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

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ABSTRACT

Fungal spoilage of bread remains an unsolved issue in bread making. This work aims 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 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); calcium propionate and sorbic acid are widely used as preservatives in pre-packed and sliced 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 produce metabolites with antifungal activity; however, 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 produced in primary carbohydrate metabolism has antifungal activity but also impacts flavour and texture of bread (Gerez et al., 2009; Drews, 1953; 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). Co-fermentation of *L. diolivorans* and *L. buchneri*

produced propionic acid in sourdough; however, propionic acid also impacts bread flavour when added at effective concentrations (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., 2009a and 2009b; Vermeulen et al., 2006). Hydroxylated unsaturated fatty acids have antifungal activity in bread but their accumulation to active concentrations in sourdough remains to be demonstrated (Black et al., 2013; Liang et al., 2017). *In situ* preservative effects of lactic acid bacteria have often been attributed to synergistic activities of uncharacterized compounds (Axel et al., 2017; Mandel et al., 2013).

Plant-derived antifungal compounds support the antifungal activity of bacterial metabolites. For example, hop extract was recently demonstrated to be an effective antifungal ingredient in bread making (Nionelli et al.,2018); compounds with antifungal activity isolated from legume flours (*Pisum sativum*, *Phaseolus vulgaris*) were also successfully employed to extend the mould-free shelf life of wheat bread (Rizzello et al., 2017 and 2015). Flaxseeds have a high oil content with a high proportion of linoleic acid, a substrate for enzymatic or microbial conversion to hydroxy-fatty acids (Black et al., 2013). The microbial and enzymatic conversion products of free linoleic acid, 10-hydroxy-12-octadecenoic and coriolic acids, respectively, have similar antifungal activity (Black et al., 2013; Liang et al., 2017).

The use of multiple antifungal metabolites to exploit synergies improves the antifungal effect of sourdough while minimizing the impact of organic acids on bread flavour (Ryan et al., 2008; Zhang et al., 2010). However, synergistic effects of different antifungal metabolites have not been systematically assessed by comparison of the correlation of *in vitro* MIC and *in situ* preservative effects (Axel et al., 2017). This study therefore aimed to compare the inhibitory concentration of antifungal compounds to their antifungal effect in bread. Antifungal compounds were assessed in 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. Representative of common fungal spoilage of bread, *Aspergillus niger* FUA5001 and *Penicillium roqueforti* FUA5005, were used as target strains for the antifungal assay. *P. roqueforti* is an isolate from mouldy bread with high resistance to antifungal interventions (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.

114 *2.3 Sourdough fermentation and bread preparation*

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

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

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

136 *2.4 Bread challenge test against* P. roqueforti *and* A. niger

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

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

Acetic acid was determined by HPLC with an Aminex HPX-87 column (300 mm × 7.8 mm, Biorad, 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 injection volume was 10 μL. A refractive index detector and UV detector (210 nm) were used for detection. For sample preparations, 2 g of bread was diluted with 10 mL of MilliQ water and incubated for 3 h at 80 °C. After centrifugation, 7% perchloric acid were added and the solution incubated at 4 °C overnight. Precipitated protein was removed by centrifugation. The samples were filtered before injection in the column.

3. Results

3.1 MIC of preservatives and combination effects

The individual MIC for each of the five compounds was tested *in vitro* against the two indicator strains *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 mM for *A. niger* and *P. roquefortii*, respectively), followed by propionic acid (1.3 ± 0.2 and 12.0 ± 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).

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

3.2 Antifungal effect of organic acids addition to bread

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

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

3.3. Antifungal effect of sourdough addition to bread

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

sourdough, 0.03% to 0.15% ricinoleic acid, corresponding to 1 to 5 mM, were added to bread produced with *L. hammesii* wheat and flaxseed sourdoughs. Sourdough fermented with addition of 2% linoleic acid, the substrate for formation of the antifungal 10-hydroxy-12-octadecenoic acid by *L. hammesii*, was additionally evaluated. Addition of 0.08 or 0.15% ricinoleic acid increased the shelf life of wheat bread challenged with *A. niger* or *P. roquefortii* to more than 12 days (Figure 2); addition of 0.03% ricinoleic acid was effective only against *A. niger*. Addition of linoleic acid to sourdoughs fermented with *L. hammesii* did not delay fungal growth (Fig. 2). An extension of the shelf life by sourdough in combination with ricinoleic acid was not observed in wheat bread with flaxseed sourdough; the increase of the average shelf life was less than experimental error (Figure 2).

4. Discussion

Bread is subject to rapid deterioration after baking**.** Fungal spoilage is one of the main causes of bread spoilage**.** Moreover, formation of mycotoxins production by filamentous fungi represents a health risk (Sirot et al., 2013). *P. roquefortii*, one of the challenge strains used in this study, is resistant to biological or chemical preservation; this organism also often occurs as spoilage agent in bread (Axel et al., 2017). An inoculum of 100 – 1000 spores per slice of bread (Nionelli et al., 2018, Ryan et al., 2011, Zhang et al., 2010) is substantially higher than the environmental contamination in industry practice. Environmental mould contamination is difficult to control and to reproduce, however, studies on the mould-free shelf life of bread consistently demonstrate that spoilage by environmental contaminants is substantially slower and more readily controlled by preservatives when compared to bread challenged with *Penicillium* spp. (Axel et al., 2015; Belz et al., 2012; Black et al., 2013). Challenge studies with *P. roqueforti* therefore represent a worst case scenario but nevertheless allow allow comparative assessment of different sourdoughs or additives.

We compared the *in vitro* MIC of antifungal bacterial metabolites and preservatives. Phenyllactic acid has the weakest antifungal activity. Inhibition of fungal growth at pH 4.5 was observed only at

concentrations exceeding 30 mmol / L, corresponding to 5 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., 2009a; Vermeulen et al., 2006). The combination of different organic acids displays additive rather than synergistic activity when adjusting for the pH; therefore, phenyllactate is not likely to make a contribution to inhibition of fungal growth in bread.

Propionic acid, 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 is estimated at 4.74; acetic acid, sorbic acid, and propionic acid have a pKa of 4.75, 4.76, and 4.90, respectively. Their activity in sourdough bread with pH < 5.0 is thus 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. Of note, the Joint Food and Agriculture Organization / World Health Organization Expert Committee on Food Additives established an acceptable daily intake of castor oil, the primary source of ricinoleic acid, of 0.7 mg/kg body weight. However, ricinoleic acid is approved for use in food and the acceptable daily intake of ricinoleic acid was estimated to be much higher, 2.4 g per person and day (Burdock et al., 2006).

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 delays fungal spoilage (Drews, 1953); however, excess 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 (Kaditzky et al., 2008). Acetic acid in concentrations of 10 – 30 mmol kg^{-1} has a beneficial impact on bread flavor (Hansen and Schieberle, 2005); the current study demonstrates that this range of acetic acid concentration also substantially contributes to the mould-free storage life of bread.

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). We extended prior observations by demonstrating additive activity of sourdough or acetic acid with propionic acid, ricinoleic acid and sorbic acid. The antifungal effect of acetic acid in combination with other antifungal organic acids is attributable to the additive antifungal activity of organic acids (Tables 3 and 4). In combination with acetic acid or sourdough, the propionate or sorbate 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. Remarkably, ricinoleic acid was effective only in combination with sourdough.

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 0.15% 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%.

Sucrose addition to sourdough did not substantially increase the acetate concentration in bread. The availability of substrates for co-factor regeneration in wheat sourdough supports formation of 10 – 20 mmol / g acetate in wheat sourdough; the acetate concentration can be increased by addition of sucrose (Korakli et al., 2001). With a sourdough addition of 10% - 30%, the carry-over of acetic acid from sourdough accounts for only 2 – 6 mmol / g and most of the acetic acid that is present in bread, 10 – 20 mmol / g, was produced after the final mixing in the bread dough where sucrose levels were not different. Heterofermentative lactobacilli produce acetate rather than ethanol as long as electron acceptors are available (Korakli et al., 2001; Stolz et al., 1995). In artisanal and industrial practice, the sourdough addition to bread dough ranges from as little as 3% for high acidity, long time fermented type II sourdoughs to more than 30% for metabolically active type I sourdoughs with a relatively high pH and low acidity (Brandt, 207; Gänzle and Zheng, 2018; Lacaze et al., 2007). Independent on the level of addition, however, antifungal compounds present in sourdough are diluted three-fold to more than 10-fold. Sourdoughs that are propagated in bakeries typically are fermented to warrant a high metabolic activity of lactobacilli in bread dough (Gänzle and Zheng, 2018; Tang et al., 2017), however, a substantial proportion of industrial sourdough products does not warrant metabolic activity of sourdough microbiota in bread dough (Brandt, 2007, Lacaze et al., 2007). In brief, the impact of sourdough on the mould-free shelf life of bread necessitates metabolic activity of sourdough microbiota during proofing and hence depends strongly on the sourdough technology employed,

Replacement of wheat with other substrates for sourdough fermentation and / or baking significantly impacts the mould-free shelf life of bread (Axel et al., 2016 and 2015). Different substrates support formation of different levels of organic acids (Axel et al., 2015) and are a potential source of plant bioactives with antifungal activity (Gänzle, 2014). We explored the use of flaxseed sourdough; flaxseed

304 is rich in linoleic acid (Dubois et al., 2007) and may support the enzymatic or microbial formation of
305 antifungal hydroxy fatty acids from linoleic acid. In addition, flaxseed offers health benefits in relation
306 to cardiovascular diseases that are derived from its high fibre content and the content of ω-3 fatty acids
307 (Caligiuri et al., 2014; Cunnane et al., 1995; Kajla et al., 2015). Fungal growth on bread produced with
308 flaxseed or flaxseed sourdoughs was equal or faster when compared to the wheat counterparts. Bread
309 produced with flaxseed sourdoughs contained higher levels of acetate than the corresponding wheat
310 breads; however, flaxseed also contains mucilage with high water binding capacity (Kaewmanee et al.,
311 2014). Hydrocolloids may increase the water activity of bread and hence accelerate fungal spoilage. Our
312 data suggest that linoleic acid bound in triglycerides does not support formation of the antifungal 10-
313 hydroxy-12-octadecaenoic acid by *L. hammesii* in flaxseed sourdoughs. Bacterial hydration of free
314 unsaturated fatty acids is a mechanism of detoxification (Volkov et al., 2010) and past studies aiming to
315 convert plant oil to bioactive lipids by lactic acid bacteria employed lipase to achieve hydrolysis of
316 triglycerides (Ogawa et al., 2005).

317 In conclusion, we demonstrate that the *in vitro* MIC of bacterial metabolites and preservatives matches
318 the *in situ* antifungal effect. We also demonstrated that the accumulation of antifungal metabolites in
319 sourdough is a difficult proposition – because sourdough is used at a dosage of only 10 –to 20%,
320 antifungal metabolites are relevant only if they are produced in bread dough, or if the concentration of
321 antifungal metabolites in sourdough exceeds the MIC by a factor of at least 5 – 10. Acetic acid is the
322 most significant antifungal metabolite of lactobacilli, mainly because it is rapidly produced during
323 mixing and proofing of the bread dough and is thus present in bread at concentrations close to the MIC.
324 Irrespective of the presence of antifungal metabolites, however, the use of sourdough greatly enhances
325 the activity of weak organic acids through the reduction of pH, and allows to exploit additive antifungal
326 activities of different organic acids. We have demonstrated additive activity of sourdough use with sorbic

acid, propionic acid, and ricinoleic acid. In addition, the study provides a conceptual template for the exploration of synergistic or additive effects of sourdough with other antifungal additives or ingredients.

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465

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.

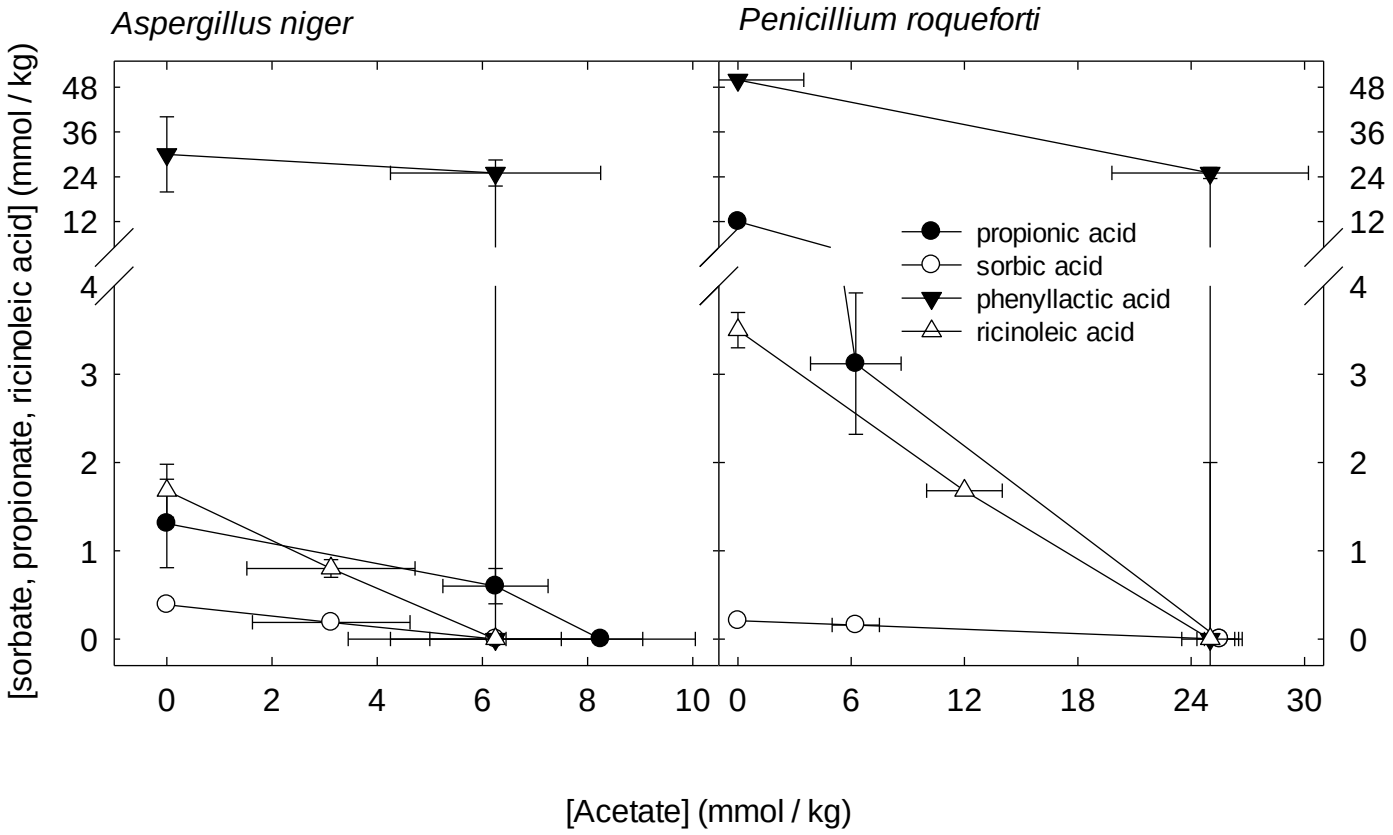

Figure 1. Minimum inhibitory concentration of acetic acid in combination with sorbic acid, propionic acid, phenyllactic acid, or ricinoleic acid. The minimum inhibitory concentrations were evaluated at a pH of 4,50. The results are shown as means ± standard deviations of three independent experiments.

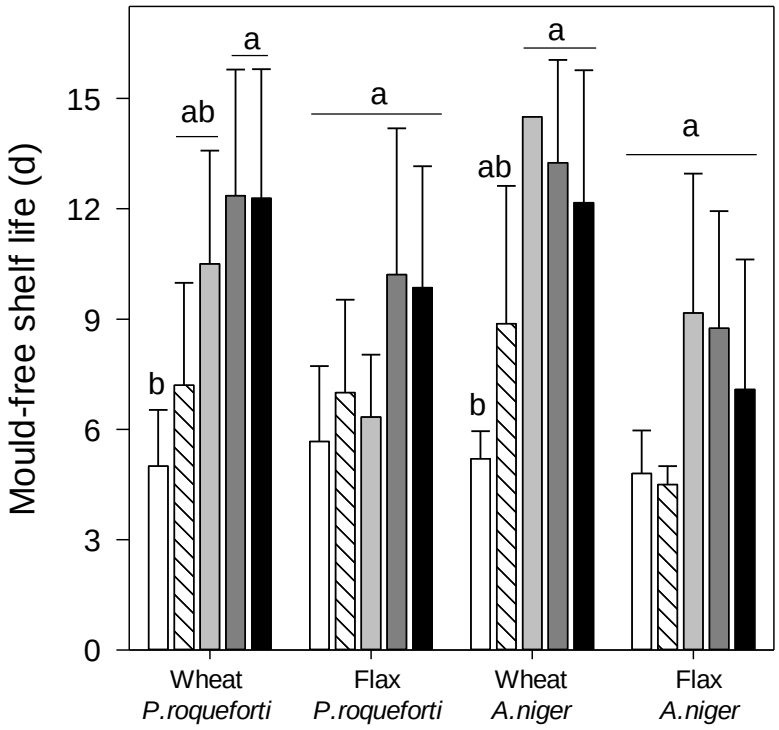

Figure 2. Effect of sourdough in combination with ricinoleic acid on the mould-free shelf life of bread. Control bread was produced without addition of sourdough (white bars); *L. hammesii*-fermented sourdough bread was produced with addition 2% linoleic acid during sourdough fermentation (white hatched 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.