metabolite of lactobacilli, mainly because it is rapidly produced during mixing and proofing of the bread dough and is thus present in bread at concentrations close to the MIC. Irrespective of the presence of antifungal metabolites, however, the use of sourdough greatly enhances the activity of weak organic acids through the reduction of pH, and allows to exploit additive antifungal activities of different organic acids. We demonstrated additive activity of sourdough use with sorbic acid, propionic acids, and ricinoleic acid; in addition, the study provides a conceptual template for exploration of synergistic or additive effects of sourdough with other antifungal additives or ingredients.

References

Anonymous, 2018a. Maple Leaf Foods Food Manifesto, http://mapleleaf.com/our-food-promise/; accessed on May 9th, 2018.

Anonymous, 2018b. Transparency and Simplicity, clean label consumer study, downloaded from https://www.cargill.com/food-beverage/na/clean-label-white-paper-info-page accessed on May 9th, 2018

Axel, C., Brosnan, B., Zannini, E., Peyer, L.C., Furey, A., Coffey, A., Arendt, E.K. 2016. Antifungal activities of three different *Lactobacillus* species and their production of antifungal carboxylic acids in wheat sourdough. Appl. Microbiol. Biotechnol. 100, 1701-1711.

Axel, C., Röcker, B., Brosnan, B., Zannini, E., Furey, A., Coffey, A., Arendt, E.K. 2015. Application of *Lactobacillus amylovorus* DSM19280 in gluten-free sourdough bread to improve the microbial shelf life. Food Microbiol. 47, 36-44.

330 Axel, C., Zannini, E., Arendt, E.K. 2017. Mold spoilage of bread and its biopreservation: A review of
331 current strategies for bread shelf life extension. Crit. Rev. Food Sci. Nutr. 57, 3528–3542.

332 Belz, M.C., Mairinger, R., Zannini, E., Ryan, L.A., Cashman, K.D., Arendt, E.K. 2012. The effect of
333 sourdough and calcium propionate on the microbial shelf-life of salt reduced bread. Appl. Microbiol.
334 Biotechnol. 96, 493-501.

335 Black, B.A., Zannini, E., Curtis, J.M., Gänzle, M.G., 2013. Antifungal hydroxy fatty acids produced
336 during sourdough fermentation: Microbial and enzymatic pathways, and antifungal activity in bread.
337 Appl. Environ. Microbiol. 79, 1866–1873.

338 Caligiuri, S.P.B., Aukema, H.M., Ravandi, A., Guzman, R., Dibrov, E., Pierce, G.N., 2014. Flaxseed
339 consumption reduces blood pressure in patients with hypertension by altering circulating oxylipins via
340 an α-linolenic acid-induced inhibition of soluble epoxide hydrolase. Hypertension 64, 53–59.

341 Cunnane, S.C., Hamadeh, M.J., Liede, A.C., Thompson, L.U., Wolever, T.M.S., Jenkins, D.J.A. 1995.
342 Nutritional attributes of traditional flaxseed in healthy young adults. Am. J. Clin. Nutr. 61, 62–68.

343 Dal Bello, F., Clarke, C.I., Ryan, L.A.M., Ulmer, H., Schober, T.J., Ström, K., Sjögren, J., van
344 Sinderen, D., Schnürer, J., Arendt, E.K., 2007. Improvement of the quality and shelf life of wheat bread
345 by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. J. Cereal Sci., 45, 309–
346 318.

347 Decimo, M., Quattrini, M., Ricci, G., Fortina, M.G., Brasca, M., Silvetti, T., Manini, F., Erba, D.,
348 Criscuoli, F., Casiraghi, M.C., 2017. Evaluation of microbial consortia and chemical changes in
349 spontaneous maize bran fermentation. AMB Express, 7, 205.

350 Denyer, S.; Baird, R (Eds). 2006. Guide to Microbiological Control in Pharmaceuticals and Medical
351 Devices, second Ed. CRC Press, Boca Raton.

352 Dubois, V., Breton, S., Linder, M., Fanni, J., Parmentier, M. 2007. Fatty acid profiles of 80 vegetable
353 oils with regard to their nutritional potential. Eur. J. Lipid Sci. Technol. 109, 710–732.

354 Gänzle, M.G. 2015. Lactic metabolism revisited: Metabolism of lactic acid bacteria in food
355 fermentations and food biotechnology. Curr. Opin. Food Sci. 2, 106–117.

356 Gänzle, M.G., Hertel, C., Hammes, W.P., 1999. Resistance of *Escherichia coli* and *Salmonella* against
357 nisin and curvacin A. Int. J. Food Microbiol. 48, 37–50.

358 Gerez, C.L., Torino, M.I., Rollán, G., Font de Valdez, G. 2009. Prevention of bread mould spoilage by
359 using lactic acid bacteria with antifungal properties. Food Control 20, 144–148.

360 Gobbetti, M., Rizzello, C.G., Di Cagno, R., De Angelis, M., 2014. How the sourdough may affect the
361 functional features of leavened baked goods. Food Microbiol. 37, 30–40.

362 Gray, J.A., Bemiller, J.N. 2003. Bread staling: molecular basis and control. Comp. Rev. Food Sci. Food
363 Saft.2, 1 - 21.

364 Hammes, W.P., Gänzle, M.G., 1998. Sourdough breads and related products, in: Wood, B.J.B. (Ed.),
365 Microbiology of Fermented Foods. Springer US, Boston, MA, pp. 199–216.

366 Hansen, Å., Schieberle, P. 2005. Generation of aroma compounds during sourdough fermentation:
367 applied and fundamental aspects. Trends Food Sci. Technol. 16, 85–94.

368 Kaditzky, S., Seitter, M., Hertel, C., Vogel, R.F., 2008. Performance of *Lactobacillus sanfranciscensis*
369 TMW 1.392 and its levansucrase deletion mutant in wheat dough and comparison of their impact on
370 bread quality. Eur. Food Res. Technol. 227, 433–442.

371 Kaewmanee, T., Bagnasco, L., Benjakul, S., Lanteri, S., Morelli, C.F., Speranza, G., Cosulich, M.E.
372 2014. Characterisation of mucilages extracted from seven Italian cultivars of flax. Food Chem. 148, 60-
373 69.

374 Kajla, P., Sharma, A., Sood, D.R., 2015. Flaxseed—a potential functional food source. J. Food Sci.
375 Technol. 52, 1857–1871.

376 Korakli, M., Rossmann, A., Gänzle, M.G., Vogel, R.F. 2001. Sucrose metabolism and
377 exopolysaccharide production in wheat and rye sourdoughs by *Lactobacillus sanfranciscensis*. J. Agric.
378 Food Chem. 49, 5194-5200.

379 Liang, N., Cai, P., Wu, D., Pan, Y., Curtis, J.M., Gänzle, M.G. 2017. High-speed counter-current
380 chromatography (HSCCC) purification of antifungal hydroxy unsaturated fatty acids from plant-seed
381 oil and *Lactobacillus* cultures. J. Agric. Food Chem. 65, 11229–11236.

382 Lind, H., Jonsson, H., Schnürer, J. 2005. Antifungal effect of dairy propionibacteria - Contribution of
383 organic acids. Int. J. Food Microbiol. 98, 157–165.

384 Magnusson, J., Schnürer, J. 2001. *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 produces a
385 broad-spectrum proteinaceous antifungal compound. Appl. Environ. Microbiol. 67, 1–5.

386 Mandel, V., Sen, S.K., Mandel, N.C. 2013. Production and partial characterisation of an inducer-
387 dependent novel antifungal compound(s) by *Pediococcus acidilactici* LAB 5. J. Sci Food Agric. 93,
388 2445 – 2453.

389 Ogawa, J., Kishino, S., Ando, A., Sugimoto, S., Mihara, K., Shimizu, S. 2005. Production of
390 conjugated fatty acids by lactic acid bacteria. J. Biosci. Bioeng. 100, 355–364.

391 Quattrini, M., Bernardi, C., Stuknytė, M., Masotti, F., Passera, A., Ricci, G., Vallone, L., De Noni, I.,
392 Brasca, M., Fortina, M.G. 2018. Functional characterization of *Lactobacillus plantarum* ITEM 17215:

A potential biocontrol agent of fungi with plant growth promoting traits, able to enhance the nutritional value of cereal products. Food Res. Int. 106, 936–944.

Rizzello, C.G., Lavecchia, A., Gramaglia, V., Gobbetti, M., 2015. Long-term fungal inhibition by *Pisum sativum* flour hydrolysate during storage of wheat flour bread. Appl. Environ. Microbiol. 81, 4195–4206.

Rizzello, C.G., Verni, M., Bordignon, S., Gramaglia, V., Gobbetti, M., 2017. Hydrolysate from a mixture of legume flours with antifungal activity as an ingredient for prolonging the shelf-life of wheat bread. Food Microbiol. 64, 72–82.

Ryan, L.A., Zannini, E., Dal Bello, F., Pawlowska, A., Koehler, P., Arendt, E.K. 2011. *Lactobacillus amylovorus* DSM 19280 as a novel food-grade antifungal agent for bakery products. Int. J. Food Microbiol. 146, 276-283.

Ryan, L.A.M., Dal Bello, F., Arendt, E.K., 2008. The use of sourdough fermented by antifungal LAB to reduce the amount of calcium propionate in bread. Int. J. Food Microbiol. 125, 274–278.

Ryan, L.A.M., Dal Bello, F., Czerny, M., Koehler, P., Arendt, E.K., 2009. Quantification of phenyllactic acid in wheat sourdough using high resolution gas chromatography-mass spectrometry. J. Agric. Food Chem. 57, 1060–1064.

Salminen, A., Latva-Kala, K., Randell, K., Hurme, E., Linko, P., Ahvenainen, R., 1996. The effect of ethanol and oxygen absorption on the shelf-life of packed sliced rye bread. Packag. Technol. Sci. 9, 29–42.

Sirot, V., Fremy, J.M., Leblanc, J.C. 2013. Dietary exposure to mycotoxins and health risk assessment in the second French total diet study. Food Chem. Toxicol. 52, 1–11.

414 Smith, J.P., Daifas, D.P., El-Khoury, W., Koukoutsis, J., El-Khoury, A. 2004. Shelf life and safety
415 concerns of bakery products - A review. Crit. Rev. Food Sci. Nutr. 44, 19–55.

416 Stolz, P., Vogel, R.F., Hammes, W.P., 1995. Utilization of electron acceptors by lactobacilli isolated
417 from sourdough. Zeitschrift für Leb. und Forsch. 201, 402–410.

418 Stratford, M., Eklund, T., 2003. Organic acids and esters, in: Russell, N.J., Gould, G.W. (Eds.), Food
419 Preservatives. Springer US, Boston, MA, pp. 48–84.

420 Valcheva, R., Korakli, M., Onno, B., Ivanova, I., Ehrmann, M.A., Dousset, X., Gänzle, M.G., Vogel,
421 R. 2006. *Lactobacillus hammesii* sp. nov., isolated from French sourdough. Int. J. System. Evol.
422 Microbiol., 55, 763–767.

423 Vermeulen, N., Gánzle, M.G., Vogel, R.F., 2006. Influence of peptide supply and cosubstrates on
424 phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM20451^{T} and *Lactobacillus plantarum*
425 TMW1.468. J. Agric. Food Chem. 54, 3832–3839.

426 Volkov, A., Liavonchanka, A., Kamneva, O., Fiedler, T., Goebel, C., Kreikemeyer, B., Feussner, I.,
427 2010. Myosin cross-reactive antigen of *Streptococcus pyogenes* M49 encodes a fatty acid double bond
428 hydratase that plays a role in oleic acid detoxification and bacterial virulence. J. Biol. Chem. 285,
429 10353–10361.

430 Zhang, C., Brandt, M.J., Schwab, C., Gänzle, M.G., 2010. Propionic acid production by cofermentation
431 of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough. Food Microbiol. 27, 390–395.

432

Table 1 Ingredients in bread formulation with chemical preservatives and their combinations.

Ingredients (g)	Control	Ca-propionate	Phenyllactate	Sorbic acid	Ricinoleic acid	Acetic acid	Ca-propionate + acetic acid	Sorbic acid + acetic acid
Wheat flour	100	100	100	100	100	100	100	100
Water	60	60	60	60	60	60	60	60
Yeast	2	2	2	2	2	2	2	2
Salt	2	2	2	2	2	2	2	2
Canola oil	2	2	2	2	2	2	2	2
Calcium propionate	-	0.25	-	-	-	-	0.058	-
3-Phenyllactate	-	-	0.42	-	-	-	-	0.002
Sorbic acid	-	-	-	0.01	-	-	-	-
Ricinoleic acid	-	-	-	-	0.5	-	-	-
Lactic acid	-	-	-	-	-	0.18	-	-
Acetic acid	-	-	-	-	-	0.25	0.037	0.037

Table 2 Ingredients of sourdough bread. Wheat or flaxseed sourdoughs were fermented with *L. hammesii*, or *L. plantarum* or *L. brevis*. 10% of the experimental sourdough was added to bread dough.

Ingred. (g)	Non-fermented control		Sourdough (*L. brevis*, *L. hammesii* or *L. plantarum*)						*L. hammesii* wheat sourdough				*L. hammesii* flaxseed sourdoug			
	Wheat	Flax	Wheat	Flax	Wheat + sucrose	Flax + sucrose	Prop.[1)]	sorbic acid	Linoleic acid	Ricinoleic acid			Linoleic acid	Ricinoleic acid		
Wheat	100	90	90	90	90	90	90	90	90	90	90	90	90	90	90	
Flaxseed		10														
Water	60	60	50	50	50	50	50	50	50	50	50	50	50	50	50	
Yeast	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	
Salt	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	
Canola oil	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	
Prop.							0.058									
Sorb.								0.002								
Ricinol.										0.037	0.075	0.15		0.037	0.075	
Linol.									2				2			
Sucrose[2)]					0.8	0.8										
Sourd.[3)]			20	20	20	20	20	20	20	20	20	20	20	20	20	

[1)] Prop. = Ca propionate; sorb. = sorbic acid; ricinol. = ricinoleic acid; linol. = linoleic acid. [2)] Sucrose was added to the sourdough.

[3)] Sourdough, prepared with 10 g water and 10 g flaxseed flour or wheat flour and sucrose as indicated.

Table 3. Effect of preservatives alone or in combination on the mould-free shelf life of bread. Preservatives were added as indicated in Table 2 to match their MIC *in vitro*. Data are shown as means ± standard deviations of three independent experiments. Values in the same row that do not share a common superscript differ significantly ($p<0.05$).

Additive	Control	3-PLA	Ricinoleic acid	Acetic acid	Prop.	Sorb.	Prop. + acetic	Sorb. + acetic
pH	5.4±0.1^{a}	4.4±0.0^{b}	5.3±0.0^{a}	4.4±0.0^{b}	5.4±0.0^{a}	5.1±0.1^{a}	4.8±0.7^{ab}	4.9±0.5^{ab}
Indicator	Bread mould-free shelf life (d)							
A. niger	3.6±1.1^{b}	5.3±0.5^{b}	4.3±1.1^{b}	9.7±0.5^{a}	8.3±1.1^{a}	10.0±1^{a}	8.5±0.7^{a}	6.0±0.0^{ab}
P. roqueforti	4.3±0.1^{b}	5.0±1.0^{b}	4.7±1.1^{b}	9.3±0.5^{a}	8.0±1.0^{a}	9.0±0.7^{b}	7.5±0.3^{ab}	6.5±0.7^{ab}

PLA = 3 phenyllactic acid; Prop. = Ca propionate; sorb. = sorbic acid

Table 4 Effect of sourdough on the pH and the mould-free shelf life of bread. The sourdough was fermented with *L. hammesii*, *L. plantarum* or *L. brevis*, with or without addition of 4% sucrose; *L. hammesii* sourdough was combined with calcium propionate (3.1 mM) or sorbic acid (0.16 mM). The challenge test was with two indicator strains. Data are shown as means ± standard deviations of three independent experiments. Values obtained for different breads with the same indicator strain differ significantly if they do not share a common superscript ($p<0.05$).

	Not fermented	***L. hammesii***	***L. plantarum***	***L. brevis***	***L. hammesii* + propionate**	***L. hammesii* + sorbic acid**	***L. hammesii* + sucrose**	***L. plantarum* + sucrose**	***L. brevis* + sucrose**
					A. niger				
Wheat	3.0±0.6^{c}	4.8±0.3^{b}	4.3±0.6^{b}	4.7±0.6^{b}	10.5±0.7^{a}	7.0±1.4^{a}	5.5±0.7^{b}	5.0±0.0^{b}	9.0±0.0^{a}
Flaxseed	3.0±0.0^{c}	5.0±0.6^{b}	3.6±0.6^{c}	3.7±0.6^{c}	n.d.	n.d.	6.5±0.0^{b}	5.0±0.0^{b}	9.0±0.0^{a}
					P. roqueforti				
Wheat	5.3±0.6^{b}	5.3±0.6^{b}	5.0±0.0^{b}	5.0±0.0^{b}	8.3±0.3^{a}	5.5±0.7^{b}	6.5±0.7^{ab}	5.5±0.7^{b}	8.5±0.7^{a}
Flaxseed	3.3±0.6^{c}	5.0±0.0^{b}	3.6±0.6^{c}	4.3±0.6^{c}	n.d.	n.d.	6.5±0.7^{ab}	5.5±0.7^{b}	8.5±0.0^{a}
					pH				
Wheat	5.4±0.6^{a}	4.3±0.1	4.3±0.1	4.3±0.0	4.2±0.2	4.1±0.2	4.5±0.3	4.3±0.1	4.6±0.2
Flaxseed	5.3±0.1^{a}	4.6±0.1	4.5±0.1	4.5±0.6	n.d.	n.d.	4.4±0.0	4.3±0.0	4.3±0.1

n.d., not determined.

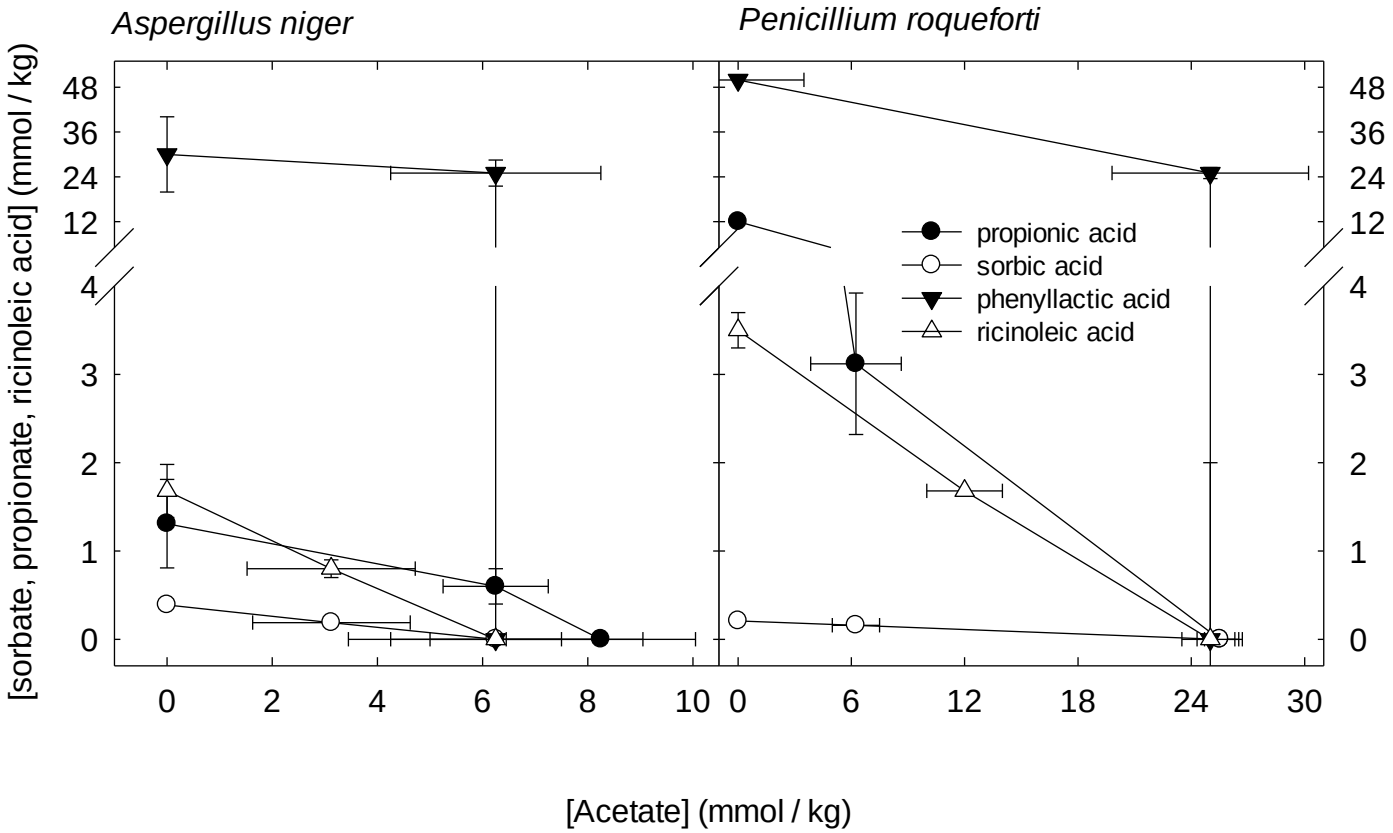

Figure 1. Minimum inhibitory concentration of acetic acid in combination with sorbic acid, propionic acid, phenyllactic acid, or ricinoleic acid. The minimum inhibitory concentrations were evaluated at a pH of 4,50. The results are shown as means ± standard deviations of three independent experiments.

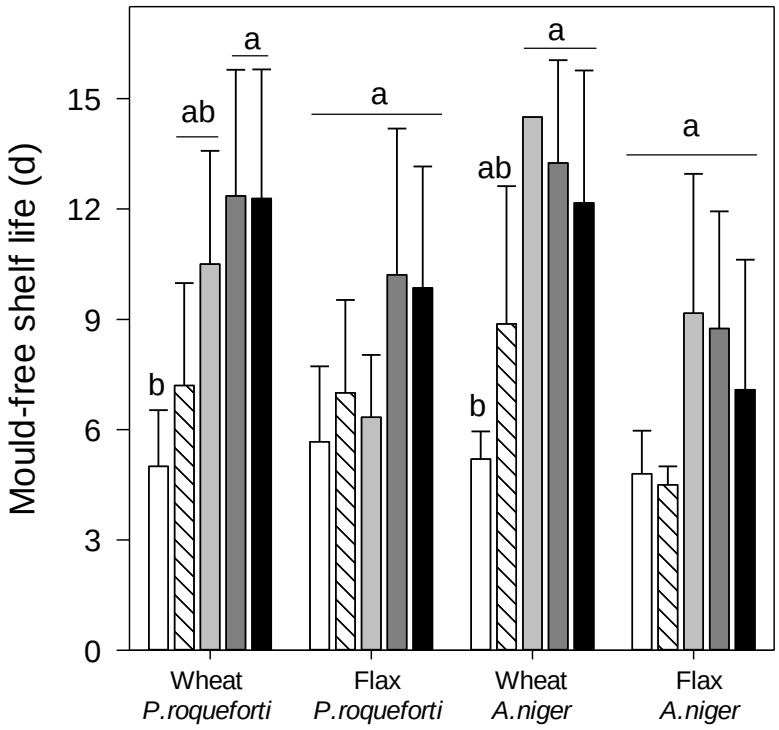

Figure 2. Effect of sourdough in combination with ricinoleic acid on the mould-free shelf life of bread. Control bread was produced without addition of sourdough (white bars); *L. hammesii*-fermented sourdough bread was produced with addition 2% linoleic acid during sourdough fermentation (white hatched bars); or with addition of 0.03% (gray bars), 0.08% (dark gray bars) or 0.15% ricinoleic acid (black bars) added at the bread stage. Experiments were done with wheat sourdough or flaxseed sourdough as indicated and *Penicillium roqueforti* and *Aspergillus niger* were used as challenge organisms. Data are shown as mean ± standard deviations of seven independent experiments. Values produced with the same sourdough and challenged with the same organism differ ($p<0.05$) if they do not share a common superscript.