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Quantification of Phenyllactic Acid in Wheat Sourdough Using High Resolution Gas Chromatography−Mass Spectrometry

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In this study, high-resolution gas chromatography−mass spectrometry (HRGC−MS) was successfully used to quantify the level of phenyllactic acid produced by *Lactobacillus plantarum* strains during sourdough fermentation. Investigation of samples collected during fermentation revealed that the production of phenyllactic acid occurs throughout the growth of *L. plantarum* in sourdough, but the highest production rate was observed during the logarithmic growth phase. The highest amount, that is, 33.47 mg of phenyllactic acid/kg of dough, was measured in sourdough fermented by the antifungal strain *L. plantarum* FST 1.7. Sourdoughs fermented by different *L. plantarum* strains contained different amounts of phenyllactic acid, thus indicating that the production is strain-dependent. Phenylacetic acid was also detected during sourdough analysis, thus showing that the HRGC-MS protocol developed is suitable for the detection not only of phenyllactic acid, but also of a broader range of phenolic acids that are highly relevant, but present in very low amounts in sourdough.

KEYWORDS: Gas chromatography; mass spectrometry; phenyllactic acid; *Lactobacillus*; sourdough; stable isotope dilution assay

INTRODUCTION

Among the natural preservation systems, the use of lactic acid bacteria in the form of sourdough has a long tradition (*1*, *2*). In the past, the preservative activity of sourdough lactic acid bacteria was mainly attributed to the production of lactic and acetic acid, with their concurrent pH drop, and only recently the range of antimicrobial metabolites produced by lactic acid bacteria has been studied in detail. These studies have led to the identification of a number of different compounds which inhibit the growth of spoilage organisms (*3*−*6*).

Among the compounds produced by sourdough lactic acid bacteria, 2-hydroxy-3-phenyl-propionic acid, that is, phenyllactic acid, was recently shown to be a strong antifungal agent, with previous studies showing no activity against human and animal cell lines (*7*). Besides its antimicrobial properties, phenyllactic acid has been detected in a number of foods and it is one of the most abundant aromatic acids found in honey (*8*). When present at low concentrations, phenyllactic acid does not affect the odor of a food product, unlike other compounds like acetic acid (*4*). Therefore, phenyllactic acid represents a promising natural device for controlling contaminants in food systems such as bread.

We have recently identified phenyllactic acid as one of the key compounds responsible for the antifungal activity of sourdough fermented by *Lactobacillus plantarum* strains, but the actual level produced was not measured (*6*, *9*). By using HPLC, measured levels of about 30 mg of phenyllactic acid/kg were found in *L. plantarum* sourdoughs (*10*). More recently, liquid chromatography−mass spectrometry (LC-MS) was used to quantify phenyllactic acid levels in back slopped sourdoughs (*11*). The main drawback of these techniques is however their low resolution, and thus an approach to accurately measure the level of phenyllactic acid in food systems is still lacking.

In this study, we have used high resolution gas chromatography−mass spectrometry (HRGC−MS) to accurately measure the level of phenyllactic acid produced by lactic acid bacteria during sourdough fermentation. Comparison of the levels produced by different *L. plantarum* strains revealed the strain-dependent production of phenyllactic acid in lactobacilli. The developed protocol allowed also the quantification of phenylacetic acid produced during sourdough fermentation.

MATERIALS AND METHODS

Chemicals. Phenyllactic acid was purchased from Bachem (Weil am Rhein, Germany). All other chemicals were purchased from Sigma (Munich, Germany).

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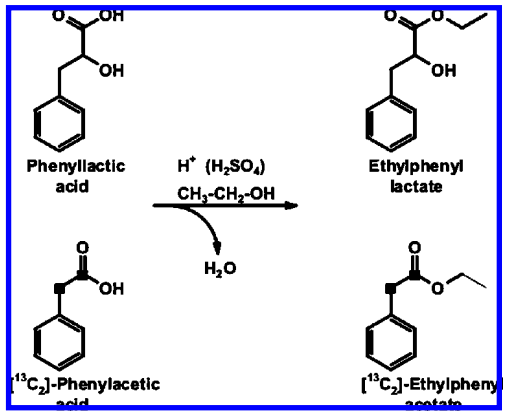

Figure 1. Acid catalyzed conversion of phenyllactic acid and [$^{13}C_2$]-phenylacetic acid (internal standard) to the respective ethyl esters in wheat sourdough prior to extraction and analysis by GC-MS. ■: [^{13}C]-label.

***Lactobacillus* Cultures.** The antifungal strains *L. plantarum* FST 1.7 (LP 1.7) (*6*) and *L. plantarum* FST 1.9 (LP 1.9) (*9*), as well as *L. plantarum* DSM 20174^T (LPTS) were routinely grown on mMRS5 medium (*12*) at 30 °C for 48 h under microaerophilic conditions.

Sourdough Fermentation. Wheat sourdough fermentations were performed as described previously (*6*). Briefly, an overnight culture was used to inoculate (at 1% level) 80 mL of mMRS5 broth and was incubated overnight at 30 °C. Cells were harvested by centrifugation at 4000*g* for 10 min, washed twice, and resuspended in 40 mL of sterile tap water, to give a final concentration of ca. 5×10^9 CFU/mL of water. Six hundred grams of wheat flour, 599 mL of sterile tap water, and 1 mL of cellular suspension, giving a dough yield ((weight of flour + weight of water)/(weight of flour) × 100) of 200, were mixed to homogeneity for 1 min with a Kenwood mixer. Doughs were incubated at 30 °C for 48 h. Bacterial cell counts, pH, and total titratable acidity (TTA) were evaluated as previously described (*6*). Control fermentations with the addition of antibiotics as well as lactic and acetic acids were performed as previously described (*9*). These controls were performed to ensure that phenyllactic acid accumulation was due to metabolic activity of *L. plantarum* strains.

Quantification of Phenyllactic Acid in Sourdough by HRGC-MS. The level of phenyllactic acid present in sourdough was determined using an extraction from sourdough of phenyllactic acid and the stable isotopically labeled internal standard phenylacetic acid ([$^{13}C_2$]-phenylacetic acid). Initially 1 g of sourdough was spiked with the internal standard [$^{13}C_2$]-phenylacetic acid (1 mL; $c = 607$ μg/mL in water). Acids were esterified as ethyl esters by mixing the sourdough with 10 mL of ethanol (99.9%) and 15 drops of sulfuric acid (97%) followed by incubation for 1 h at 80 °C under stirring at 200 rpm (**Figure 1**). After cooling, 20 mL of water was added. Each sample was extracted 5 times with 50 mL of dichloromethane. Sodium sulfate (50 g) was added and the mixture was stirred at 200 rpm for 10 min, filtered (0.45 μm), and the dichloromethane phase was then collected. After removal of the solvent at 50 °C, the residue was dissolved in 5 mL of dichloromethane and subjected to HRGC-MS analysis.

HRGC-MS analysis was performed as previously described (*13*) using a gas chromatograph type HP 5890 series II (Agilent, Waldbronn, Germany) coupled to a MAT-95S mass spectrometer (Finnigan, Bremen, Germany) using a 30 m × 0.32 mm i.d., 0.25 μm, fused silica capillary DB-FFAP (J&W Scientific, Folsom, CA, USA). The flow rate of the carrier gas helium was 2 mL/min. Samples were applied at 40 °C by the cold-on-column technique (*13*). After 2 min, the temperature was increased at a rate of 6 °C/min to 230 °C and held at 230 °C for 5 min. Mass spectra were generated in the electron impact mode (MS/EI) at 70 eV. The most intense mass traces for phenyllactic acid and [$^{13}C_2$]-phenylacetic acid were extracted from the mass chromatograms and used for quantitation. The response factor (mass to signal intensity ratio) of both compounds was calculated by analyzing phenyllactic acid/[$^{13}C_2$]-phenylacetic acid mixtures in the following mass ratios: 0.013:1, 0.02:1, 0.025:1, 0.033:1, 0.05:1, 0.066:1, 0.1:1, 0.2:1, and 1:1. To ensure that both phenyllactic acid and [$^{13}C_2$]-phenylacetic acid were extracted and ethyl esterified to the same extent in sourdough, known levels of each compound were added to dough and were analyzed as described above. The limit of accurate quantification of phenyllactic acid was defined as the lowest level at which phenyllactic acid could be accurately quantified in dough with good peak form and signal intensity (signal-to-noise ratio higher than 5:1).

Accumulation of Phenyllactic Acid during Sourdough Fermentation. The HRGC-MS protocol described above was used to quantify the amount of phenyllactic acid accumulating during sourdough fermented with the antifungal strain LP 1.7. Sourdough samples were collected after 0, 8, 11, 24, and 48 h of fermentation. Since the production of specific antifungal compounds can vary among different strains of the same species (*14*), the production of phenyllactic acid was investigated in wheat sourdoughs fermented by two additional *L. plantarum* strains, that is, LP 1.9 and LPTS. Sourdough samples were collected after 0, 24, and 48 h of fermentation and investigated as described above.

For all investigated doughs, three sourdough samples were taken from three independent fermentations at each time point, with means and standard deviations being determined for each set of data.

RESULTS AND DISCUSSION

In the context of using lactic acid bacteria as a natural tool for improving the shelf life of food products, we have recently described the use of *L. plantarum* strains to produce sourdough which improves the shelf life of wheat bread (*6*, *9*). Among the different compounds isolated, phenyllactic acid was indicated as a major compound responsible for the antifungal activity of *L. plantarum* sourdough. In this work we have successfully applied HRGC-MS to accurately measure the amount of phenyllactic acid produced by *L. plantarum* during sourdough fermentation.

Sourdough Fermentation. Wheat sourdoughs were fermented with three different *L. plantarum* strains, and cell counts, pH, and TTA were determined. Overall, similar cell counts, pH, and TTA values were measured, independent from the strain used (data not shown). *Lactobacillus* cell counts of ca. 1×10^9 CFU/g of dough were reached after 11 h of fermentation, and the values were constant until the end of fermentation, that is, after 48 h. Analysis of the bacteria growing on mMRS5 agar containing bromocresol green (to allow visual differentiation among different strains) revealed that only one strain was responsible for the sourdough fermentation (data not shown). At the end of fermentation, the doughs were characterized by a pH of ca. 3.7 and TTA of ca. 14. The rate of acidification of the doughs (pH drop and TTA increase) did not differ significantly between the selected *L. plantarum* strains.

Quantification of Phenyllactic Acid by Using HRGC-MS. The amount of phenyllactic acid produced by *L. plantarum* during sourdough fermentation was determined using HRGC−MS. In general GC−MS techniques are time-consuming and require convoluted sample preparation that involves metabolite extraction and derivatization to improve volatility. To overcome these pitfalls, we developed a simple and rapid sample preparation protocol, using a relatively short treatment time, involving the acid catalyzed ethyl esterification of phenyllactic acid. Phenylacetic acid was chosen as the internal standard because of its structural similarity to phenyllactic acid and because it is commercially available as a [$^{13}C_2$]-labeled compound. The use of a commercially available internal standard removed the need to synthesis an isotopically labeled internal standard. To render them volatile and make them soluble in organic solvents, phenyllactic acid and [$^{13}C_2$]-phenylacetic acid were esterified with ethanol in the sourdough by acid catalysis (**Figure 1**). Analysis of the EI spectra from phenyllactic acid and [$^{13}C_2$]-phenylacetic acid showed signals at *m*/*z* 194 and *m*/*z* 166, which correspond to the molecular weights of the ethyl esters of

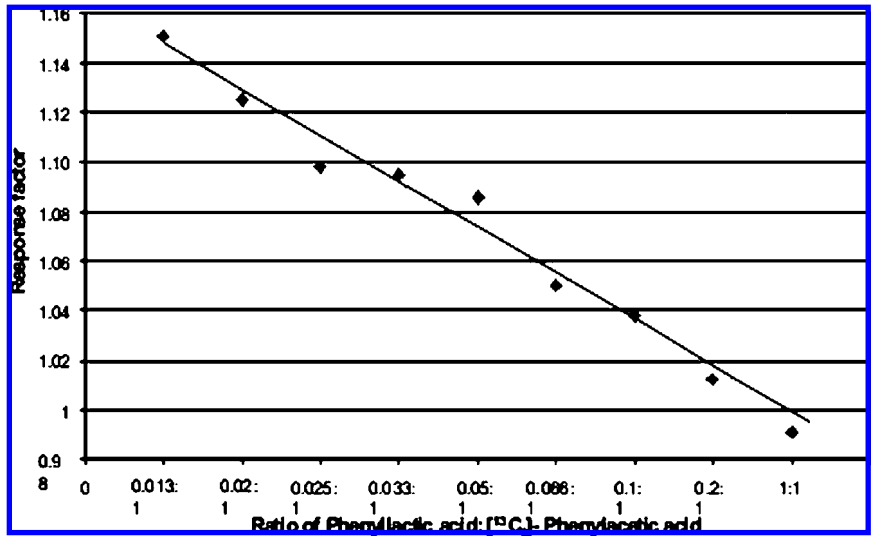

Figure 2. Calibration curve of mixtures of phenyllactic acid and [$^{13}C_2$]-phenylacetic acid versus response factor.

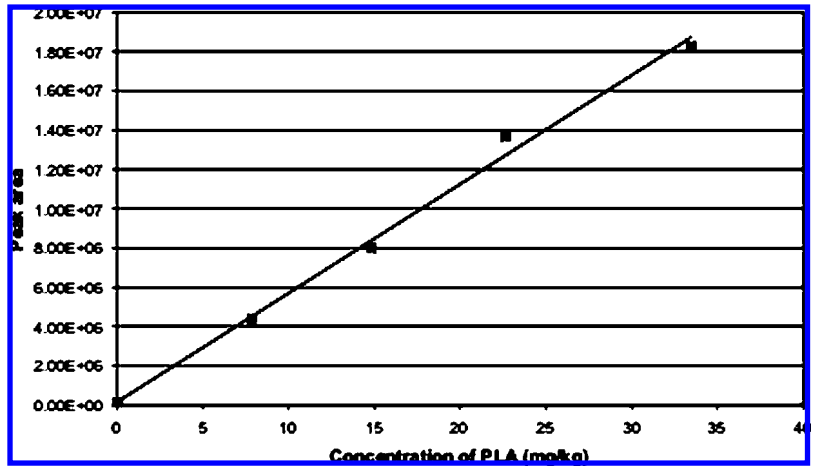

Figure 3. Calibration curve of phenyllactic acid concentration versus peak area.

phenyllactic acid and [$^{13}C_2$]-phenylacetic acid, respectively. Phenyllactic acid and [$^{13}C_2$]-phenylacetic acid showed elution times of 1359.6 and 1002 s, respectively. Besides the signals corresponding to the molecular weight, the most intense ions generated by EI ionization were *m*/*z* 176 and *m*/*z* 164 for phenyllactic acid and [$^{13}C_2$]-phenylacetic acid, respectively. Therefore, these fragment ions were used for quantification.

To ensure the accuracy of the method developed, the response factors (mass to area ratios) obtained with fixed phenyllactic acid/[$^{13}C_2$]-phenylacetic acid mixtures were determined (**Figure 2**). The corresponding R^2 value was 0.984. Both compounds were found during multiple esterifications to be extracted and ethyl esterified in equal yields from sourdough. After extraction with dichloromethane, the ethyl esterified samples were found to be below the detection limit in the residual sourdough. The limit of accurate quantification was also determined and found to be 10 μg of phenyllactic acid/kg of sourdough, that is, ca. 60 nM. This value is in good agreement with the lower limit of detection of metabolites reported for GC−MS (*15*). At concentrations lower than this value, poor peak form and signal-to-noise ratio reduced the accuracy of quantification. Because of the time dependent increase of phenyllactic acid during sourdough fermentation response factors of 1.151, 1.098, 1.090, and 1.074 were used for phenyllactic acid quantification in sourdough fermented by LP 1.7 for 8, 11, 24, and 48 h, respectively. To evaluate the accuracy of phenyllactic acid quantification the peak area was graphed against the level of phenyllactic acid quantified over the 48 h of fermentation by LP 1.7 (**Figure 3**) and the corresponding R^2 value was 0.994. The response factors for sourdoughs fermented for 24 and 48 h were 1.208 and 1.145 or 1.104 and 1.095 for LP 1.9 or LPTS, respectively.

The phenyllactic acid content of sourdough has been recently determined using HPLC (*10*) and LC-MS (*11*), and the reported values are in good agreement with the results of our study. However, even though both HPLC and LC-MS can be used for quantification of phenyllactic acid, HRGC-MS was found to offer significant improvements, in particular with respect to sample preparation time and overall sensitivity. While the HPLC method described involved an overnight treatment prior to extraction (*10*), in our study the ethyl esterification performed prior to the extraction required only 1 h, thus giving a significant improvement with respect to preparation time. Concerning the use of LC-MS for quantification of phenyllactic acid, direct injection into an ion trap LC-MS of phenyllactic acid solutions of 50 mg/mL as well as 50 mg/L were found to give a peak with a low signal-to-noise ratio (<5:1), and therefore the use of LC-MS was regarded as not satisfactory. The low resolution of LC-MS is most probably due to the fact that electrospray ionization of organic acids produces negative ions that are not detected with the same degree of sensitivity as positive ions (*15*).

Quantification of Phenyllactic Acid during Sourdough Fermentation. Phenyllactic acid formation in sourdough has

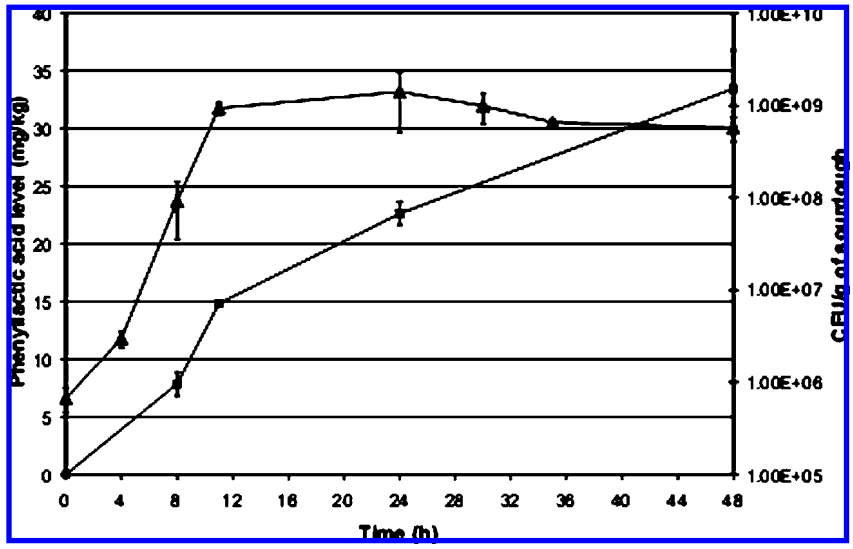

Figure 4. Comparison of cell counts and production of phenyllactic acid in wheat sourdough fermented by *L. plantarum* FST 1.7 (LP 1.7) at 30 °C. Squares, phenyllactic acid production; triangles, *L. plantarum* cell counts. *At the beginning of fermentation (0 h), phenyllactic acid was below the limit of accurate quantification and its value is reported as 0.

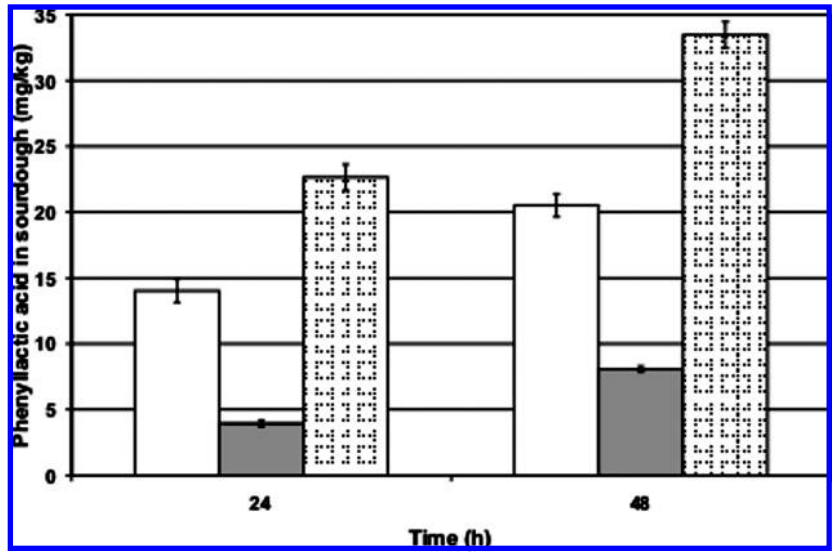

Figure 5. Production of phenyllactic acid in wheat sourdough fermented for 24 and 48 h by different *L. plantarum* strains. White bar, *L. plantarum* DSM 20174^T (LPTS); gray bars, *L. plantarum* FST 1.9 (LP 1.9); black stripes bar, *L. plantarum* FST 1.7. (LP 1.7).

been reported to be affected by a number of different factors, among which type of starter, fermentation conditions, and raw material all exert a strong influence (*14*). The HRGC-MS protocol described above was used to accurately quantify the level of phenyllactic acid produced by LP 1.7 during wheat sourdough fermentation. At the beginning of fermentation (t = 0), the level of phenyllactic acid was below the accurate quantification limit (10 μg of phenyllactic acid/kg of dough) and its value was therefore expressed as 0. The levels of phenyllactic acid increased throughout the fermentation reaching a maximum level of 33.47 mg of phenyllactic acid/kg of sourdough after 48 h (**Figure 4**). The metabolite accumulated most rapidly during the logarithmic growth phase; that is, 44% of the total amount was produced during the first 11 h of fermentation. To the best of our knowledge, this is the first study linking the production of phenyllactic acid with the kinetic of growth of lactic acid bacteria during sourdough fermentation. Phenyllactic acid was not detected (values below the limit of accurate quantification) in the control doughs (data not shown).

To determine if the production of phenyllactic acid is a common trait among *L. plantarum* strains, two additional strains were used to ferment sourdough, and HRGC-MS was used to measure the amount of phenyllactic acid accumulated in samples taken after 24 or 48 h of fermentation. Overall, each different sourdough showed specific contents of phenyllactic acid (**Figure 5**). These results thus confirm that the synthesis of phenyllactic acid, and its relative accumulation, varies among different *L. plantarum* strains (*14*). LPTS produced 14.04 and 20.52 mg of phenyllactic acid/kg of dough, after 24 and 48 h of fermentation, respectively. The lowest level of phenyllactic acid was measured in sourdough fermented by the antifungal strain LP 1.9, with 3.95 and 8.08 mg of phenyllactic acid/kg of dough being detected after 24 and 48 h of fermentation, respectively. In challenge studies against the common bread spoilage molds *Aspergillus niger*, *Fusarium culmorum*, *Penicillium expansum*, and *Penicillium roqueforti* we have recently shown that the sourdough fermented by LP 1.7 or LP 1.9 significantly increases the shelf life of wheat bread, and the activity was linked to the production of phenyllactic acid by the strains (*6*, *9*). However, using a similar approach we found that addition of LPTS sourdough did not increase the shelf life of wheat bread to the same extent (data not shown), even though relatively high

amounts of phenyllactic acid were detected. These results thus confirm that, while phenyllactic acid is a strong antifungal compound produced by *L. plantarum* strains (*3*, *6*, *9*), other compounds are concomitantly required to achieve an increase in the shelf life of bread.

HRGC−MS analysis of sourdough also revealed the presence of phenylacetic acid at levels of microgram per kilogram of sourdough (data not shown), demonstrating the high sensitivity of the developed method. Such levels are below the detection limit of both HPLC (*10*) and LC−MS (*11*). Therefore, this result suggests that acid catalyzed ethyl esterification in combination with HRGC−MS can be used to detect and quantify a broader range of phenolic acids that are highly relevant, but present in very low amounts in sourdough.

ABBREVIATIONS USED

LP 1.7, *Lactobacillus plantarum* FST 1.7; LP 1.9, *Lactobacillus plantarum* FST 1.9; LPTS, *Lactobacillus plantarum* DSMZ 20174^T.

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