Antifungal hydroxy-fatty acids produced during sourdough fermentation:
  microbial and enzymatic pathways, and antifungal activity in bread

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ABSTRACT

Lactobacilli convert linoleic acid to hydroxy fatty acids; however, this conversion has not been demonstrated in food fermentations and it remains unknown whether hydroxy fatty acids produced by lactobacilli have antifungal activity. This study aimed to determine whether lactobacilli convert linoleic acid to metabolites with antifungal activity, and to assess whether this conversion can be employed to delay fungal growth on bread. Aqueous and organic extracts from seven strains of lactobacilli grown in mMRS or sourdough were assayed for antifungal activity. *L. hammesii* exhibited increased antifungal activity upon addition of linoleic acid as substrate. Bioassay-guided fractionation attributed the antifungal activity of *L. hammesii* to a mono-hydroxy C18:1 fatty acid. Comparison of its antifungal activity to other hydroxy fatty acid revealed that the mono-hydroxy fraction from *L. hammesii* and coriolic (13-hydroxy-9,11-octadecadienoic) acid were the most active with a MIC of 0.1 - 0.7 g L⁻¹. Ricinoleic (12-hydroxy-9-octadecenoic) acid was active at a MIC of 2.4 g L⁻¹. *L. hammesii* accumulated the mono-hydroxy C18:1 fatty acid in sourdough to a concentration of 0.73 ± 0.03 g L⁻¹. Generation of hydroxy fatty acids in sourdough also occurred through enzymatic oxidation of linoleic acid to coriolic acid. The use of 20% sourdough fermented with *L. hammesii*, or the use of 0.15% coriolic acid in breadmaking increased the mold-free shelf life by 2 – 3 days or from 2 to more than 6 days, respectively. In conclusion, *L. hammesii* converts linoleic acid in sourdough and the resulting mono-hydroxy octadecenoic acid exerts antifungal activity in bread.

KEYWORDS:

Antifungal activity, lactobacillus, coriolic acid, hydroxy-fatty acids, sourdough.
INTRODUCTION

Sourdough bread has an extended mold-free storage-life compared to conventionally leavened products (1, 2) and the presence of metabolites from specific strains of lactobacilli contributes to the prolonged storage-life of sourdough bread (3, 4, 5). While the fermentation microbiota of traditional sourdough is controlled by the fermentation conditions and the choice of raw materials, the industrial production of sourdough often relies on single strains of lactobacilli with defined metabolic properties (6, 7). To date, cyclic dipeptides, phenyllactic acid, acetic and propionic acids, and short-chain hydroxy-fatty acids have been identified as antifungal metabolites of sourdough lactobacilli (8, 9, 10). However, these compounds are either not produced in effective quantities in sourdough fermentations, or adversely affect the quality of the product when produced in active concentrations. Cyclic dipeptides, such as 2,5-diketopiperazines, are produced in quantities 1000-fold below the MIC against molds, and are accompanied by bitter or metallic flavors if present in higher quantities (11). Similarly, the amount of phenyllactic acid produced in sourdough is 1000 times less than the required amount for activity (8, 12, 13). Cooperative metabolism in sourdough of Lactobacillus buchneri and Lactobacillus diolivorans produced acetic and propionic acids in concentrations of 4 and 3 g L\(^{-1}\) respectively, in sourdough (10). Acetic and propionic acid formation during sourdough fermentation contributed to mold inhibition in bread (10), however, their concentrations remain below the MIC for mold inhibition at 7.2 g L\(^{-1}\) and 4.4 g L\(^{-1}\), respectively, and increased concentrations adversely affect sensory properties of bread.

Pseudomonas aeruginosa transformed linoleic acid to a mixture of mono-, di- and tri-hydroxy fatty acids with antifungal activity against wide range of crop fungal pathogens (14, 15).
However, P. aeruginosa is not suitable for use in food fermentations. Lactic acid bacteria also convert linoleic acid to hydroxy fatty acids (16, 17); however, this conversion was not demonstrated in food fermentations and it remains unknown whether hydroxy fatty acids produced by lactobacilli have antifungal activity. Hence, the aim of this study was to determine whether lactobacilli convert linoleic acid to metabolites with antifungal activity, to assess whether this conversion can be achieved in sourdough fermentation, and to determine whether conversion of linoleic acid in sourdough delays fungal spoilage of bread. The screening of lactobacilli focused on sourdough isolates that were previously shown to convert linoleic and oleic acids to hydroxylated metabolites (18).

**MATERIALS AND METHODS**

**Chemicals and standards.** 9-cis-12-cis-Octadecadienoic (linoleic) acid, 9-cis-12-hydroxy-octadecenoic (ricinoleic) acid, 12-hydroxystearic acid, octadecanoic (stearic) acid, 9-cis-octadecenoic (oleic) acid and distearin with > 99% purity, were purchased from Nu-Chek Prep, Inc. (Elysian, MN). 9,10-Dihydroxystearic acid (> 90%) was supplied by Pfaltz and Bauer (Waterbury, CT). Cysteine-HCl (≥ 98%), trizma hydrochloride (> 99%), lipoxidase from *Glycine max* (soybean) type I-B (≥ 50,000 units mg⁻¹) were purchased from Sigma-Aldrich, (St. Louis, MO). Fisher Scientific (Ottawa, Canada) supplied microbiological media, HPLC grade chloroform, methanol and acetic acid. Solvents were of analytical grade unless specified otherwise.

**Strains and growth conditions.** Lactobacillus sanfranciscensis ATCC27651, Lactobacillus reuteri LTH2584, Lactobacillus pontis LTH2587, Lactobacillus hammesii DSM16381, Lactobacillus plantarum TMW1460 and TMW1701 were cultivated on modified DeMan-
Lactobacilli were incubated under microaerophilic conditions (1% \( \text{O}_2 \), balance \( \text{N}_2 \)) for 24 h at 37 °C (\( L. \text{reuteri} \)) or 30 °C (all other strains). \textit{Mucor plumbeus FUA5003}, \textit{Aspergillus niger FUA5001} or \textit{Penicillium roqueforti FUA5005} (10) were used as target organisms for antifungal assays. Fungal cultures were grown on malt extract agar medium at 25 °C for 72 h and spores were harvested as described (10). Spore counts were standardized to \( 10^4 \) or \( 10^2 \) spores mL\(^{-1} \) with a haemocytometer (Fein-Optik, Jena, Germany).

**Screening of antifungal activity.** Strains were inoculated into 15 mL of mMRS and incubated for 24 h. The cells were washed twice with 0.85% NaCl and resuspended in 10 mL of 0.85% NaCl. For each strain, 5% (v/v) washed cells were inoculated into 20 mL mMRS broth. Additionally, linoleic acid at 0, 2 or 4 g L\(^{-1} \) was added. Inoculated broth was incubated with shaking at 120 rpm for 8 days. After 1, 4 and 8 days of incubation, the culture supernatant was collected by centrifugation and sterilized by filtration. Organic extracts of culture supernatants were obtained by extraction with chloroform 2:1 (v/v). The organic phase was collected by centrifugation and the upper layer was again extracted in the same manner. Organic extracts were dried under nitrogen gas and tested for antifungal activity.

Sourdough was prepared by mixing 10 g white wheat flour and 10 mL tap water, and inoculation with 5% (v/v) lactobacilli suspended in saline. Linoleic acid (0, 2, or 4 g kg\(^{-1} \)) was added as a substrate and dough was incubated at 30 °C, or 37 °C for 8 d. At days 1, 4 and 8, 2 g samples of sourdough were removed for extraction and analysis. All sourdough fermentations were routinely characterized with regards to cell counts and pH to verify growth of lactobacilli, and to ensure the identity of fermentation microbiota with the inoculum. Aqueous extracts were obtained by centrifugation (4000 × g for 10 min). To obtain organic extracts, 2 mL of water and 1.5 mL isopropanol were added to 2 g sourdough. The pH of the mixture was adjusted to 2.5 ± 0.5.
0.05 using 5 M HCl, followed by addition of NaCl to saturation to obtain phase separation. Solids were removed by centrifugation, and the organic phase was collected.

**Antifungal activity assay.** The assay used to determine MIC values were performed as serial two-fold dilutions using a microtiter plate well method described by Magnusson and Schnürer (19). Microtiter plates were inoculated with mMRS broth containing $10^4$ spores mL$^{-1}$ of *A. niger*, *M. plumbeus* or *P. roqueforti* and incubated at 25 °C. The MIC was determined as the lowest concentration of sample inhibition growth. Organic solvents in the samples were removed by evaporation under a laminar flow hood prior to the addition of fungal spores. Experiments were performed in triplicate.

**Combined Liquid Chromatography / Atmospheric Pressure Photo Ionization Mass Spectrometry (LC/APPI-MS).** Underivatized organic extracts were analyzed by LC/APPI-MS. Separations were conducted on an Agilent 1200 series LC system (Agilent Technologies; Palo Alto, CA) at 25 °C using a YMC PVA-Sil column (150 mm × 2.0 mm I.D., 5 µm, Waters Ltd.; Mississauga, Canada). Lipid samples dissolved in chloroform were eluted using an injection volume of 5 µL and a gradient of (A) hexane with 0.2% acetic acid and (B) isopropanol with 0.2% acetic acid at a flow rate of 0.2 mL min$^{-1}$. The gradient was as follows: 0 min 99% A; 20 min 70% A; 20.1 min 99% A; for a total run time of 27 min. Negative ion APPI-MS was performed on a QStar® Elite hybrid orthogonal Q-TOF mass spectrometer coupled to a PhotoSpray® source with Analyst® QS 2.0 software (Applied Biosystems/MDS Sciex; Concord, Canada). The source and mass spectrometer conditions were: nebulizer gas 70 (arbitrary units), auxillary gas 20, curtain gas 25, ionspray voltage -1300 V, source temperature 400 °C,
declustering potential (DP) -35 V, focusing potential -130 V and DP2 -13 V with a scanning mass range of m/z 50-700.

**Identification and quantitation of antifungal compounds.** For isolation of antifungal compounds, mMRS was fermented with *L. hammesii* or *L. sanfranciscensis* and 4 g L⁻¹ linoleic acid for 4 days. Cultures were extracted twice with two volumes chloroform/methanol 85:15 (v/v). The organic phase was then dried under vacuum at 30 °C and was stored at -20 °C under nitrogen gas. Up to 25 mg of extracted sample was loaded onto a conditioned Sep-Pak 500 mg silica cartridge (Waters Ltd.; Mississauga, Canada), washed with 20 mL of chloroform, and hydroxy fatty acids were eluted with 10 mL 50% isopropyl alcohol in chloroform (v/v). The hydroxy fatty acid fraction was dried under nitrogen and dissolved in chloroform at 30 mg mL⁻¹.

For further fractionation according to hydroxyl group number, semi-preparative high performance liquid chromatography was performed on an Agilent 1200 series LC system (Agilent Technologies; Palo Alto, CA). An injection volume of 100 µL was loaded onto a Zorbax Rx-SIL semi-prep column (9.4 mm × 250 mm I.D., 5 μm, Agilent Technologies). Separations were performed at 23 °C at a flow of 3 mL min⁻¹ a gradient analogous to the analytical column described above. Separations were monitored by a DAD at 210 nm, and confirmed by splitting the post-column flow to an ELSD at 60 °C with 3.5 standard L min⁻¹ nitrogen gas. Fractions were collected in 0.1 min time-slices, analyzed by mass spectrometry for purity, and assessed for their antifungal activity by MIC assays in triplicate.

For the further fractionation of C18:1 mono-hydroxy fatty acids, a Supelcosil LC-18-DB column (10mm × 250 mm I.D., 5 μm, Sigma-Aldrich; Oakville, Canada) was employed with a 3 mL min⁻¹ flow rate and a gradient of 50% acetonitrile and 50% water at 0 min, increasing to 100%
acetonitrile at 35 min. A total of 20 µL was injected of a 1 mg mL⁻¹ fatty acid extract in chloroform. Fractions were collected from 13 – 15.5 min and LC/APPI-MS confirmed the absence of other compounds prior to assessment for antifungal activity.

For relative quantification, peak areas percentages of hydroxy fatty acids were compared to the peak area of the same hydroxy fatty acid in extracts from cultures of *L. hammesii* supplemented with linoleic acid. mMRS and sourdough were fermented for 4 days with *L. hammesii* or *L. sanfranciscensis*, or were chemically acidified with 4:1 lactic/acetic acid (v/v) to pH 3.5. Linoleic acid was added at either 0 or 4 g L⁻¹. mMRS was extracted directly with methanol/chloroform as described above. Wheat doughs were extracted using the Bligh and Dyer method (20). Briefly, the wheat dough was lyophilized and to the dried sample 1:2:0.8 chloroform/methanol/water (v/v/v) was added. The mixture was homogenized and left at ambient temperature for 1 h. One part each chloroform and water was added for phase separation, the solution was mixed again and the lower phase was collected. Each broth and dough lipid extract was dried under nitrogen and reconstituted in chloroform/methanol 85:15 (v/v) to 1 mg mL⁻¹ with 5 µg mL⁻¹ distearin added as an internal standard. All samples were then analyzed by LC/APPI-MS and the Analyst® software was used to determine peak areas. Each peak was normalized using the response of the distearin internal standard. For optimization of lactobacilli metabolites over time, the same fermentations were prepared and sampled at 12 h intervals over 8 days. All relative quantifications were performed in triplicate independent experiments with a minimum of three technical repeats.

For absolute quantification of the antifungal fatty acid in sourdough starter, dough and bread, 200 mg lyophilized samples were extracted by the Bligh and Dyer method (20). The lyophilized samples were first spiked with 150 µg of ricinoleic acid standard to measure extraction recovery.
Each extraction was adjusted to a volume of 5 mL with chloroform after 25 µg of distearin internal standard was added. An external standard of ricinoleic acid was used to construct a calibration curve, with the assumption that ionization efficiency was similar to the unknown mono-hydroxy C18:1 product. All samples from triplicate independent experiments were analyzed in duplicate by LC/APPI-MS.

**Enzymatic production of coriolic acid.** To test the activity of different components of C18 fatty acids, 9-cis-11-trans-13-hydroxy-octadecadienoic (coriolic) acid was produced in a one-step method (18). Linoleic acid was added at to a concentration of 3 mM to a 0.1 M Trizma® hydrochloride buffer (pH 9.0) containing cysteine in a 4:1 molar ratio to linoleic acid. After the addition of 0.16 g L⁻¹ lipoxygenase, the reaction was carried out under a gentle stream of oxygen at room temperature for 5 min and then transferred to an incubator at 25 °C with shaking speed of 150 rpm for 25 min. At the end of incubation period, the buffer was adjusted to pH 2 with 1 N HCl and extracted three times with chloroform containing 15% (v/v) methanol. Coriolic acid was purified from unreduced peroxide fatty acids by semi-preparative silica chromatography as outlined above. LC/APPI-MS confirmed the identity and the preparation and the absence of contaminants.

**Sourdough fermentation and bread preparation.** *L. hammesii* and *L. sanfranciscensis* were used to prepare sourdough bread. Cells from an overnight culture in mMRS were washed twice and suspended in sterile tap water to a concentration of 10⁹ CFU mL⁻¹. Sourdough was prepared by mixing white wheat flour, sterile tap water and culture in a ratio of 2:1:1 (w/w/w), and 4 g kg⁻¹ linoleic acid to homogeneity. The dough was fermented at 30 °C for 2 days. Samples were taken after 0, 1 and 2 d for analysis of cell counts, pH-values, and the concentration of organic
acids and ethanol (10). The identity of the fermentation microbiota and the inoculum was verified by observation of a uniform and matching colony morphology metabolite profiles.

During sourdough fermentation, cell counts for *L. hammesii* and *L. sanfranciscensis* reached 9 log CFU mL\(^{-1}\) after 24 h and remained constant after 48 h. The pH values of sourdough after 24 h of fermentation were 3.3 ± 0.1 (*L. sanfranciscensis*) and 3.4 ± 0.1 (*L. hammesii*). The pH values remained after 24 h of fermentation; *L. hammesii* and *L. sanfranciscensis* produced 64.9 ± 6.1 and 70.6 ± 2.4 mmol lactate (kg sourdough\(^{-1}\)), respectively, and 11.1 ± 1.6 and 12.5 ± 2.3 mmol acetate (kg sourdough\(^{-1}\)), respectively.

Dough was prepared with bread formulations shown in Table 1. Bread was prepared with 20% addition of sourdough; non-acidified dough, chemically acidified dough and dough supplemented with 0.4% (w/w) calcium propionate or 0.15% (w/w) coriolic acid were prepared as references. Dough was mixed for 8 min (Kitchen Aid, K45SS, Hobart, OH) and proofed for 25 min at 30 °C and 85% relative humidity in a proofer (Cres-Cor, 12711, Cleveland OH). After the first proof, the dough was molded, placed into tins and proofed under the same conditions for an additional 105 min. Dough was baked in a convection oven (Bakers Pride Canada, X-300L, Lachine, Canada) at 180 °C for 25 min. The loaves were cooled to room temperature on racks for 120 min, where samples were taken for antifungal testing, pH determination and quantification of antifungal compounds by LC/APPI-MS.

Determination of bread pH was measured by homogenizing a 10-fold dilution of bread crumb in deionized water. Growth of mold on bread was measured by slicing the bread in 25 mm thick uniform slices under sterile conditions and placing into sealed sterile plastic bags with filter tips inserted to allow the exchange of oxygen. Bread slices were inoculated with a spore suspension containing 10\(^2\) spores mL\(^{-1}\) in a 0.9% NaCl (w/v), 0.1% (w/v) Tween-80 solution. The spore
suspension was sprayed five times in each corner of the bread slice and once in the middle delivering 89.1 ± 3.1 µL spore suspension for *P. roqueforti* and 90.3 ± 3.3 µL *A. niger* spore suspension with each spray. Additional samples were sliced in an open baking area to allow environmental contamination without inoculation. Slices were incubated for 15 days at 20 ºC and monitored every 12 h. The time to visible mycelial growth was reported as mold-free shelf life.

The effect of sourdough fermentation was determined in triplicate independent experiments (triplicate sourdough fermentation and baking). Statistical analysis was done using the SAS 9.3 with Tukey’s pairwise multiple comparison test. Significant differences were reported at a confidence level of *p* ≤ 0.05.

**RESULTS**

**Selection of sourdough lactobacilli with antifungal activity.** Seven strains of lactobacilli that are known to convert linoleic acid (18) were screened for antifungal activity to identify lactobacilli that specifically convert linoleic acid to antifungal metabolites. Inhibitory activity of culture supernatant or organic extracts from cultures in mMRS or sourdough was investigated against *A. niger* and *M. plumbeus*. The antifungal activity increased from 1 to 4 days of incubation and was maintained until 8 days (data not shown). Table 2 shows the activity of culture supernatants after 4 d fermentation with *A. niger* as indicator strain. Comparable results were obtained with *M. plumbeus* (Table S1 of the online supplemental material). *L. plantarum* TMW1460, *L. reuteri* LTH2584 and *L. hamnesii* DSM16381 exhibited the strongest activity in both mMRS broth and sourdough medium. However, *L. hamnesii* was the only strain where higher concentrations of linoleic acid lead to a stronger antifungal effect. Organic extracts exhibited higher activity compared to the culture supernatants (Table 3). The strongest activity of
the organic extract from mMRS broth was from *L. reuteri*, *L. pontis* and *L. harnesii*; the strongest activity in sourdough extracts was observed with *L. sanfranciscensis*, *L. plantarum* and *L. harnesii*. Again, *L. harnesii* exhibited a strong effect in either media and addition of linoleic acid increased the antifungal effect against *A. niger* (Table 3) and *M. plumbeus* (Table S2 of the online supplemental material). Overall, results indicate that *L. harnesii* converts linoleic acid to a hydrophobic compound with antifungal activity.

**Preliminary characterization of antifungal compounds.** Antifungal compounds were fractionated from organic extract of *L. harnesii*. Corresponding extracts from *L. sanfranciscensis* were also fractionated for comparison. The organic extract from both strains in mMRS media was shown by normal phase LC/APPI-MS analysis to be a mixture of carbon 18 fatty acid isomers with from 0 to 3 hydroxyl groups and 0 to 3 double bonds (Figure 1). Detection with ELSD revealed that mono-hydroxy fatty acids in extracts from cultures of *L. harnesii* accounted for greater than 90% of the peak area in the chromatogram (data not shown). The peak area for mono-hydroxy fatty acids in extracts from *L. harnesii* was 6.5 times larger compared to extracts from *L. sanfranciscensis*; di- and tri-hydroxy fatty acids were at or below the limit of detection for LC/ELSD for both strains (data not shown). Fatty acids were fractionated by hydroxyl group number and tested for antifungal activity. Mono-hydroxy fatty acids from either *L. harnesii* or *L. sanfranciscensis* exhibited antifungal activity (Table 4). The MIC of di-hydroxy and tri-hydroxy fatty acids was greater than 20 g L\(^{-1}\). LC/MS analysis of the mono-hydroxy fraction from *L. harnesii* indicated that it consisted almost exclusively of a single compound. The main compound in the mono-hydroxy fraction of *L. harnesii* produced an [M-H]\(^{-}\) ion with \(m/z\) 297.2403, indicating a mono-hydroxy C18:1 fatty acid with the
composition C$_{18}$H$_{33}$O$_3$. Contrary to this, the same fraction from *L. sanfranciscensis* consisted of many mono-hydroxy fatty acids and isomers.

Because mono-hydroxy fatty acids from *L. hammesii* or *L. sanfranciscensis* differed in their antifungal activity, the influence of fatty acid structure on antifungal activity was examined. The mono-hydroxy fraction from *L. hammesii* and coriolic acid were the most active with a MIC of 0.7 and 0.1 g L$^{-1}$ using *A. niger* and *P. roquefortii* as indicator strains, respectively. The MIC of ricinoleic acid against *A. niger* was 2.4 g L$^{-1}$. Oleic and stearic acids and saturated hydroxy fatty acids exhibited no antifungal activity (Table 4). The C18:1 mono-hydroxy fatty acid from *L. hammesii* was purified by reverse phase chromatography to attribute antifungal activity to a single compound. The purified compound inhibited *A. niger* with a MIC of 0.7 ± 0.2 g L$^{-1}$.

**Quantification of conversion products.** In sourdough, hydroxy fatty acids may be produced by enzymatic or chemical oxidation in addition to microbial metabolism. Particularly the oxidation of linoleic acid by lipoxygenase, followed by chemical reduction to coriolic acid may contribute to the pool of hydroxy fatty acids (18). To distinguish between chemical, enzymatic and microbial conversions, a quantification of fatty acids in organic extracts from mMRS and sourdough was performed. Since authentic standards were not available, hydroxy fatty acids were quantified relative to the concentration of the same compounds in culture supernatants of *L. hammesii*. Relative quantification was performed in extracts from cultures of *L. hammesii* or *L. sanfranciscensis* in mMRS and sourdough, and chemically acidified controls (Table 5). Peak areas for each fatty acid were expressed as a percentage of the peak area of the same fatty acid in the supernatant of *L. hammesii* grown mMRS with 4 g L$^{-1}$ linoleic acid.
Mono-hydroxy octadecenoic acid concentrations in fermentations with *L. hammesii* including linoleic acid were 20-fold higher compared to controls containing no linoleic acid, or chemically acidified controls without bacterial metabolism (Table 5), demonstrating that it is a microbial metabolite from linoleic acid. The relative concentration of the mono-hydroxy C18:1 antifungal metabolite was higher in mMRS than in sourdough and more abundant in fermentations with *L. hammesii* compared to *L. sanfranciscensis*. Absolute quantification of the mono-hydroxy C18:1 from *L. hammesii* revealed that it is produced in sourdough to a concentration of $0.73 \pm 0.03$ g kg$^{-1}$, a level that is equivalent or higher than the MIC.

Culture supernatants from mMRS without the addition of linoleic acid show a high amount of mono- and di-hydroxy saturated fatty acids (Table 5). These products thus likely result from metabolism of other fatty acids, i.e. the hydration of oleic acid (17), a component of Tween 80. Sourdough fermentations with added linoleic acid generated a large amount of di-hydroxy octadecenoic acid, suggesting that flour-derived enzymes or microbial conversion of fatty acids present in dough play a role in their formation. The relative concentration of mono-hydroxy fatty acids with two or three double bonds and di-hydroxy fatty acids with two or three double bonds were high in the chemically acidified controls, and their absolute concentration was low (Table S3 of the online supplementary material). This result indicates that these compounds result from chemical or enzymatic oxidation rather than microbial metabolism.

**Concentration of hydroxy fatty acids in dough and bread.** Initially, the fermentation time to achieve high concentrations of C18 hydroxy fatty acids was optimized. Sourdough was fermented with *L. hammesii* and *L. sanfranciscensis* for up to 8 d and samples were taken every 12 h for analysis with LC-APPI/MS. The peak area for the antifungal C18:1 hydroxy fatty acid peaked at 2 d of fermentation for *L. hammesii* and *L. sanfranciscensis* and remained at a constant
level throughout subsequent incubation (data not shown). Therefore, sourdoughs for use in bread making were fermented for 2 d. Bread dough prepared with *L. hammesii* sourdough contained 0.13 ± 0.02 g kg⁻¹ mono-hydroxy C18:1 after proofing. After baking, 0.11 ± 0.02 g kg⁻¹ remained, corresponding to a loss of 14%. Coriolic acid was also present in the bread fermented with *L. hammesii* at a concentration of 0.13 ± 0.03 g kg⁻¹, confirming its formation from linoleic acid, enzymes and reducing agents in the wheat flour. Bread supplemented with 1.5 g kg⁻¹ coriolic acid contained 1.2 ± 0.09 g kg⁻¹ coriolic acid after proofing and 1.1 ± 0.02 g kg⁻¹ coriolic acid after heating, corresponding to a baking loss of 12%. The pH value of bread was 5.3 ± 0.1 for non-acidified control and bread supplemented with calcium propionate or coriolic acid. Chemically acidified bread and sourdough bread fermented with *L. sanfranciscensis* or *L. hammesii* had pH values of 4.1 ± 0.1, 4.3 ± 0.1 and 4.4 ± 0.1, respectively.

**Effect of sourdough fermentation and coriolic acid on fungal spoilage of bread.** The effect of sourdough fermentation on fungal spoilage was evaluated by challenge with two different fungal strains and after environmental contamination. Sourdough bread was compared to bread prepared in a straight dough process without additives, and to bread with 0.4% calcium propionate or 0.15% coriolic acid. Chemical acidification or addition of sourdough fermented with *L. sanfranciscensis* had no effect on growth of *A. niger* or *P. roqueforti* compared to the control. Environmental contaminants, however, were inhibited by inclusion of sourdough fermented with *L. sanfranciscensis* (Figure 2). Bread prepared with *L. hammesii* sourdough inhibited growth of all molds relative to the control, and delayed growth of environmental contaminants when compared to *L. sanfranciscensis* sourdough or the chemically acidified control. Coriolic acid supplemented bread exhibited inhibitory effects against all molds when
compared to the control, and was as effective as 0.4% calcium propionate for bread inoculated
with *P. roqueforti* and environmental contaminants.

**DISCUSSION**

This study demonstrated that *L. hammesii* DSM16381, an isolate from sourdough (21), converts
linoleic acid to a mono-hydroxy octadecenoic acid with antifungal activity. Thus, hydroxy fatty
acids produced by food fermenting lactic acid bacteria (16, 17, 18, 22) exhibit antifungal activity.
Moreover, linoleic acid metabolism by lactic acid bacteria was previously not observed in food
fermentations (16, 17). *L. hammesii* produced higher quantities of the mono-hydroxy C18:1 fatty
acid than *L. sanfranciscensis*, and demonstrated higher antifungal activity than other lactobacilli.
Moreover, coriolic acid exhibits antifungal activity and its activity is comparable to the hydroxy
fatty acid produced by *L. hammesii*. Sourdough bread prepared with *L. hammesii* delayed fungal
spoilage of bread and coriolic acid delayed fungal spoilage of bread for up to 15 days.

Lactobacilli hydrate linoleic acid to 13-hydroxy-9-octadecenoic acid or 10-hydroxy-12-octadecenoic acid (16, 22). The hydratase of lactic acid bacteria converting linoleic and oleic
acids to hydroxy fatty acids was recently characterized (17, 23). Hydratases of lactobacilli
produce predominantly 10-hydroxy-octadecenoic acid (23). In *Lactobacillus acidophilus*, the
proportion of hydroxy-fatty acids in the cytoplasmic membrane increased at a higher growth
temperature (24), suggesting a role of hydroxy fatty acids in membrane homeostasis.
Correspondingly, over-expression of the hydratase in lactic acid bacteria increased their heat
resistance (25). Cells change the fatty acid composition of the plasma membrane in response to
altered environmental conditions to maintain a liquid-crystalline state (26, 27). Hydroxy-C18:1-fatty acids decreased the phase transition temperature of the membrane, stabilizing the liquid–
crystalline state (28). Under direct comparison, the same unsaturated fatty acid had much less impact on membrane properties (28).

The antifungal activity of hydroxy fatty acids (14) is likely also linked to their interaction with membranes. Partitioning of hydroxy fatty acids into fungal membrane has been proposed to increase membrane permeability (29, 30, 31). Our results demonstrate that antifungal activity is highly dependent on fatty acid structure. Unsaturated mono-hydroxy fatty acids exhibited antifungal activity but saturated hydroxy fatty acids or unsaturated fatty acids were not active. This suggests that at least one double bound and one hydroxyl group along a C18 aliphatic chain are required for antifungal activity. Remarkably, the mono-hydroxy C18:1 fatty acid produced by L. hammesii had higher activity than the mixture of mono-hydroxy fatty acids extracted from L. sanfranciscensis. Moreover, the 13-hydroxy-9cis,11trans-octadecadienoic (coriolic) acid had higher antifungal activity than 12-hydroxy-9-cis-octadecenoic (ricinoleic) acid. The trans configuration in coriolic acid has no effect on antifungal activity (32, 33), indicating that the exact positioning and configuration of hydroxyl groups and double bonds also affects antifungal activity.

The antifungal activity of metabolites from lactic acid bacteria in bread has, to date, not been attributed to a single compound, but rather to their synergistic activity with substrate- or yeast-derived compounds (4, 5, 8, 10). This study demonstrated that enzymatic and microbial activities generate antifungal hydroxy fatty acids from linoleic acid. Coriolic acid, the product of enzymatic conversion of linoleic acid, has antifungal activity that is equivalent to the linoleic acid metabolite from L. hammesii. Conversion of linoleic acid to coriolic acid depends on lipoxygenase activity to generate fatty acid peroxides and thiols to reduce fatty acid peroxides to hydroxy fatty acids (18); both lipoxygenase activity and low-molecular weight thiols are present
in wheat (sourdough) (34, 35). Chemical and enzymatic oxidation generates additional hydroxy fatty acids in wheat dough, although identification of all of the isomeric structures has not yet been achieved. Microbial conversion of linoleic acid during growth of *L. hammesii* and *L. sanfranciscensis* produced C18:1 mono hydroxy fatty acids. Mono-hydroxy-C18:1-fatty acids produced at the dough stage were relatively stable with a baking loss of less than 15% after baking. The concentration of the antifungal hydroxy C18:1 fatty acid from *L. hammesii* in bread was at or below the MIC; nevertheless, bread prepared with *L. hammesii* sourdough delayed growth of *A. niger*, *P. roquefortii*, and environmental contaminants. The comparison to bread prepared with *L. sanfranciscensis* sourdough indicates that microbial conversion of linoleic acid contributes to the antifungal activity of sourdough. The antifungal activity of 0.15% coriolic acid – a concentration exceeding the MIC against *A. niger* and *P. roquefortii* two to tenfold – was comparable to the preservative effect of 0.4% calcium propionate. The bitter taste threshold level for di- and tri-hydroxy fatty acids was 4 and 2 g L⁻¹, respectively (36); mono-hydroxy fatty acids have a higher taste threshold (37). Mono-hydroxy fatty acids thus delay or prevent fungal spoilage of bread without adverse impact on the sensory properties of bread.

In conclusion, *L. hammesii* converts linoleic acid to a mono-hydroxy octadecenoic acid with antifungal activity. This conversion was observed in sourdough fermentations supplemented with linoleic acid but generation of hydroxy fatty acids in sourdough also occurred through enzymatic or chemical oxidation. Mono-hydroxy octadecenoic acid in combination with substrate derived coriolic acid inhibited mold growth on sourdough bread. The use of coriolic acid and antifungal metabolites from linoleic acid as natural antifungals is not limited to food preservation. Antifungal metabolites from lactobacilli may complement or substitute these fungicides for use in seed treatment and crop protection (38).
ACKNOWLEDGEMENTS

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REFERENCES


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Figure 1. LC/APPI-MS extracted ion chromatogram (XIC) overlay of organic extract of sourdough fermented with *L. hammeii* in presence of 4 g L⁻¹ linoleic acid (Panel A) and extract of sourdough fermented with *L. sanfranciscensis* in presence of 4 g L⁻¹ linoleic acid (Panel B). Shown are the [M-H]⁻ ions of m/z 279 corresponding to linoleic acid (LA); m/z 293 – 299 corresponding to saturated, mono-, di- and triunsaturated mono-hydroxy C18 fatty acids (solid line); m/z 309 – 315 corresponding to saturated, mono-, di- and triunsaturated di-hydroxy C18 fatty acids (dotted line); and m/z 325 – 331 corresponding to saturated, mono-, di- and triunsaturated tri-hydroxy C18 fatty acids (dashed line). Separations were performed on a Waters YMC silica column.

Figure 2. Mold free shelf life of bread (Cont.), chemically acidified bread (Chem. acid.), sourdough bread fermented with *L. sanfranciscensis* (*L. sanfran.* or *L. hammeii* (*L. ham.*), and bread supplemented with 0.15% coriolic acid (0.15% CA) or 0.4% Ca-propionate (0.4% Prop.). Sourdough was supplemented with 4 g kg⁻¹ linoleic acid and fermented with *L. hammesii* or *L. sanfranciscensis*. Bread slices were inoculated with *A. niger*, *P. roquefortii*, or contaminated by environmental fungal spores during handling, and stored until visible mold growth, or for 15 days. Data are shown as means ± standard deviation of triplicate independent experiments. Values for bread inoculated with the same mold that do not share a common superscript are significantly different (p <0.05).
Figure 1. Black et al.

LC/APPI-MS extracted ion chromatogram (XIC) overlay of organic extract of sourdough fermented with *L. hammesii* in presence of 4 g L\(^{-1}\) linoleic acid (Panel A) and extract of sourdough fermented with *L. sanfranciscensis* in presence of 4 g L\(^{-1}\) linoleic acid (Panel B).

Shown are the [M-H]\(^{-}\) ions of m/z 279 corresponding to linoleic acid (LA); m/z 293 – 299 corresponding to saturated, mono-, di- and triunsaturated mono-hydroxy C18 fatty acids (solid line); m/z 309 – 315 corresponding to saturated, mono-, di- and triunsaturated di-hydroxy C18 fatty acids (dotted line); and m/z 325 – 331 corresponding to saturated, mono-, di- and triunsaturated tri-hydroxy C18 fatty acids (dashed line). Separations were performed on a Waters YMC silica column.
Figure 2. Mold free shelf life of bread (Cont.), chemically acidified bread (Chem. acid.), sourdough bread fermented with *L. sanfranciscensis* (*L. sanfran.*) or *L. hammesii* (*L. hamm.*), and bread supplemented with 0.15% coriolic acid (0.15% CA) or 0.4% Ca-propionate (0.4% Prop.). Sourdough was supplemented with 4 g kg⁻¹ linoleic acid and fermented with *L. hammesii* or *L. sanfranciscensis*. Bread slices were inoculated with *A. niger*, *P. roquefortii*, or contaminated by environmental fungal spores during handling, and stored until visible mold growth, or for 15 days. Data are shown as means ± standard deviation of triplicate independent experiments. Values for bread inoculated with the same mold that do not share a common superscript are significantly different (p <0.05).
Table 1. Bread formulation.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Non-acidified</th>
<th>Chemically acidified</th>
<th>Fermented</th>
<th>Propionate</th>
<th>Coriolic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flour</td>
<td>200</td>
<td>200</td>
<td>180</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Water</td>
<td>130</td>
<td>130</td>
<td>110</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td>Salt</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Yeast</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Acid mix(a))</td>
<td>0</td>
<td>1.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sourdough(b))</td>
<td>0</td>
<td>0</td>
<td>40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Calcium propionate</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>Coriolic acid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

\(a\) Mixture of lactic and acetic acid (4:1 v/v) to yield a dough pH of 3.9 ± 0.5. \(b\) Fermented by either *L. hammesii* or *L. sanfranciscensis* supplemented with 4 g kg\(^{-1}\) linoleic acid.
Table 2. MIC of aqueous extracts from cultures in mMRS and sourdough with differing levels of linoleic acid. Samples were extracted at 4 days of fermentation and tested for activity using *Aspergillus niger* as an indicator. MIC analysis was performed after 2 days of indicator growth and data are shown as means ± standard deviation of triplicate independent experiments.

<table>
<thead>
<tr>
<th>Starter culture</th>
<th>MIC (mL·L⁻¹) mMRS Broth</th>
<th>MIC (mL·L⁻¹) Sourdough</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linoleic acid concentration</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 g·L⁻¹</td>
<td>2 g·L⁻¹</td>
</tr>
<tr>
<td><em>L. sanfranciscensis</em> ATCC 27051</td>
<td>83±0</td>
<td>70±17</td>
</tr>
<tr>
<td><em>L. plantarum</em> TMW 1460</td>
<td>70±20</td>
<td>9±2</td>
</tr>
<tr>
<td><em>L. plantarum</em> TMW 1701</td>
<td>56±20</td>
<td>10±0</td>
</tr>
<tr>
<td><em>L. reuteri</em> LTH 2584</td>
<td>42±0</td>
<td>9±2</td>
</tr>
<tr>
<td><em>L. pontis</em> LTH 2587</td>
<td>70±20</td>
<td>83±0</td>
</tr>
<tr>
<td><em>L. hammesii</em> DSM 16381</td>
<td>14±5</td>
<td>17±5</td>
</tr>
</tbody>
</table>
Table 3. MIC of organic extracts from both mMRS growth medium and sourdough starter with differing levels of linoleic acid added for conversion. Samples were extracted at 4 days of fermentation and tested for activity using *Aspergillus niger* as an indicator. MIC analysis was performed after 2 days of indicator growth and data are shown as means ± standard deviation of triplicate independent experiments.

<table>
<thead>
<tr>
<th>Starter culture</th>
<th>MIC (mL·L⁻¹) mMRS Broth Linoleic acid concentration</th>
<th>MIC (mL·L⁻¹) Sourdough Linoleic acid concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 g·L⁻¹  2 g·L⁻¹  4 g·L⁻¹</td>
<td>0 g·L⁻¹  2 g·L⁻¹  4 g·L⁻¹</td>
</tr>
<tr>
<td><em>L. sanfranciscensis</em> ATCC 27051</td>
<td>2±0  2±0  6±2</td>
<td>1±0  5±2  6±2</td>
</tr>
<tr>
<td><em>L. plantarum</em> TMW 1460</td>
<td>2±0  8±0  8±0</td>
<td>2±0  2±0  3±1</td>
</tr>
<tr>
<td><em>L. plantarum</em> TMW 1701</td>
<td>6±2  2±0  3±1</td>
<td>4±0  1±0  8±0</td>
</tr>
<tr>
<td><em>L. reuteri</em> LTH 2584</td>
<td>1±0  1±0  3±1</td>
<td>5±2  8±0  3±1</td>
</tr>
<tr>
<td><em>L. pontis</em> LTH 2587</td>
<td>8±0  1±0  8±0</td>
<td>2±0  2±0  2±0</td>
</tr>
<tr>
<td><em>L. hammesii</em> DSM 16381</td>
<td>4±1  2±0  1±0</td>
<td>6±2  5±2  1±0</td>
</tr>
</tbody>
</table>
Table 4. MIC of fatty acids isolated from culture supernatants of *L. hammesii* and *L. sanfranciscensis*, reference compounds, and enzymatically produced coriolic acid. MIC analysis was performed after 3 days of growth with *Aspergillus niger* or 5 days growth with *Penicillium roqueforti*, as an indicator strain; data are shown as means ± standard deviation of triplicate independent experiments. The MIC of di- and tri-hydroxy-fatty acids from *L. hammesii* and *L. sanfranciscensis* was higher than 20 g L⁻¹.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (g L⁻¹)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. niger</strong></td>
<td><strong>P. roqueforti</strong></td>
<td></td>
</tr>
<tr>
<td>Mono-OH-fatty acids <em>L. hammesii</em></td>
<td>0.7 ± 02</td>
<td>0.1 ± 0.06</td>
</tr>
<tr>
<td>Mono-OH-fatty acids <em>L. sanfranciscensis</em></td>
<td>5.9 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Coriolic acid</td>
<td>0.7 ± 0.2</td>
<td>0.1 ± 0.08</td>
</tr>
<tr>
<td>Ricinoleic acid</td>
<td>2.4 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>4.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>&gt; 20</td>
<td></td>
</tr>
<tr>
<td>Stearic acid</td>
<td>&gt; 20</td>
<td></td>
</tr>
<tr>
<td>12-OH-stearic acid</td>
<td>&gt; 20</td>
<td></td>
</tr>
<tr>
<td>9, 12 di-OH-stearic acid</td>
<td>&gt; 20</td>
<td></td>
</tr>
</tbody>
</table>
Table 5. Relative quantitation of C18 hydroxy fatty acids in mMRS and sourdough by LC-MS.

Hydroxy fatty acid concentrations are expressed relative to the concentration of the same compound in the *L. hammesii* mMRS + LA sample. Data are shown as means ± standard deviation of triplicate independent experiments. LA, addition of 4 g L⁻¹ linoleic acid.

<table>
<thead>
<tr>
<th>Strain / matrix</th>
<th>Mono-OH C18 fatty acids</th>
<th>Di-OH C18 fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>L. hammesii</em> / mMRS + LA⁹</td>
<td>100±12</td>
<td>100±3</td>
</tr>
<tr>
<td><em>L. hammesii</em> / mMRS</td>
<td>258±43</td>
<td>1±0</td>
</tr>
<tr>
<td><em>L. sanfranciscensis</em> / mMRS + LA</td>
<td>190±31</td>
<td>30±3</td>
</tr>
<tr>
<td><em>L. sanfranciscensis</em> / mMRS</td>
<td>341±51</td>
<td>0±0</td>
</tr>
<tr>
<td><em>L. hammesii</em> / dough + LA</td>
<td>31±6</td>
<td>29±1</td>
</tr>
<tr>
<td><em>L. hammesii</em> / dough</td>
<td>15±2</td>
<td>15±1</td>
</tr>
<tr>
<td><em>L. sanfranciscensis</em> / dough + LA</td>
<td>8±2</td>
<td>3±0</td>
</tr>
<tr>
<td><em>L. sanfranciscensis</em> / dough</td>
<td>7±2</td>
<td>4±1</td>
</tr>
<tr>
<td>Chemically acidified / mMRS + LA</td>
<td>5±1</td>
<td>1±0</td>
</tr>
<tr>
<td>Chemically acidified / dough + LA</td>
<td>2±1</td>
<td>0±0</td>
</tr>
</tbody>
</table>

⁹) The concentration of fatty acids extracted from *L. hammesii* grown in mMRS in presence of linoleic acid was used as a reference (100%).

a) Number of hydroxy groups on C18 fatty acids. b) Number of double bonds on C18 fatty acids.