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Antifungal Lipids Produced by Lactobacilli and Their Structural Identification by Normal Phase LC/Atmospheric Pressure Photoionization−MS/MS

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Supporting Information

ABSTRACT: *Lactobacillus hammesii* converts linoleic acid into an antifungal hydroxy fatty acid. High speed counter-current chromatography (HSCCC) using a hexane/ethyl acetate/methanol/water solvent system [3.5:1.5:3:2 (v/v/v/v)] allowed isolation of the antifungal hydroxy fatty acid. A method was developed for characterization of antifungal hydroxy fatty acids using normal phase liquid chromatography combined with atmospheric pressure photoionization−tandem mass spectrometry (LC/APPI-MS/MS). The position of unsaturations and hydroxyl groups was determined directly from crude lipid extracts and their hydroxylated derivatives. The antifungal compounds were identified as a racemic mixture of 10-hydroxy-*cis*-12-octadecenoic and 10-hydroxy-*trans*-12-octadecenoic acid. Additionally, HSCCC and LC/APPI-MS/MS methods were used to elucidate the pathway of conversion of linoleic acid by *Lactobacillus sanfranciscensis*, *Lactobacillus plantarum*, and *L. hammesii* to hydroxy fatty acids and conjugated linoleic acid. This study links previously reported 10-hydroxy-12-octadecenoic acid producing *Lactobacillus* strains to antifungal activities.

KEYWORDS: *hydroxy fatty acids, conjugated linoleic acid, antifungal, Lactobacillus hammesii, high speed counter-current chromatography, atmospheric pressure photoionization mass spectrometry*

INTRODUCTION

Lactobacillus species are widely used in food fermentation.[1] Lactobacilli convert linoleic acid into hydroxy fatty acids and conjugated linoleic acid (CLA).[2−5] The first step of this conversion, the hydration of linoleic acid to 13-hydroxy-9-octadecenoic acid or 10-hydroxy-12-octadecenoic acid, is catalyzed by hydratases.[3,6,7] The subsequent synthesis of conjugated fatty acids from hydroxy fatty acids involves enzymes that have not yet been characterized in purified form.[2,8] However, CLA formation by lactobacilli has been observed in buffer fermentations but not in growing cultures.[2,4,8,9] Overall, information on the physiological role of linoleic acid conversion in lactobacilli remains scarce.[10]

The biological activities of CLA and hydroxy fatty acids are highly related to lipid structure. The active isomers of CLA include *cis*-9,*trans*-11 and *trans*-10,*cis*-12; both of these isomers elicit different biological and physiological effects including changes in immune response, insulin sensitivity, and body fat composition.[11−14] However, conflicting results have arisen in human studies as to the healthful effects of CLA.[15] Only the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers have been studied, while others remain untested or elicit concern.[16]

Hydroxy fatty acids produced by lactobacilli exhibit antifungal activity.[17] Similar to the biological activity of CLA, only specific hydroxy fatty acids display antifungal action.[17] For example, the antifungal activity of 13-hydroxy-*cis*-9,*trans*-11-octadecadienoic (coriolic) acid produced by the conversion of lipoxygenases, as well as a hydroxy fatty acid metabolite produced by *Lactobacillus hammesii*, were substantially higher than that of 12-hydroxy-*cis*-9-octadecenoic (ricinoleic) acid. Furthermore, saturated hydroxy fatty acids have been shown to be inactive.[17] This difference in activity appears to relate to the location of a hydroxyl group and the unsaturation(s) along the 18 carbon chain.

Because subtle structural differences of linoleic acid metabolites from lactobacilli strongly affect their biological activity, it is necessary to fully elucidate these fatty acid metabolite structures. This can be challenging because many isomeric forms may be present, and the most abundant forms are not necessarily the most active. Combined results from infrared spectroscopy, NMR, and mass spectrometry (MS) have been used for the analysis of lipid metabolites from lactobacilli.[4,18] However, relatively large amounts and/or purified samples are required for complete identification. Analysis of hydroxy fatty acids and CLA isomers by gas chromatography coupled to mass spectrometry (GC/MS) requires sample derivatization, long run times, and ultimately, geometric isomers are still difficult to differentiate.[6,7,19] Alternatively, liquid chromatography−mass spectrometry (LC/MS) can be used for the analysis of fatty acids with the advantage that purification and derivatization is not always necessary.[20] In addition, the use of atmospheric pressure photo ionization (APPI) sources, available for many LC/MS systems, has been shown to have high ionization efficiency for nonpolar

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compounds such as lipids but with less matrix effect compared to electrospray ionization.[21]

The aim of this study was to develop methods for the isolation and structure elucidation of antifungal compounds produced by lactobacilli. The preparative purification of antifungal monohydroxy fatty acids was achieved using high-speed counter-current chromatography (HSCCC), an alternative to semipreparative HPLC for the fractionation of complex extracts.[22] A normal phase LC/APPI-MS/MS method was developed for the analysis of the antifungal hydroxy fatty acids and applied to elucidate the structure of these compounds produced by select strains of lactobacilli. In this way, it was hoped to investigate the pathway of conversion from linoleic acid into hydroxy fatty acids and other metabolites by lactobacilli.

MATERIALS AND METHODS

Chemicals and Standards. *cis*-9-*cis*-12-Octadecadienoic (linoleic) acid, ricinoleic acid, 12-hydroxy octadecanoic acid, distearin, 7-nonadecenoic acid, 7-nonadecenoic methyl ester, *cis*-9-*cis*-12-*cis*-15-octadecatrienoic (linolenic) methyl ester, linolenic acid, and CLA methyl ester (*cis*-9,*trans*-11 and *trans*-10,*cis*-12 mixture) all with >99% purity were purchased from Nu-Chek Prep, Inc. (Elysian, MN). 9,10-Dihydroxy octadecanoic acid (>90% purity) was supplied by Pfaltz and Bauer (Waterbury, CT). 2-Amino-2-methyl-1-propanol (95% purity), butyronitrile, 4-methylmorpholine-*N*-oxide (97% purity) and osmium tetroxide 2.5 wt % solution in 2-methyl-2-propanol were from Sigma Aldrich (St. Louis, MO). HPLC grade solvents (chloroform, methanol, hexane, isopropyl alcohol, acetonitrile, acetic acid) as well as sulfuric acid, formic acid, and microbiological media were obtained from Fisher Scientific (Ottawa, Canada).

Strains and Growth Conditions. *L. hammesii* DSM16381, *Lactobacillus sanfranciscensis* ATCC27651, and *Lactobacillus plantarum* TMW1460 were cultivated in modified DeMan−Rogosa−Sharpe (mMRS) broth containing 10 g L^{-1} maltose, 5 g L^{-1} fructose, and 5 g L^{-1} glucose with a pH of 6.2. Lactobacilli were incubated under microaerophilic conditions (1% O_2, balance N_2) at 30 °C for 24 h. The cells were washed twice with 0.85% NaCl and resuspended in 10 mL of 0.85% NaCl and 20 mL mMRS broth containing 4 g L^{-1} linoleic acid were inoculated with 5% inoculum. Alternatively, cultures were inoculated in mMRS media containing 4 g L^{-1} monohydroxy octadecenoic acid, linolenic acid, CLA, or ricinoleic acid. Media that were incubated without inoculum, and cultures grown without addition of lipid substrates served as controls. Inoculated media were incubated with shaking at 120 rpm for 48 h at 30 °C.

Extraction of Lipids. After incubation, cells were removed by centrifugation and filtration with a 0.45 μm filter. Supernatants were extracted three times with two volumes of chloroform/methanol 85:15 (v/v), and the organic phase was collected after each extraction. The organic solvent was removed under vacuum at 30 °C, and the residue was stored at −20 °C under nitrogen until analysis.

High-Speed Counter-Current Chromatography. HSCCC was performed using a model TBE-300B high-speed counter-current chromatograph with 300 mL column capacity and 0.03 mm i.d. tubing (Tauto Biotech, Shanghai, China), equipped with a 501 PrimeLine solvent delivery module (Analytical Scientific Instruments, El Sobrante, CA), a VUV-24 Visacon UV−Vis detector (Reflect Scientific Inc., Orem, UT), and a model CHF 122SC fraction collector (Avantec Toyo Kaisha Ltd., Tokyo, Japan).[23] The solvent system consisting of hexane/ethyl acetate/methanol/water 3.5:1.5:3:2 (v/v/v/v) was selected for separation of lipids extracted from cultures of lactobacilli. The solvent mixture was usually prepared in 2 L batches and allowed to separate at room temperature overnight.[22] The upper phase of the biphasic solvent system was used as stationary phase, and the lower phase was used as mobile phase. A retention of 70 ± 1.6%, (v/v) of the stationary phase was achieved on introduction of the mobile phase, which was pumped at 3 mL min^{-1} in the head-to-tail mode.[22] The sample was dissolved to give a concentration of 25 mg mL^{-1} in 10 mL of solvent comprised of 5 mL of the upper phase and 5 mL of the lower phase; the entire 10 mL of sample solution was injected onto the HSCCC column. After a run time of 180 min at a rotor speed of 1000 rpm, all of the hydroxy fatty acids eluted and the mobile phase was switched to be the upper phase to facilitate elution of linoleic acid and CLA. HSCCC separations were monitored at 210 nm with a UV detector. Fractions were collected at 3 min intervals and were analyzed by normal phase LC/APPI-MS as described below. After analysis, fractions containing only the C18:1 monohydroxy fatty acid were combined. This combined fraction was used for derivatization, method development analysis, and lactobacilli pathway determination.

Preparation of Hydroxylated Derivatives. The procedure for the vicinal hydroxylation of double bonds was based on the method described by Moe and Jensen.[24] Approximately 1 mg of fatty acid was dissolved in 100 μL of chloroform, and 200 μL of a 1% (w/v) solution of 4-methylmorpholine-*N*-oxide in methanol and 10 μL of osmium tetroxide were added. The sample was flushed with nitrogen gas and reacted at 60 °C for 2 h. After cooling, the sample was dissolved in 1 mL of chloroform and passed through a conditioned Sep-Pak 500 mg silica solid-phase cartridge (Waters Ltd., Mississauga, Canada). Hydroxylated fatty acids were then eluted using 20 mL of chloroform/methanol/formic acid (10:1:0.1 v/v/v). The sample was dried under nitrogen gas and resuspended in chloroform/methanol (85:15 v/v) at a concentration of 0.1 mg mL^{-1} prior to LC/APPI-MS/MS analysis.

Preparation of Fatty Acid Methyl Ester Derivatives. Esterification of free fatty acids was performed using an acid catalyst as outlined by Christie.[25] Fatty acid (10 mg) was dissolved in 1 mL of methanol, and 2 mL of 1% (v/v) sulfuric acid in methanol was added. The sample was reacted at 50 °C for 2 h with intermittent shaking. After the reaction was complete, 5 mL of a 5% (w/w) sodium chloride solution was added and 5 mL of hexane was used to extract fatty acid methyl esters (FAME) twice. The hexane layer was washed with a 4 mL solution of 2% potassium bicarbonate (w/w). The hexane layer was collected and dried over anhydrous sodium sulfate and evaporated under nitrogen gas. FAME were resuspended to a concentration of 0.1 mg mL^{-1} in isopropyl alcohol for analysis by ozonolysis/APPI-MS or in hexane for silver ion LC/APPI-MS/MS.

Ozonolysis/Atmospheric Pressure Photo Ionization-Mass Spectrometry. In-line ozonolysis/APPI-MS analysis was performed on the pure monohydroxy octadecenoic FAME fraction from HSCCC for the determination of double bond positions as described by Sun et al.[26] Briefly, the in-line system consisted of a 0.5 L Omnifit solvent bottle, a three-valve bottle cap, and 15 cm of gas permeable 0.5 mm o.d., 0.25 mm i.d. Teflon AF-2400 tubing (Biogeneral Inc., San Diego, CA). The Teflon tubing was inserted into the ozone filled solvent bottle, with one end connected to the LC autosampler and the other end directly coupled to the APPI ion source of the mass spectrometer. Samples in 3 μL aliquots were injected into a 0.2 mL min^{-1} flow of hexane/isopropyl alcohol (98:2, v/v) in the Teflon tubing. After being subjected to ozonolysis, FAME were directly analyzed by APPI/MS operating in positive ion mode.

Combined Liquid Chromatography/Atmospheric Pressure Photo Ionization Tandem Mass Spectrometry. Underivatized fatty acid extracts, hydroxylated fatty acids, and FAME were analyzed by LC/APPI-MS/MS. All liquid chromatography was conducted on an Agilent 1200 series LC system (Agilent Technologies, Palo Alto, CA). Separations for underivatized and vicinal hydroxylated fatty acid derivatives were conducted on a 150 mm × 2.0 mm i.d., 5 μm YMC PVA-Sil column (Waters Ltd., Mississauga, Canada) at 25 °C. Lipid samples were injected (injection volume, 2 μL) onto the column and eluted with a gradient of (A) 0.2% acetic acid in hexane and (B) 0.2% acetic acid in isopropyl alcohol 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 including equilibration. Silver ion chromatography was performed on lipid fractions as FAME using a 250 mm × 2.0 mm i.d., 5 μm Varian ChromSpher 5 Lipids column (Varian Inc., Palo Alto, CA) at 25 °C. The separation used an isocratic flow of 0.3 mL min^{-1} hexane containing 0.14% butyronitrile, a run time of 20 min, and an injection volume of 2 μL.

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Negative and positive ion APPI-MS/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). For negative ion-mode, the source and mass spectrometer conditions were: nebulizer gas 70 (arbitrary units), auxiliary 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 scan range of m/z 50−700. Collision induced dissociation (CID) was used with nitrogen at a collision energy of 35 eV for underivatized fatty acids and 40 eV for vicinal hydroxylated fatty acids. The conditions used for positive-ion mode were: nebulizer gas 50 (arbitrary units), auxiliary gas 10, curtain gas 25, ionspray voltage 1300 V, source temperature 380 °C, DP 30 V, focusing potential 130 V, and DP2 5 V with a scan range of m/z 100−1000. CID spectra of FAME were obtained using a collision energy of 20 eV.

Positive ion APPI-MS analysis of the FAME ozonolysis products were run under the same conditions except for a source temperature of 375 °C, DP 35 V, focusing potential 150 V, and DP2 10 V and a scan range of m/z 100−1300. For analysis of FAME CLA, the flow was split 1:1 postcolumn between a UV detector at 242 nm and the mass spectrometer in order to distinguish CLA from linoleic acid.

Preparation of 4,4-Dimethyloxazoline Derivatives and Gas Chromatography/Mass Spectrometry Analysis. 4,4-Dimethyloxazoline (DMOX) derivatives of fatty acids were prepared as described by Fay and Richli.[27] Briefly, 0.25 g of 2-amino-2-methyl-1-propanol was added to 1 mg of pure fatty acid sample. The samples were flushed with nitrogen gas and reacted at 180 °C for 2 h. After cooling to ambient temperature, the reaction mixture was dissolved in 5 mL of dichloromethane and washed with 3 mL of water. The water layer was then extracted with 2 mL of dichloromethane. The combined dichloromethane layer was dried with anhydrous sodium sulfate and evaporated under a stream of nitrogen gas. The derivatized DMOX fatty acid sample was dissolved in hexane to a concentration of 0.1 mg mL^{-1} prior to analysis by GC/MS using an Agilent 7890A gas chromatograph and Agilent 5975C mass-selective detector (Agilent Technologies Inc., Palo Alto, CA). A 2 μL sample volume was injected with a 50:1 split onto a 30 m × 0.32 mm i.d., 0.25 μm film thickness HP-5 capillary column (J & W Scientific Inc., Folsom, CA) at 300 °C. The helium flow was kept constant at 1 mL min^{-1}. The oven program was as follows: the initial column temperature of 100 °C was increased to 285 °C at 10 °C min^{-1}, after which, immediately to a final temperature of 300 °C at 15 °C min^{-1} for 3 min. Electron impact ionization was used at 70 eV electron energy and a mass scan range of m/z 40−500. An Agilent Chem Station E.02.02.1431 was used for data analysis.

Quantification of Metabolites. For the quantitation of hydroxy fatty acids, 200 μL of the supernatant from the centrifugation and filtration of bacterial cultures was extracted twice with 15% methanol in chloroform (v/v). The samples were spiked with 150 μg of a ricinoleic acid standard to measure extraction recovery. Extracts were adjusted to a volume of 5 mL with chloroform after addition of 25 μg of distearin as an internal standard. Calibration curves were established with 10-hydroxy-12-octadecenoic acid that was purified from *L. hammesii* cultures by HSCCC. The identity and purity of the standard was verified to be >99% by normal phase and reversed phase LC/MS, LC/ELSD, and GC/MS. All samples were analyzed by negative ion LC/APPI-MS from triplicate independent experiments with triplicate technical repeats.

For the quantification of CLA in bacterial cultures, 2 mL of the supernatant was extracted twice with 15% methanol in chloroform (v/v) in the presence of 100 μg of 7-nonadecenoic acid recovery standard. The resulting extract was dried under nitrogen gas and methylated as described above. After the extraction of FAME, 50 μg of linolenic methyl ester internal standard was added to the 10 mL total volume of hexane. External calibration curves were established with 7-nonadecenoic methyl ester and CLA methyl ester standards. All samples were analyzed by positive ion LC/APPI-MS from triplicate independent experiments with triplicate technical repeats.

RESULTS

Development of a High-Speed Counter-Current Chromatography Method for Isolation of Antifungal Fatty Acids. In our previous communication[17] we identified antifungal activity from a hydroxy fatty acid fraction extracted from *L. hammesii*. To isolate a sufficient quantity of this antifungal fraction for structural analyses and further testing, a HSCCC method was developed. A two-phase solvent system was selected on the basis of recommendations by Ito.[22] Various combinations of biphasic solvent systems were equilibrated following this, linoleic acid, 12-hydroxy octadecanoic acid, or 9,10-dihydroxy octadecanoic acid standards were added, mixed, and left to settle at room temperature. Aliquots of the upper and lower phases were removed and analyzed by LC/MS, and relative peak areas from these analyses were used to determine the partition coefficient ($K_{U/L}$). The $K_{U/L}$ of each analyte was calculated for each solvent system containing different ratios of hexane/ethyl acetate/methanol/water. The solvent system selected for the separation of lipids extracted from cultures of lactobacilli was hexane/ethyl acetate/methanol/water 3.5:1.5:3:2 (v/v/v/v) with $K_{U/L}$s for linoleic acid, 12-hydroxy octadecanoic acid, and 9,10-dihydroxy octadecanoic acid of 400, 1.4 and 1.0, respectively.

Using this solvent system, fractions were collected every 3 min and analyzed by flow injection APPI-MS (Supporting Information Figure 1). Over the elution times of interest, two major compounds were observed. The first, with a nominal m/z of 295, was shown previously to be a lipid oxidation product.[17] This eluted close to the antifungal monohydroxy fatty acid of m/z 297 (Supporting Information Figure 1). The fractions that did not contain the compound with m/z 295 were all combined to give the purified extract containing the monohydroxy fatty acid. A 250 mg injection of crude lipid extract from *L. hammesii* fermentation yielded >55 mg of purified monohydroxy fatty acid in each HSCCC separation.

Analysis of Hydroxy Fatty Acid Fractions by Liquid Chromatography/Atmospheric Pressure Photo Ionization-Mass Spectrometry. Because the crude lipid extracts from fermentations of lactobacilli contain complex mixtures of fatty acids with various degrees of hydroxylation and unsaturation, a sophisticated analytical approach was needed to separate and identify individual compounds. Initially, LC separations in both normal and reversed phase modes were compared. It was found that a normal phase separation using hexane and isopropyl alcohol with acetic acid on a polymeric (polyvinyl alcohol) silica phase resulted in complete separation of mono-, di-, and tri- hydroxy fatty acids and unhydroxylated compounds (Figure 1A). Separation of many isomeric hydroxy fatty acids was also achieved. However, such normal phase separations can be less amenable to analysis using electrospray ionization methods without postcolumn makeup flows of polar solvents, ideally aqueous. To overcome this, in the present work we have exclusively used atmospheric pressure photoionization which does not require postcolumn solvent addition to assist in ionization. In addition, the hexane in the mobile phase acts as an APPI dopant due its low ionization potential, so no addition of toluene or other dopant is required. Thus, it was found that normal phase LC/APPI-MS was highly suitable for the separation and mass spectrometric identification of hydroxy fatty acids. However, it should be noted that the APPI-MS response to linoleic acid and other nonhydroxylated fatty

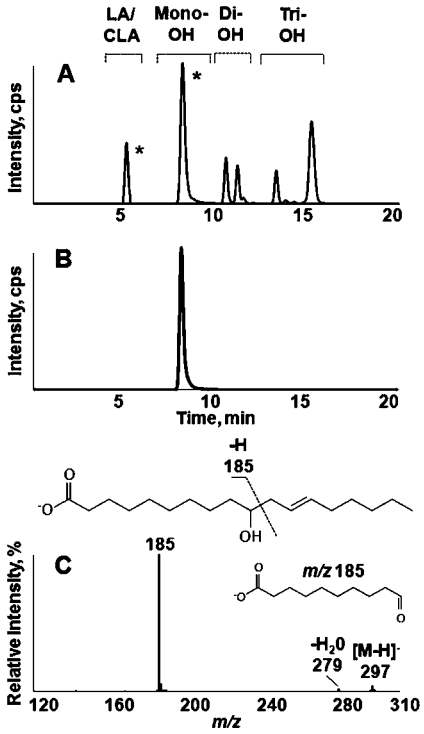

Figure 1. Silica LC/APPI-MS analysis of deprotonated underivatized fatty acids from *L. hammesii*. (A) XIC of *m/z* 279−331.5, encompassing the crude lipid extract profile before fractionation by HSCCC. Compounds that were produced by microbial transformation are labeled with an asterisk. (B) XIC of *m/z* 279−331.5, after fractionation by HSCCC. A single peak was collected, with an elution time of 8.4 min and a *m/z* 297.2403 ($C_{18}H_{33}O_3$). (C) MS/MS spectra of the compound isolated by HSCCC.

acids were considerably lower than those for hydroxylated compounds.

The LC/APPI-MS chromatogram of the lipid extract from *L. hammesii* before and after HSCCC fractionation was compared in parts A and B of Figure 1. Only a single peak with retention time of 8.4 min was observed in the HSCCC purified material, demonstrating the complete separation from the linoleic acid substrate as well as from more highly hydroxylated metabolites. The negative ion APPI mass spectrum averaged across the single peak seen in Figure 1B showed a single ion of nominal *m/z* 297 in addition to a background ion at *m/z* 59 assigned to acetic acid from the mobile phase. The measured mass for the observed $[M - H]^-$ ion at *m/z* 297.2410 corresponded to the exact mass of a monohydroxy octadecenoic acid (calculated *m/z* 297.2403).

Identification of the Hydroxyl Group Location. After collection by HSCCC, the antifungal hydroxy fatty acid produced by *L. hammesii* was analyzed by negative ion LC/APPI-MS/MS to identify the position of the hydroxyl group. The MS/MS spectrum of the $[M - H]^-$ ion at *m/z* 297 showed two main fragment ions at *m/z* 279 and 185 (Figure 1C). The ion at *m/z* 279 was due to loss of water from $[M - H]^-$, which was consistent with the presence of one hydroxyl group.[28] The fragment ion of *m/z* 185.1183, identified from the accurate mass as having an elemental composition of $C_{10}H_{17}0_3$, indicated the likely formation of an aldehyde at the Δ^{10} position of the hydroxyl fatty acid.[29] This implies that the hydroxyl group position was at Δ^{10}. However, the mass spectrum, which showed limited fragmentation, gave no indication of the location of the double bond.

The hydroxy group position was further confirmed by derivatization of the fatty acid to a DMOX derivative for subsequent analysis by GC/MS in the electron impact mode of ionization. Derivatives such as DMOX can be used in mass spectrometry to localize the charge, resulting in charge-remote fragmentation along the hydrocarbon chain.[30] This allows for simple interpretation of the mass spectra to determine the positions of functional groups and unsaturations. The mass spectrum of the DMOX derivative of the hydroxy fatty acid isolated from *L. hammesii* was shown in Figure 2. The intense

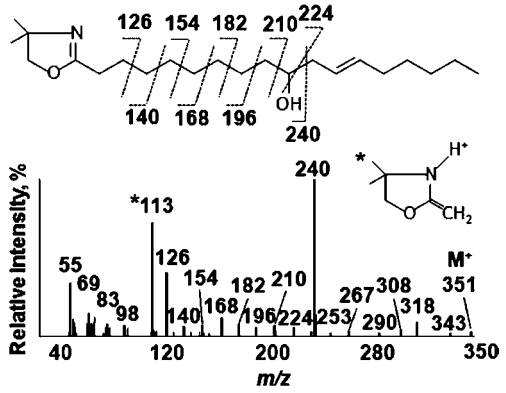

Figure 2. GC/EI-MS spectrum of 4,4-dimethyloxazoline derivative of monohydroxy C18:1 fatty acid from *L. hammesii*. The proposed McLaffertty ion at *m/z* 113 is designated with an asterisk.

ion at *m/z* 113 was characteristic of mass spectra of DMOX derivatives of fatty acids due to a McLafferty rearrangement ion.[30] The series of fragment ions separated by 14 mass units going from *m/z* 126 to *m/z* 210 indicate successive CH_2 groups, i.e., a saturated hydrocarbon chain. A 30 mass unit difference occurred between fragmentations *m/z* 210 and 240 (with intermediate ion at *m/z* 224), indicating the presence of a hydroxyl group as shown in Figure 2. The general features of this spectrum including the high abundance of the ion at *m/z* 240 resulting from fragmentation adjacent to the hydroxyl group are consistent with that seen for the isomeric DMOX derivative of 9-hydroxy-12-octadecenoic acid.[31] Thus, analysis of the DMOX derivative of the fatty acid metabolite from *L. hammesii* confirms the hydroxyl group to be located at Δ^{10}. However, although the double bond location was shown to be closer to the end of the chain than hydroxyl group, consistent with the MS/MS spectrum, its location cannot be determined from the spectrum of the DMOX derivative.

Identification of Double Bond Location. The position of the double bond in the hydroxy fatty acid from *L. hammesii* was identified after vicinal hydroxylation. Following this derivatization procedure, analysis by LC/APPI-MS resulted in two peaks from the monohydroxy fatty acid with retention times of 13.2 and 13.5 min (Figure 3A). The MS/MS spectra of the $[M - H]^-$ ions from both peaks showed identical fragmentation patterns (only one of which is shown in Figure 3), suggesting stereoisomers. The exact mass of the deprotonated molecule of *m/z* 331.2484 and the consecutive triple water loss ions at *m/z* 313, 295, and 277 (Figure 3B) indicated the presence of three hydroxyl groups, consistent with the addition of hydroxyl groups across the double bond (Figure 3 inset). The fragment ion at *m/z* 185 in the MS/MS spectrum was the result of

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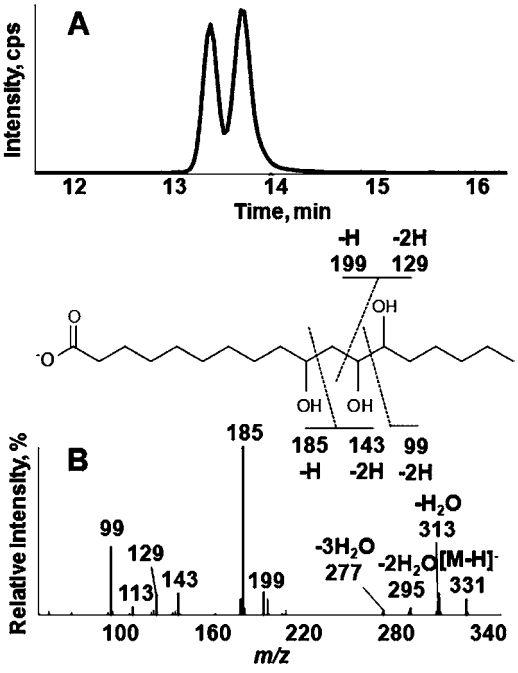

Figure 3. LC/APPI-MS analysis of deprotonated vicinal hydroxylation derivatized fatty acid from *L. hammesii*, exact mass m/z 331.2484 ($C_{18}H_{35}O_5$). (A) Silica separation of the hydroxylated monohydroxy C18:1 fatty acid collected from HSCCC, two peaks eluting at 13.2 and 13.5 min. (B) LC/APPI-MS/MS spectra of 10-hydroxy-12-octadecenoic acid, representative of both peaks.

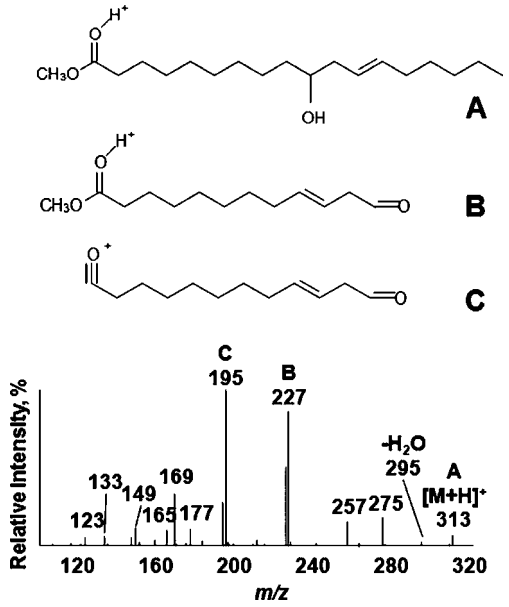

Figure 4. Ozonolysis/APPI-MS spectra of protonated methyl esters from *L. hammesii*. (A) $[M + H]^+$ m/z 313.2737. (B) Fragment ion m/z 227.1642, proposed structure. (C) Fragment ion m/z 195.1380, proposed structure.

fragmentation adjacent to the original hydroxy group, consistent with the underivatized fatty acid spectrum in Figure 2C. Considering the overall structure of a hydroxy fatty acid, a fragment ion at m/z 99.0815 ($C_6H_{11}O$) can only arise via cleavage that incorporates a single hydroxyl group. This cannot be the original hydroxy group because this would have required multiple cleavages along the chain and was thus highly unlikely. Furthermore, the fragment ion at m/z 129 and complementary ion at m/z 199 located the second of the vicinal hydroxyl groups in the derivative, as shown in Figure 3. Hence, the MS/MS spectra indicated that the double bond was located at the Δ^{12} position in the hydroxy fatty acid.

The position of the double bond at Δ^{12} was further confirmed by analysis of the methyl ester derivative of the hydroxy fatty acid using in-line ozonolysis/APPI-MS.[26] In this method, ozonolysis which cleaves double bonds, resulting in characteristic aldehydes that were indicative of double bond positions, was performed in-line with APPI-MS. The degree of ozonolysis was adjusted such that both the molecular ion $[M + H]^+$ and ozonolysis fragments were seen. In the ozonolysis/APPI-MS spectrum of the FAME derivative of the monohydroxy fatty acid from *L. hammesii*, the $[M + H]^+$ ion and subsequent water loss indicated a methylated monohydroxy fatty acid (Figure 4A). The abundant ion at m/z 227 corresponded to the formation of an aldehyde following ozonolysis and subsequent neutral loss of water. This was indicative of a double bond at Δ^{12} (Figure 4B). The ion at m/z 195 was due to a further methanol loss from the protonated aldehyde ion at m/z 227, which was often observed in previous ozonolysis experiments[26] (Figure 4C). Because oxidative cleavage of ozonolysis only occurs across carbon−carbon double bonds, in-line ozonolysis/APPI-MS unambiguously assigned the double bond position at Δ^{12}, in agreement with the vicinal hydroxylation result.

In summary, LC/APPI-MS/MS analysis identified the antifungal hydroxy fatty acid produced by *L. hammesii* to be 10-hydroxy-12-octadecenoic acid; the hydroxyl group and double bond locations were confirmed using GC/MS and in-line ozonlysis/MS.

Identification of Geometric Isomers. As described above, normal phase separation of the purified hydroxylated derivative of 10-hydroxy-12-octadecenoic acid gave a pair of peaks with identical mass spectra (Figure 3). Therefore, silver ion chromatography was employed to investigate the possibility that these stereoisomers arose through the *syn* addition of hydroxyl groups by OsO_4 to both the *cis* and *trans* geometric isomers of the unsaturated hydroxy fatty acid. The purified 10-hydroxy-12-octadecenoic acid from *L. hammesii* was collected by HSCCC, derivatized to FAME, and analyzed by silver ion LC/APPI-MS/MS. The extracted ion chromatogram (XIC) of the $[M + H]^+$ ion at m/z 295 gave two well-resolved peaks, as shown in Figure 5. The MS/MS spectra of the $[M + H]^+$ ions were identical for each of the two peaks (Figure 5), implying isomeric compounds. It is well-known that the interaction between silver ions and double bonds in the *trans* configuration are weaker than those with *cis* double bonds.[32] Thus, the two peaks eluting at 15.0 and 17.7 min can be identified as methylated *trans* and *cis* 10-hydroxy-12-octadecenoic acid, respectively. The *cis* isomer accounted for 53 ± 5% of the total peak area of the m/z 295 extracted ion chromatogram, while the *trans* isomer accounted for 47.4 ± 5%. This indicates that the 10-hydroxy-12-octadecenoic acid produced by *L. hammesii* was a racemic mixture of *cis* and *trans* isomers.

Analysis of the Lipid Extract from *L. plantarum* by Liquid Chromatography/Atmospheric Pressure Photo Ionization-Mass Spectrometry. The normal phase LC/APPI-MS/MS technique was further applied to analyze the crude lipid extract of *L. plantarum*, fermented in broth supplemented with linoleic acid. Comparison to cell free or

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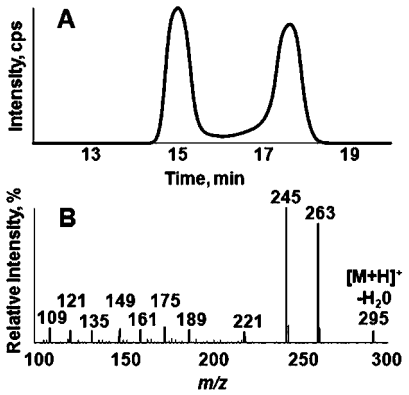

Figure 5. (A) XIC of a silver ion LC/APPI-MS chromatogram of methyl ester geometric isomers from *L. hammesii*, product ion of *m/z* 295.2. (B) MS/MS spectra representative of both methylated *trans* isomer at 15.0 min and methylated *cis* isomer at 17.7 min.

substrate free controls revealed that *L. plantarum* produced three hydroxy fatty acids and several CLA isomers. Two of these hydroxy fatty acids (one mono- and one dihydroxy compound) were not produced by *L. hammesii* under similar conditions (compare Figures 6A and 1A). Without fractionation or derivatization, LC/APPI-MS of the *L. plantarum* lipid extract shows a peak at 11.8 min ($[M - H]^-$ at *m/z* 315.2535, $C_{18}H_{35}O_4$); the MS/MS spectrum of this peak (Figure 7A) identifies it as 10,13-dihydroxy octadecanoic acid. The second compound eluting at 7.8 min in Figure 6A was identified as a

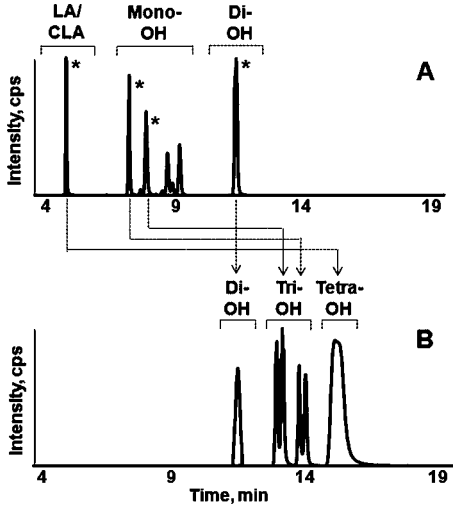

Figure 6. Silica LC/APPI-MS chromatograms of crude lipid extract from *L. plantarum*. (A) XIC of *m/z* 279−331.5, underivatized fatty acids. CLA and linoleic acid (LA) coeluted at 5.1 min. Compounds produced from microbial transformation were a secondary monohydroxy C18:1 fatty acid eluting at 7.8 min and 10-hydroxy-12-octadecenoic acid eluting at 8.4 min, both with an exact mass of *m/z* 297.2403 ($C_{18}H_{33}O_3$) and a saturated dihydroxy fatty acid eluting at 11.8 min with a mass of *m/z* 315.2535 ($C_{18}H_{35}O_4$). Compounds that were produced by microbial transformation are labeled with an asterisk. (B) XIC of *m/z* 279−348.5, hydroxylated derivatives of crude lipid extract. Resulting peaks consisted of a saturated dihydroxy fatty acid with a retention time of 11.8 min, *m/z* 315.2535 ($C_{18}H_{35}O_4$), saturated trihydroxy fatty acids, two pairs of peaks at 13.2 and 13.5 min and 14.0 and 14.3 min with a *m/z* of 331.2484 ($C_{18}H_{35}O_5$), and an unresolved group of saturated tetra-hydroxy fatty acids with a mass of *m/z* 348.2517 ($C_{18}H_{36}O_6$).

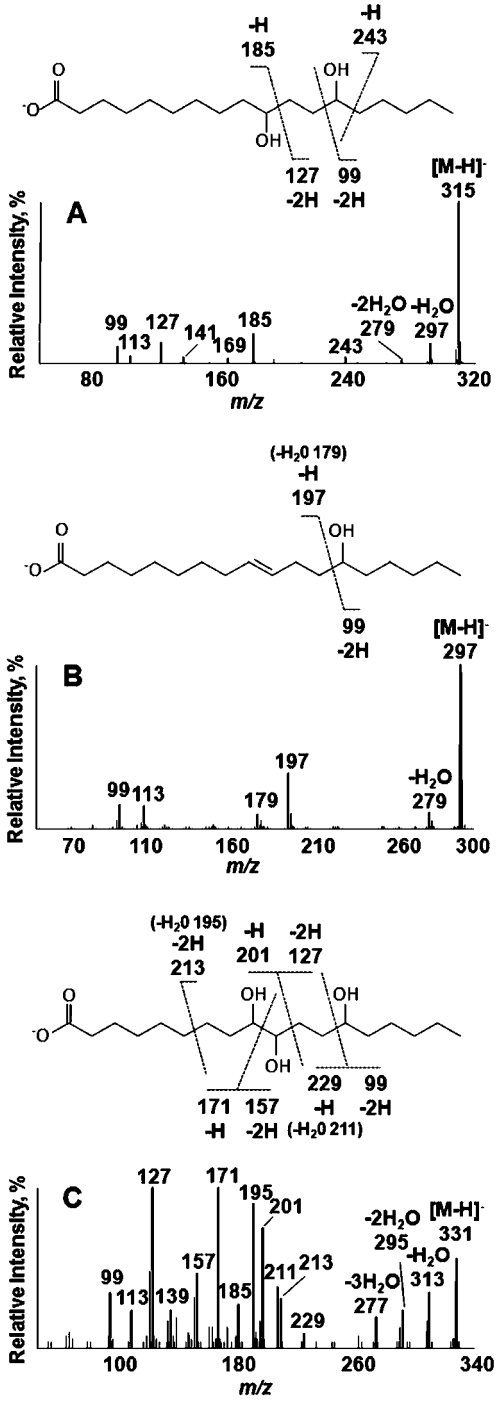

Figure 7. APPI-MS/MS spectra of hydroxy fatty acids from *L. plantaurm*. (A) Underivatized 10,13-dihydroxy octadecenoic acid eluting at 11.8 min, *m/z* 315.2535 ($C_{18}H_{35}O_4$). (B) Underivatized fatty acid eluting at 7.8 min, *m/z* 297.2403 ($C_{18}H_{33}O_3$). (C) Vicinal hydroxylation derivatized 13-hydroxy-9-octadecenoic acid, representative of both peaks at 14.0 and 14.3 min, *m/z* 331.2484 ($C_{18}H_{35}O_5$).

monohydroxy octadecenoic acid isomer (*m/z* 297.2403, $C_{18}H_{33}O_3$); its MS/MS spectrum (Figure 7B) was distinct to that of 10-hydroxy-12-octadecenoic acid (Figure 1C). The fragment ion at *m/z* 99.0815 ($C_6H_{11}O$) and complementary ion at *m/z* 197.1547 ($C_{12}H_{21}O_2$) identified the hydroxyl group position as Δ^{13}, but the MS/MS spectrum can only locate the double bond to between Δ^2 and Δ^{12}. Hence, vicinal hydroxylation of the whole crude lipid extract was performed.

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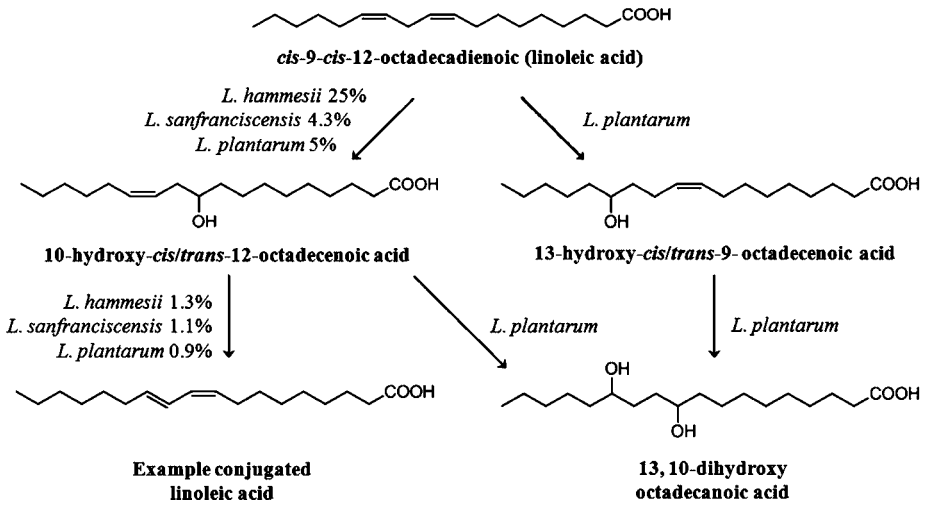
Scheme 1. A Proposed Scheme of Two Alternate Pathways for the Bioconversion of Linoleic Acid by Lactobacilli after 2 d[a]



[a]Each percentage represents the amount of compound converted of the original 4 g L^{-1} linoleic acid used for supplementation. Reactions are not reversible.

The LC/APPI-MS chromatogram (Figure 6B) of the resulting hydroxylated derivatives showed that while 10,13-dihydroxy octadecanoic acid was unaffected (retention time 11.8 min), the unsaturated compounds, such as linoleic acid/CLA and the monohydroxy fatty acids, were shifted to longer elution times due to the addition of hydroxyl groups across double bonds. Thus, monohydoxy C18:1 compounds were converted to diastereomers of saturated trihydroxy fatty acids (m/z 331.2484, $C_{18}H_{35}O_5$). At the same time, CLA isomers were converted to tetra-hydroxy derivatives. Additionally, from the *L. plantarum* fermented crude lipid extract, a separate peak pair resulted from the derivatization at retention times of 14.0 and 14.3, each with an identical MS/MS spectrum (Figure 7C), which indicates the double bond position at Δ^9 from diagnostic fragment ions at m/z 201, 171, and 157. Hence, in the case of the monohydroxy C18:1 fatty acid produced by *L. plantarum*, the combination of normal phase LC/APPI-MS/MS with vicinal hydroxylation was used to identify 13-hydroxy-*cis*/*trans*-9-octadecenoic acid, without fractionation of the crude lipid extract.

Pathway of Conversion. Conversion of linoleic acid by lactobacilli produced *cis*/*trans* 10-hydroxy-12-octadecenoic acid and CLA (Scheme 1). *L. hammesii* converted 25% (1.0 ± 0.1 g L^{-1}) of the substrate to 10-hydroxy-12-octadecenoic acid; other lactobacilli tested also produced this metabolite, including *L. sanfranciscensis* and *L. plantarum*, which converted 4.3% (0.17 ± 0.0 g L^{-1}) and 5% (0.2 ± 0.0 g L^{-1}), respectively. All strains produced racemic mixtures of the *cis* and *trans* isomers. After 48 h of fermentation with *L. hammesii*, *L. sanfranciscensis*, and *L. plantarum*, CLA isomers were observed (Supporting Information Figure 2) and accounted for 1.3% (0.1 ± 0.0 g L^{-1}), 1.1% (0.04 ± 0.01 g L^{-1}), and 0.9% (0.04 ± 0.0 g L^{-1}) of the initial substrate, respectively (Scheme 1). *L. plantarum* converted linoleic acid to *cis*/*trans* 13-hydroxy-9-octadecenoic acid and 10,13-dihydroxy octadecanoic acid in addition to 10-hydroxy-12-octadecenoic acid and CLA. These metabolites were not found in lipid extracts from cultures of *L. hammesii* and *L. sanfranciscensis*.

To determine whether the conversion of linoleic acid to hydroxy fatty acids and CLA was reversible, cultures were grown in presence of 4 g L^{-1} CLA or purified *cis*/*trans* 10-hydroxy-12-octadecenoic acid. 10-Hydroxy-12-octadecenoic acid was converted to CLA by all three strains, but formation of linoleic acid from 10-hydroxy-12-octadecenoic acid was not observed. CLA was not further transformed by any of the three strains. Hence, the conversion of linoleic acid to hydroxy fatty acids and further to CLA was found to be irreversible.

The enzymatic transformation of ricinoleic acid by *L. hammesii*, *L. sanfranciscensis*, or *L. plantarum* resulted in a saturated dihydroxy fatty acid as the sole product. Conversion of linolenic acid yielded a 10-hydroxy diunsaturated fatty acid as the sole product, indicating a preference for hydration of double bond located at Δ^9. These results confirm that hydratases of lactobacilli accept alternative substrates, however, only linoleic acid supported the formation of CLA.

DISCUSSION

In this study, LC/APPI-MS/MS was used to effectively elucidate the structure of antifungal 10-hydroxy-*cis*-12-octadecenoic and 10-hydroxy-*trans*-12-octadecenoic acids and to quantify metabolites of linoleic acid. APPI-MS was sensitive and allowed for the observation of underivatized fatty acids and their hydroxyl-derivatives from bacterial extracts. The ionization energy of the mobile phase itself was low enough that a postcolumn addition of a dopant was not necessary for the photoionization of fatty acids. No derivatization was required for both the identification of hydroxyl group positioning and the quantification of deprotonated fatty acids via silica LC/APPI-MS and tandem mass spectrometry. However, the addition of hydroxyl groups across the unsaturations was necessary to determine the position of the double bonds.

HSCCC was utilized as an efficient semipreparative separation for antifungal metabolites from lactobacilli extracts. The identification of hydroxy fatty acids with silica LC/APPI-MS/MS did not require a prestep fractionation with HSCCC, as the chromatography produced baseline resolution from crude lipid extracts of food and media fermented with lactobacilli.[17] Thus, when compared to previous fractionation and spectroscopic identification methods used for lactobacilli extracts,[4] much less effort was required for the fractionation and identification of hydroxy fatty acids by the methods described

above. In addition, the data were more informative than that obtained from GC/MS methods.[6,7,33]

10-Hydroxy-12-octadecenoic acid inhibits growth of *Aspergillus niger* and *Penicillium roqueforti*, and conversion of linoleic acid to 10-hydroxy-12-octadecenoic acid during growth of *L. hammesii* in sourdough delayed fungal spoilage of bread.[17] The *cis*/*trans* configuration of hydroxy fatty acids did not influence their antifungal activity.[34] However, the positioning of hydroxyl groups and double bonds strongly affected the antifungal activity of hydroxy fatty acids.[17,34] The LC/APPI-MS/MS methods that were developed in this study were essential for the identification of these antifungal metabolites of linoleic acid.

The first step of the conversion of linoleic acid by lactic acid bacteria is catalyzed by hydratases. Hydratases of lactic acid bacteria recognize oleic, linoleic, linolenic, and ricinoleic acid as substrates. They preferentially hydrate double bonds at Δ^9 and are specific for free fatty acids.[2,6,7] Our data thus conforms to known properties of hydratases and additionally demonstrates that the conversion is irreversible. Remarkably, hydratases of lactic acid bacteria do not hydrate *trans* double bonds[6,7] even though unsaturated hydroxy fatty acids were produced as racemic mixture of *cis* and *trans* isomers (this study). Linoleic acid was converted to 10,13-dihydroxystearic acid by *L. plantarum* via 10-hydroxy-12-octadecenoic acid and 13-hydroxy-9-octadecenoic acid but not by the other strains used in this study. It remains unknown whether the observed differences in the profile of fatty acid metabolites from different lactobacilli relate to functional differences of the hydratase enzymes or other species- or strain-specific differences.

A majority of reports on CLA formation by lactobacilli used buffer fermentations with high cell densities.[2−4] This study provides data on linoleic acid metabolites produced by growing cells, representing physiological conditions. Our observations demonstrate that hydroxy fatty acids are the major product of conversion while CLA levels remain low. Lactobacilli increase the proportion of hydroxy fatty acids in membrane lipids in response to environmental stress,[35] indicating a physiological role in the protection against heat stress and membrane-active inhibitors.[10] A physiological role of CLA, or the presence of CLA in bacterial membranes, remains to be demonstrated. Moreover, formation of CLA by purified enzymes from lactic acid bacteria has not yet been demonstrated. Recently, CLA formation from hydroxy fatty acids by *L. plantarum* was attributed to the combined activity of an alcohol dehydrogenase and acetolactate decarboxylase.[36] Taken together, CLA production from hydroxy fatty acids likely occurs as an enzymatic side reaction of metabolic enzymes that moonlight as linoleic acid isomerases. Comparable to the formation of hydroxy fatty acids by lactobacilli, the formation CLA by lactobacilli yields a mixture of *cis*-9,*trans*-11 and *trans*-9,*trans*-11 geometric isomers.[33,36] However, the previous analytical methods were not sufficiently sensitive and/or specific, and many isomers were potentially not accounted for. To date, the biological function of most CLA isomers remains unknown.[37] In this work, at least five as yet unidentified CLA isomers were shown to be produced by *L. hammesii* and *L. plantarum*. The analytical methods developed in this study can in future be used in optimizing the production of antifungal fatty acids in food fermentations while monitoring the production of CLA isomers of unknown biological activity.

The antifungal activity of 10-hydroxy-12-octadecenoic acid has initially been demonstrated in sourdough bread.[17] However, lactobacilli are commonly utilized in food fermentation,[1,17] and production of 10-hydroxy-12-octadecenoic acid was observed for several other food-fermenting lactobacilli.[2,17] Antimicrobial metabolites produced by lactobacilli during fermentation are considered as "natural" and have the potential to be used in many food applications. Within this study, we demonstrate a rapid and sensitive identification method as well as the means to collect increased quantities of 10-hydroxy-12-octadecenoic acid.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Flow injection APPI-MS analysis of 3 min fractions collected from HSCCC separations of *L. hammesii* lipid extracts. Ag^+ LC/APPI-MS XIC of protonated m/z 295.2, conjugated linoleic acid methyl ester isomers from *L. hammesii*. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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