Lactobacillus casei expressing methylglyoxal synthase causes browning and heterocyclic amine formation in Parmesan cheese extract

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ABSTRACT

Undesired browning of Parmesan cheese can occur during the latter period of ripening and cold storage despite the relative absence of reducing sugars and high temperatures typically associated with Maillard browning. Highly reactive α -dicarbonyls such as methylglyoxal (MG) are products and accelerants of Maillard browning chemistry and can result from the microbial metabolism of sugars and AA by lactic acid bacteria. We demonstrate the effects of microbially produced MG in a model Parmesan cheese extract using a strain of *Lactobacillus casei* 12A engineered for inducible overexpression of MG synthase (*mgsA*) from *Thermoanaerobacterium thermosaccharolyticum* HG-8. Maximum induction of plasmid-born *mgsA* led to 1.6 mM MG formation in Parmesan cheese extract and its distinct discoloration. The accumulation of heterocyclic amines including β -carboline derivatives arising from *mgsA* expression were determined by mass spectrometry. Potential MG-contributing reaction mechanisms for the formation of heterocyclic amines are proposed. These findings implicate nonstarter lactic acid bacteria may cause browning and influence nutritional aspects of Parmesan by enzymatic conversion of triosephosphates to MG. Moreover, these findings indicate that the microbial production of MG can lead to the formation of late-stage Maillard reaction products such as melanoidin and β -carbolines, effectively circumventing the thermal requirement of the early- and intermediate-stage Maillard reaction. Therefore, the identification and control of offending microbiota may prevent late-stage browning of Parmesan. The gene *mgsA* may serve as a genetic biomarker for cheeses with a propensity to undergo MG-mediated browning.

Key words: methylglyoxal-mediated browning, Parmesan cheese, methylglyoxal synthase, β -carboline, heterocyclic amine

INTRODUCTION

The development of brown pigmentation can materialize during the latter ripening and storage periods of Parmesan cheese, leading to the economic depreciation of affected cheeses (Divine et al., 2012; Divine and Rankin, 2013). Methylglyoxal (MG) augments and redirects the glycation of peptides, which can subsequently accelerate the formation of brown, nitrogenous, polymeric compounds known as melanoidins. The aldehydic carbonyl carbon of MG is highly electrophilic due to its oxidative state and vicinal position to a second ketonic carbonyl group, readily enhancing elimination, condensation, and other degradation mechanisms. Methylglyoxal-based browning of Parmesan proceeds as a part of the Maillard reaction; however, cold-storage temperatures and the relatively low abundance of reducing sugars do not favor nonenzymatic browning. Instead, evidence suggests that the microbial production of MG is a potential contributor to late-stage browning of Parmesan and is hypothesized to occur due to differences in either the composition or activity of the ripening microbiota caused by 2 different manufacturing styles: direct-salting and brine-salting (McDonald, 1992).

The browning defect is more prominently observed in cheeses that have undergone direct-salting of the curd before pressing, compared with the more time-intensive brine-salting of already pressed cheese. Directly salted cheeses have a uniform dispersion of salt throughout the wheel, whereas a brined cheese has a gradient concentration of salt, with less diffusion toward the center. However, it remains undetermined as to which types of organisms constitute the offending microbiota and to what extent it contributed toward browning.

Selective conditions of ripening cheese result in the decline and autolysis of starter cultures and concurrent rise of adventitious nonstarter lactic acid bacteria (NSLAB) up to 10^7 cfu·g⁻¹ of cheese (Fitzsimons et al., 2001; Solieri et al., 2012). Nonstarter lactic acid bacteria, predominated by facultative and obligate heterofermentative lactobacilli, significantly influence

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flavor maturation of long-ripened cheeses such as Parmesan. Because NSLAB exist as natural contaminants in the processing environment and within milk, their influence on cheese flavor maturation can be variable. To exert control over NSLAB microbiota of cheese, industry selects adjunct cultures with desired properties and adds these cultures to the cheese at relatively high numbers (Broadbent et al., 2011). Despite the effectiveness of this approach in achieving more consistent flavor maturation, much less is known about discriminating NSLAB that have the propensity to cause or prevent browning.

The enzymatic conversion of the glycolytic intermediate, dihydroxyacetone phosphate (**DHAP**), into MG and free phosphate is exclusively catalyzed by MG synthase in bacteria (Ferguson et al., 1998; Saadat and Harrison, 1999). During glycolysis, DHAP is converted to glyceraldehyde 3-phosphate (**GA3P**) and proceeds toward pyruvate to generate NADH and ATP. The conversion of DHAP to MG provides an alternative pathway of triosphosphate catabolism away from glycolysis, with the conversion of MG to lactic acid being uncoupled from ATP production. Because this glycolytic bypass is energy futile and produces a toxic electrophile as an intermediate, the biological function of MG synthase is much debated.

An increasingly cited explanation is that MG synthase functions as an overflow mechanism to prevent the accumulation of phosphorylated glycolytic intermediates while averting phosphate starvation of glycolysis (Cooper, 1984; Booth et al., 2003; Weber et al., 2005). The conversion of GA3P to glycerate 1,3-bisphosphate by GA3P dehydrogenase requires phosphate and NAD^+ ; therefore, the dephosphorylation of DHAP by MG synthase replenishes depleted phosphate levels. In turn, MG synthase is strongly inhibited by phosphate as well as the glycolytic intermediates 3-phosphoglycerate and phosphoenolpyruvate (Hopper and Cooper, 1972; Falahati et al., 2013). Hence, the ability to produce MG, despite its toxicity and metabolic disadvantage, may serve as another degree of control over the Embden–Meyerhof–Parnas (EMP) pathway in some bacteria.

Determining whether a cheese environment supports physiological conditions for MG production through persistent MG synthase activity has not been explored. Moreover, reaction products arising from the microbial production of MG in complex matrices such as cheese have yet to be evaluated for their contribution toward functional and nutritional aspects including browning. In this study, we employed a typical species of NSLAB, *Lactobacillus casei*, to overexpress MG synthase growing in a Parmesan cheese extract (**PCE**) to determine the extent of browning and elucidate compounds arising from microbially produced MG. We validated that

overexpressing MG synthase leads to increased production of MG, discoloration of PCE, and formation of heterocyclic amine compounds. Among these heterocyclic amines, we structurally validated the presence of β -carboline derivatives using commercially available standards.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Stock cultures of *L. casei* 12A and *Escherichia coli* MC1061 sub-cloning host were maintained at -80°C with 15% (vol/vol) glycerol in de Man, Rogosa, and Sharpe (**MRS**) and Luria-Bertani (**LB**) medium (BD Biosciences, Sparks, MD), respectively. Derivatives of pSIP411 were maintained in *L. casei* and *E. coli* using 2.5 and 500 $\mu\text{g/mL}$ of erythromycin (**Erm**), respectively. Working cultures of *L. casei* in PCE were prepared from frozen stocks by a single transfer in MRS and one successive 0.1% (vol/vol) transfer in PCE. All sub-cultures and working cultures of *L. casei* were propagated at 37°C , without shaking, for 16 to 18 h. *Escherichia coli* MC1061 was propagated in LB aerobically at 37°C overnight. The pSIP-inducer was obtained as a 98% pure synthesized peptide (Peptide 2.0, Chantilly, VA) and 100 ng/mL was added to *L. casei* cultures once optical density at 600 nm (OD_{600}) = 0.3 was reached, when applicable.

Parmesan Cheese Extract

The PCE was obtained from a 10-mo-old Parmesan cheese and processed by the Western Dairy Center at Utah State University. A Parmesan cheese block was cut into 5.08-cm cubes and processed through a Com-mitrol grinder (Urschel, Chesterton, IN) using a 0.317-cm cutting head. The ground cheese was vacuum sealed and cooled at 4°C for 24 h. A minimum of 54.4 kg of cheese was combined with 265 L of deionized water in a stainless-steel, steam-heated, jacketed vat with a total capacity of 321 L. The cheese and water mixture was continuously stirred and heated from 15.5°C to 48.8°C over 40 min and held at 48.8°C for 20 min. Cheese solids were removed from the cheese-water extract by processing through a cheesecloth. The extract (~ 265 L) was processed through 2 spiral-wound UF membranes with a molecular weight cutoff of 5,000 kDa while using an operating flow of 18.9 L/min, operating temperature of 48.8°C , and operating pressure of 100 to 1,000 kPa. The permeate was collected and recycled once with the concentrated retentate through the UF system before a final reverse osmosis filtration treatment. The reverse osmosis filtration system used the aforementioned con-

ditions albeit at an operating pressure of 3,000 to 6,000 kPa. The extract was packaged and stored at -20°C . Before use as a bacterial growth medium, 2% glucose was added to PCE, filter sterilized (0.2 μm filter, Bottle-top Nalgene, Thermo Fisher Scientific, Waltham, MA), and stored at 4°C .

Construction of MG Producing *L. casei* 12A

All primers used for the construction of *L. casei* harboring pSIP411_ *mgsA* are listed in Table 1. The MG synthase (*mgsA*) from *Thermoanaerobacterium thermosaccharolyticum* HG-8 (GenBank AF127246.1) was codon optimized for expression in *L. casei* using JCAT (Grote et al., 2005) and synthesized by The US Department of Energy Joint Genome Institute (Walnut Creek, CA; Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-15042>). The plasmid pSIP411_ *gusA* (β -glucuronidase) was obtained from Lars Axelsson (Norwegian Food Research Institute, Ås, Norway) and was used as a control as well as template DNA for the pSIP411 backbone (Sørvig et al., 2005). The plasmid pSIP411_ *mgsA* was constructed using Gibson Assembly (Gibson et al., 2009). The pSIP411 backbone (5,864 bp) and *mgsA* gene (405 bp) were amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) as per the manufacturer’s recommendation. Primers, 60 bp in length, containing 30-bp overlapping regions homologous to the pSIP411 backbone, were used to amplify *mgsA*. Amplicons were gel purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Gibson Assembly Mastermix was used for a 2-fragment assembly as per the manufacturer’s recommendations (New England Biolabs). Gibson reactions were transformed into electrocompetent *E. coli* MC1061 and transformants were selected on LB plates containing 500 $\mu\text{g}/\text{mL}$ of Erm. Positive transformants were screened by colony PCR and the sequence of pSIP411_ *mgsA* was confirmed by Sanger sequencing (University of Wisconsin, Biotechnology Center).

Recombinant plasmid pSIP411_ *mgsA* was transformed into electrocompetent *L. casei* 12A as described

by Welker et al. (2015). Briefly, cells were grown in MRS containing 2% glycine to an $\text{OD}_{600} = 0.6$, washed with ice-cold sterile deionized and distilled H_2O , and cell slurries re-suspended in 30% polyethylene glycol (PEG-8000). Cells were treated for 20 min at room temperature with 1 volume filter sterilized lithium acetate mixture (1.2 *M* sucrose, 200 *mM* lithium acetate, and 20 *mM* Tris) and 0.2 volume filter sterilized dithiothreitol before being washed once and re-suspended in PEG-8000. Electroporation was performed in 2-mm cuvettes using a Bio-Rad Gene Pulser (Hercules, CA) set to 2,500 V, 25 μF , and 400 Ω . Cells were recovered in MRS containing 0.5 *M* sucrose for 4 h before being plated onto MRS containing 2.5 $\mu\text{g}/\text{mL}$ of Erm. Positive transformants were confirmed by colony PCR.

Quantification of MG Production and Browning in PCE

Analyte concentrations of MG and 2,3 hexanedione (internal standard) were determined by derivatization with 1,2-diaminobenzene to form 2-methylquinoxaline and 2,3-diethylquinoxaline, respectively, and measured by GC-MS using methods adapted from Chaplen et al. (1996) and the Compendium of International Analysis of Methods (2010). Early stationary-phase cells of *L. casei* 12A harboring pSIP411_ *mgsA* were inoculated (0.1% vol/vol) into 50 mL of PCE supplemented with 2% glucose and 2.5 $\mu\text{g}/\text{mL}$ of Erm. Cultures were induced with 100 ng/mL of pSIP-inducer at $\text{OD}_{600} = 0.3$ before being incubated at 37°C for 24 h. Uninduced control cultures as well as *L. casei* 12A harboring pSIP411_ *gusA* (β -glucuronidase) were included alongside as controls.

The PCE supernatants were visually inspected for browning before quantification of MG using GC-MS. A browning unit (BU) was used to define visualized discoloration of PCE (defined as the spectrophotometric difference measured at 420 and 500 nm). An increase in BU represents browning of PCE. Added to each 50-mL sample was 17.5 *mM* 2,3-hexanedione. Each sample was treated with 1.94 mL of 70% perchloric acid and incubated on ice for 30 min before being centrifuged

Table 1. Primers used for the construction of pSIP411 expressing methylglyoxal synthase (*mgsA*)

Primer	Description	Sequence ¹
mgsA_F	Gibson assembly	aaatactattacaaggagatttttagccatgGTTAACTTGAACATCGCTTTGATCGCTCAC
mgsA_R		accgggggtaccgaattcctcgagtctagaTTACTTAACAGCGTTACGCCATTCCAACAA
pSIP411_F		TCTAGACTCGAGGAATTCGGTACCCCGGT
pSIP411_R		GGCTAAAATCTCCTTGTAATAGTATTTTATAGAATACATATATGCTGGCC
MCS_F	Screening	GCTCCAGATCTACCGGTTTAAT
MCS_R		CTATCGAAAGCGAAATCAAACGAA

¹Gibson primers: uppercase and lowercase nucleotides represent the annealing and overhanging regions, respectively.

at $11,400 \times g$ for 10 min at 4°C. Supernatants were separated from cell pellets and allowed to reach room temperature before being brought to a pH of 8.0 using NaOH. Added to each sample was 20 mg of 1,2-diaminobenzene. Derivatization occurred at 60°C for 3 h before samples were brought to room temperature and a pH of 2.0 using H₂SO₄. Liquid-liquid extraction was performed twice using 5 mL of dichloromethane and decanting the lower phase using a separatory funnel. Extracts were dried over 1 g of anhydrous Na₂SO₄, decanted, and syringe filtered into 2-mL GC-MS vials. Commercial solutions of 2,3-hexanedione, 2-methylquinoxaline, 2,3-diethylquinoxaline, and tablets of 1,2-diaminobenzene were purchased from Sigma-Aldrich (St. Louis, MO).

The gas chromatograph (Agilent 6890N, Agilent Technologies Inc., Palo Alto, CA) was fitted with a fused-silica capillary column (RTx-5MS, 30 m long $\times$ 0.25 mm i.d. $\times$ 0.5 μ m film thickness; Restek Corp., Bellefonte, PA) and paired with a mass selective detector (Agilent 5973 MS, Agilent Technologies Inc.). The initial temperature of the front inlet was 250°C at a flow of 40.0 mL/min, using helium as the carrier gas. The volume injected was 1 μ L, splitless. The oven temperature was held at 60°C for 1 min, then programmed to increase at a rate of 10°C/min to 220°C with a final isothermal period of 10 min. Chemstation Software (Rev. D.02.00 SP1, Agilent Technologies Inc.) was used to identify 2-methylquinoxaline and 2,3-diethylquinoxaline representing MG and 2,3-hexanedione, respectively. Analytes were confirmed by comparison of retention times to purchased standards and the selected ion monitoring method; MG: ions m/z = 117 and 144, 2,3-hexanedione: ions m/z = 158 and 171. The quantitative determination of MG and 2,3-hexanedione was performed using a multiple-point external standard curve of 2-methylquinoxaline and 2,3-diethylquinoxaline from 10 μ M to 2 mM and analyte concentrations were normalized to the maximum OD₆₀₀ achieved after growth. Statistical significance of biological triplicates from the control was determined using a paired *t*-test with α of <0.05.

Heterocyclic Amine Detection

Lactobacillus casei 12A harboring pSIP411_*mgsA* was inoculated (0.1%) into 5 mL of PCE containing 2% glucose and 2.5 μ g/mL of Erm. Experimental cultures were induced with 100 ng/mL of pSIP-inducer at OD₆₀₀ = 0.3 before being incubated at 37°C for 72 h. Cultures were centrifuged at $11,400 \times g$ for 5 min at 4°C and supernatants were passed through a 0.2 μ M filter before being frozen at -20°C until further analysis. Analysis was performed in biological triplicate.

Amicon ultra 10 kDa molecular weight cutoff filters (Millipore, Temecula, CA) were first rinsed with 18 MOhm of deionized water and then 200 μ L of sample was applied. Samples were centrifuged at $14,000 \times g$ for 20 min at room temperature and ~150 μ L of filtrate were removed. Samples were analyzed by liquid chromatography (LC)-MS using an Agilent 1969A ESI-TOF in positive ion mode coupled with a 1200 series Binary Pump SL. Sample injection volume was 5 μ L. Solvents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The column was a Phenomenex Synergi Hydro-RP (Torrance, CA), 150 mm $\times$ 2 mm held at 25°C. Sample constituents were gradient eluted over 38 min starting at 0% B held for 2 min, then increasing to 30% B over 30 min, increasing to 95% B over 6 min, holding for 1 min at 95% B, then returning to 0% B over 1 min and re-equilibrating for 5 min. The flow rate was constant at 250 μ L/min. Mass spectrometer parameters were 350°C gas temperature, 10 L/min of drying gas, 35 psig nebulizing gas, 3,600 V of electrospray ionization (ESI) voltage, fragmentor at 130 V, skimmer at 60 V, and octopole RF (radio frequency) at 250 V. Scans were acquired over the m/z range 50 to 1,700 at 1.24 scans/s averaging 10,040 transients/scan. A dual-spray source was used with the main sprayer receiving the HPLC column effluent and the second sprayer receiving internal calibrant solution at 30 μ L/min. Applied Biosystems MDS Sciex Analyst QS version 1.1 and Agilent MassHunter Workstation version 5.1.2600 was used for data acquisition and analysis (Agilent Technologies Inc., Santa Clara, CA).

Structural Identification of Heterocyclic Amines

The LC-MS/MS was performed on 8 species previously identified in PCE due to expression of *mgsA* in *L. casei* 12A. Culture conditions and sample preparations were as previously stated. The HPLC used an Agilent 1100 binary pump with column compartment and WPALS thermostatted autosampler. Mass spectrometry was performed on a Sciex QTRAP 5500 in positive ion ESI mode using the TurboIon source. The LC separation was achieved with a Synergi Hydro-RP column (Phenomenex), 150 mm $\times$ 2 mm, using solvents containing 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), both purchased as pre-mixed solvents (Thermo Fisher Scientific, LC/MS grade). Compounds were gradient eluted at 250 μ L/min with the following program: 0% B for 2 min, increase to 30% B over 30 min, increase to 95% B over 6 min, hold for 1 min, return to 0% B over 1 min and re-equilibrate for 15 min. The column was held at 25°C and the autosampler was chilled to 6°C. Injection volume was 5 μ L. The MS/MS was performed in 4 periods

targeting 2 compounds per period and collecting MS/MS data at 3 collision energies (10, 20, and 40) per compound. Data were acquired in enhanced product ion mode using the ion trap function of the instrument at a scan rate of 1,000 Da/s with unit resolution in Q1 for precursor selection and dynamic fill time with a target value of 1×10^8 cps. Source parameters were as follows: CUR 40, CAD high, IS 3250, TEM 650, GS1 45, GS2 40, DP 50, EP 10, and CES 0 (CUR = curtain gas in psi; CAD = collision gas setting; TEM = ionspray temperature; GS1 = nebulizing gas in psi; GS2 = ionspray gas in psi; DP = declustering potential in V; EP = entrance potential in V; CES = collision energy spread in V). Period 1 (0–16.5 min) precursors were 127 and 143, period 2 (16.5–22 min) precursors were 229 and 217, period 3 (22–28 min) precursors were 331, 183, and period 4 (28–50 min) precursors were 255 and 211. The LC was controlled using Agilent OpenLab CDS/Chemstation C.01.03 and the MS was controlled using Analyst 1.6.2. The MS acquisition was triggered by contact closure from the LC.

Comparison of Unknowns with Standard Compounds by LC-MS/MS

Based on retention time comparisons between standards and unknown compounds detected in PCE, targeted LC-MS/MS was performed to determine similarity between unknowns and standard compounds. Harman (Sigma-Aldrich), 1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (Sigma-Aldrich), and 1,2,3,4-tetrahydroharmane-3-carboxylic acid (Sigma-Aldrich) were initially dissolved in dimethyl sulfoxide, then diluted to suitable concentration in 0.1% formic acid, 50% acetonitrile in water as a mixed standard containing all 3 compounds. The LC-MS/MS was performed on a Thermo TSQ Quantum Discovery Max triple quadrupole mass spectrometer coupled to Agilent 1100 binary HPLC with WPALS autosampler and column compartment. The HPLC column and gradient were exactly as described above. Targeted full-scan MS/MS was performed on the standards and unknowns in their protonated forms at m/z 217.1 (1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid), 183.1 (Harman), 231.1 (1,2,3,4-tetrahydroharmane-3-carboxylic acid), and m/z 229.1, for which no standard was available. The ESI-MS was performed using the HESI-II source at 4,000 V in positive ion mode with heated electrospray at 400°C. Sheath gas was set to 30 (arbitrary units), sweep gas was at 0, auxiliary gas at 1, and heated capillary at 325°C. The MS/MS parameters for all targeted scans were 0.5 s scan time, Q1 and Q3 set to 0.7 amu FWHM (full width half max), 40 V of collision energy, and 1.5 mT of argon as collision gas. Scan ranges were

50 to 240 for m/z 217.1, 50 to 210 for m/z 183.1, 50 to 260 for m/z 231.1, and 50 to 260 for m/z 229.1. Spectra were obtained by averaging across the chromatographic peak for each precursor and then applying 7-point boxcar smoothing.

RESULTS

Using the sakacin-based inducible plasmid pSIP411 to express MG synthase (*mgsA*) from *T. thermosaccharolyticum* HG-8 in *L. casei* 12A led to the production of 1.6 mM MG in PCE (Figure 1). This was found to be a statistically significant increase in MG production compared with the uninduced *mgsA* control (0.09 mM) and both uninduced and induced *gusA* (β -glucuronidase) controls (0.009 mM) as determined using a paired *t*-test with α of <0.05 . In addition, we established that inducing expressing of *mgsA* causes visible browning of the PCE supernatant, as represented by the increase from 0.11 to 0.43 BU.

We detected 8 compounds in PCE resulting from the expression of *mgsA* in *L. casei* 12A using LC-MS (ESI-time of flight; Figure 2). Based on our analysis, the 8 compounds arising from *mgsA* expression had masses ranging from 126.0313 to 330.1211 g/mol, which were

Figure 1. Quantification of methylglyoxal production by *Lactobacillus casei* 12A expressing plasmid-born methylglyoxal synthase (*mgsA*) growing in Parmesan cheese extract (PCE). Early stationary-phase cells of *L. casei* 12A harboring pSIP411-*mgsA* were inoculated (0.1% vol/vol) into 50 mL of PCE supplemented with 2% glucose and 2.5 μ g/mL of erythromycin. Cultures were induced with 100 ng/mL of pSIP-inducer for optical density at 600 nm = 0.3 before being incubated at 37°C for 24 h. Uninduced control cultures as well as *L. casei* 12A harboring pSIP411-*gusA* (β -glucuronidase) were included alongside. Methylglyoxal concentrations were determined in PCE supernatant by derivatization with o-phenyldiamine and GC-MS. Statistical significance from the control (*) was determined using a paired *t*-test with α of <0.05 . Error bars represent SD of the mean of biological triplicates.

Figure 2. Liquid chromatography-electrospray ionization time-of-flight base-peak chromatograms depicting compounds in Parmesan cheese extract (PCE) resulting from growth of *Lactobacillus casei* 12A expressing methylglyoxal synthase (*mgsA*; top). The uninduced control culture is included (bottom). *Lactobacillus casei* 12A harboring pSIP411-*mgsA* was inoculated into 5 mL of PCE containing 2% glucose and 2.5 $\mu\text{g/mL}$ of erythromycin. Cultures were induced with 100 ng/mL of pSIP-inducer at optical density at 600 nm = 0.3 before being incubated at 37°C for 72 h. The PCE supernatants were filter sterilized (0.2 μm filter) and frozen at -20°C until further analysis. Samples were filtered (10 kDa molecular weight cutoff) and analyzed in biological triplicate. Numbers seen above peaks represent the observed ion mass [M+H]. Peaks (8) unique to methylglyoxal synthase expression are specified by black arrows.

used to predict their chemical composition using the METLIN simple search function. In an attempt to identify these compounds structurally, we used LC-MS/MS and different collision energies (10, 20, and 40 eV) to generate mass spectra. Fragment ions and their intensities generated from a collision energy of 40 eV were compared against the METLIN database using

MS/MS spectrum and fragment ion similarity search functions (Table 2).

We detected the formation of β -carboline derivatives of heterocyclic amines among the compounds formed in PCE due to overexpression of *mgsA* by *L. casei* 12A. Compound 4 ($\text{C}_{12}\text{H}_{12}\text{N}_2\text{O}_2$) was positively identified to be 1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid

(TH β CA) based on co-elution and spectral match using a commercially available standard (Figure 3A). The TH β CA was the only compound observed to form in PCE from the exogenous addition of MG (data not shown). Compound 6 (C₁₂H₁₀N₂) closely resembled Harman (1-methyl- β -carboline) based on similar mass spectra and nearly identical co-elution; however, it is likely the unknown species represents a close isomer (Figure 3B).

The commercial standard for 1,2,3,4-tetrahydroharmane-3-carboxylic acid displayed 2 separate chromatographic peaks at m/z 231 and the unknown in the sample at m/z 231 also showed 2 separate chromatographic peaks. The first of these peaks co-eluted with the first peak from the standard and showed excellent MS/MS spectral similarity (Figure 4A). Whereas the second peak at m/z 231 in the standard and in the sample showed excellent co-elution, the MS/MS spectra displayed some differences, indicating that the structures were not identical (Figure 4B). Additionally, although no standard was identified as a candidate structure for the unknown at m/z 229, it is possible that this compound is formed by inclusion of a double bond in the pyridine ring of compound 3 (removal of 2 hydrogen atoms), representing a similar species such as 1-methyl-3,4-dihydro-2H- β -carboline-3-carboxylic acid.

Spectral matches for C₁₃H₈N₄O₄, C₁₇H₁₈N₂O₅, C₉H₁₀N₄O₃S, and C₈H₁₀N₄OS were not found; however, their chemical composition implicate the formation of nitrogenous heterocycles, many of which are inferred to contain pyrazine or imidazole sub-groups. The exception to heterocyclic amine formation is the compound C₆H₆O₃, which was found to likely be either a pyrone or quinone derivative.

We outline potential MG-contributing reaction mechanisms that may contribute to the formation of heterocyclic amines (Figure 5). These reactions de-

scribe the formation of Strecker aldehydes, pyrazines, imidazo-quinoxalines, and β -carbolines. The Strecker degradation of glycine or alanine with MG, for example, can lead to the formation of formaldehyde and acetaldehyde, respectively (Nursten, 2005). Second, the formation of alkyl pyrazines is generally linked to Strecker degradation in which the α -dicarbonyl undergoes transamination, leading to an α -aminocarbonyl compound. The subsequent condensation of 2 α -aminocarbonyls leads to various substituted pyrazines which offer a low threshold nutty, toasted, or roasted aroma (Rizzi, 2008). For example, the condensation of aminoacetone, the α -aminocarbonyl derived from MG, can form 2,5-dimethyl-pyrazine. Furthermore, creatine that is naturally occurring or biosynthesized from glycine and arginine, can self-condense into creatinine and react with pyrazine and aldehyde constituents to form imidazo-quinoxalines (Jägerstad and Skog, 1991; Skog et al., 1992). Last, β -carboline compounds can be derived from indolethylamines and Strecker aldehydes in a mechanism that likely resulted in the products formed herein. The reaction mechanism begins by the primary amine of an indolethylamine (e.g., tryptamine or tryptophan) molecule performing nucleophilic attack on the carbonyl carbon of an aldehyde to form an imine intermediate. The reaction proceeds by the Pictet-Spengler reaction, in which the intermediate undergoes ring closure after condensation (Cox and Cook, 1995).

DISCUSSION

The growth of NSLAB is a critical component for the maturation of many long-ripened cheeses, yet the microbial production of MG can contribute to melanoidin formation, leading to undesired browning of Parmesan cheese and overall economic loss. Ongoing MG synthase activity is one potential source of MG in Parmesan

Table 2. Compounds detected in Parmesan cheese extract after 72 h of growth of *Lactobacillus casei* 12A expressing methylglyoxal synthase (*mgsA*)¹

Compound	Retention time (min)	Mass (g/mol)	Fragment ions m/z value (relative intensity)
1. C ₆ H ₆ O ₃	12.73	126.0313	126.9 (100); 69.2 (82.5); 81.2 (42.5); 109.1 (30); 67.2 (17.5); 87.1 (7.5); 79.2 (6.2); 73.1 (6.2); 61.2 (5); 63.2 (5); 53.3 (0.6); 55.3 (0.6)
2. C ₁₃ H ₈ N ₄ O ₄	13.36	142.0240	142.9 (100); 69.2 (95); 124.9 (31); 79.2 (18); 113.1 (6)
3. C ₁₃ H ₁₂ N ₂ O ₂	16.63	228.0902	143.0 (100); 167.0 (40); 156.0 (40); 115.1 (40); 130.0 (40); 91.1 (10); 192.6 (3)
4. C ₁₂ H ₁₂ N ₂ O ₂	18.34	216.0904	143.9 (100); 115.1 (60); 127.1 (50); 77.0 (50); 103.2 (28); 91.1 (17); 74.1 (8)
5. C ₁₇ H ₁₈ N ₂ O ₅	22.09	330.1211	256.4 (100); 210.8 (86); 169.1 (60); 238.6 (20); 198.9 (20); 142.1 (18); 294.2 (10); 155.1 (10); 130.2 (7); 330 (7); 118.3 (4)
6. C ₁₂ H ₁₀ N ₂	22.53	182.0843	115.0 (100); 89.0 (20); 140.0 (15); 140.0 (12); 127.9 (10); 181.1 (10); 168.0 (10); 154.0 (10)
7. C ₉ H ₁₀ N ₄ O ₃ S	37.41	254.0469	180.8 (100); 226.5 (40); 153.9 (40); 208.6 (35)
8. C ₈ H ₁₀ N ₄ OS	38.6	210.0573	168.8 (100); 192.6 (86); 115.1 (24); 139.9 (24); 210.6 (17); 184.8 (8)

¹Accurate mass was determined by liquid chromatography (LC) electrospray ionization time-of-flight (Agilent 1969A, Agilent Technologies, Santa Clara, CA). The mass to charge ratio (m/z) of fragment ions and their relative intensity were generated using a collision energy of 40 eV and determined by LC-MS/MS (Sciex QTRAP 5500, Sciex, Framingham, MA).

Figure 3. Liquid chromatography tandem mass spectrometry (LC-MS/MS) comparison between β -carboline derivatives formed in Parmesan cheese extract (PCE) from *Lactobacillus casei* 12A expressing methylglyoxal synthase (*mgsA*) and 1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (TH β CA) and Harman. (A) Compound 4 and TH β CA, and (B) compound 6 and Harman. The mass to charge ratio (m/z) of fragment ions and their relative abundance in samples and commercially available standards were generated using a collision energy of 40 eV and detected by LC-MS/MS (Thermo TSQ Quantum Discovery Max, Thermo Fisher Scientific, Waltham, MA). The co-elution profile of the compound and standard is depicted in the upper right-hand corner of each mass spectrum.

Figure 4. Liquid chromatography tandem mass spectrometry (LC-MS/MS) comparison between β -carboline derivatives formed in Parmesan cheese extract (PCE) from *Lactobacillus casei* 12A expressing methylglyoxal synthase (*mgsA*) and 1,2,3,4-tetrahydroharmane-3-carboxylic acid (THHCA; A) peak 1 and (B) peak 2. The mass to charge ratio (m/z) of fragment ions and their relative abundance in samples and commercially available standards were generated using a collision energy of 40 eV and detected by LC-MS/MS (Thermo TSQ Quantum Discovery Max, Thermo Fisher Scientific, Waltham, MA). The co-elution profile of the sample and the standard is depicted in the upper right-hand corner of each mass spectrum.

Figure 5. Possible methylglyoxal (MG)-contributing reaction mechanisms for the formation of heterocyclic amines. (A) Formation of aldehydes (box) from Strecker degradation of MG and AA (Nursten, 2005). (B) Formation of 2,5-dimethyl-pyrazine from condensation of MG (Rizzi, 2008). (C) Formation of imidazo-quinoxaline derivatives from the condensation of creatine to creatinine and subsequent reaction with pyrazine and aldehyde constituents (Jägerstad and Skog, 1991; Skog et al., 1992). (D) Formation of (I) Harman, (II) 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, and (III) 1,2,3,4-tetrahydroharmane-3-carboxylic acid from the Pictet-Spengler condensation of formaldehyde (R = H) or acetaldehyde (R = CH₃) with tryptamine (R₁ = H) or tryptophan (R₁ = COOH; Cox and Cook, 1995).

cheese and may exacerbate browning during ripening and cold-storage periods. We demonstrate that the microbial production of MG leads to browning and heterocyclic amine formation in PCE despite atypical conditions supporting Maillard reactions. Determining the effects of MG synthase activity in Parmesan cheese may help implicate specific MG-producing NSLAB so that industry can focus on mitigating their occurrence.

This study used PCE to simulate constituents of Parmesan that would readily react with MG. Moreover, the plasmid-born overexpression of transgenic *mgsA* was used as a tool for the in vivo production of MG in *L. casei*, a method which we find differs from the

exogenous addition of MG in respect to the amount of reaction products formed. The presence of *mgsA* is distributed in at least 17 species of *Lactobacillus* to date, according to the GenBank database (Benson et al., 2017). To detect the formation of MG adducts from bacterial overexpression of *mgsA*, we required conditions that increased the magnitude of MG production, such as the inclusion of 2% glucose and growth at 37°C. Furthermore, because MG is bacteriostatic, we used an inducible system for timely expression of *mgsA* once a relatively higher cell density was achieved. The *mgsA* gene from *T. thermosaccharolyticum* HG-8 has been previously shown to efficiently catalyze the conversion

of DHAP to MG and its nucleotide sequence was codon optimized for expression in *L. casei* (Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-15042>; Altaras et al., 2001). We demonstrate the ability of the system to cause browning in PCE as a result of MG production and determine the formation of heterocyclic amines including β -carboline derivatives, which pose larger implications toward functional, nutritional, and quality aspects of affected cheeses.

Beta-carbolines are naturally occurring hetero-tricyclic alkaloids that are also readily produced during production, processing, and storage of food. The essential skeleton is composed of a pyridine ring and indole group and is derived from Pictet-Spengler condensation of indolethylamines and aldehydes or α -ketoacids (Cox and Cook, 1995; Maryanoff et al., 2004).

Beta-carbolines are considered mutagenic and carcinogenic compounds, and have been extensively studied for their neurochemical and pharmacological action (Airaksinen and Kari, 1981; Wakabayashi et al., 1997; Husbands et al., 2001; Totsuka et al., 2004; Miralles et al., 2005; Herraiz et al., 2010). Beta-carbolines are bioactive compounds, hence dietary exposure and their presence in foodstuffs have garnered much interest. Tetrahydro- β -carbolines and the fully aromatic oxidized form, Harman, have been extensively determined to occur naturally in many commercial foods such as fruit juices, soft drinks, fermented alcoholic beverages, fermented sauces, vinegar, meats, fish, cheese, yogurt, toasted bread, and coffee (Sen et al., 1995; Herraiz and Sanchez, 1997; Herraiz, 2000a,b,c, 2002; Herraiz and Galisteo, 2002; Pfau and Skog, 2004). High temperature cooking applications associated with Maillard chemistry, such as smoking, grilling, and frying of foods, have been shown to augment the formation of β -carbolines (Herraiz and Papavergou, 2004; Louis et al., 2007; Kim and Lee, 2010; Dong et al., 2011). Smoked foods accumulate greater amounts of β -carbolines, presumably caused by a reaction between the formaldehyde present in smoke and tryptophan (Papavergou and Herraiz, 2003). Our study suggests that MG readily contributes toward the formation of heterocyclic amines such as β -carbolines in Parmesan cheese, despite the relative absence of high temperatures commonly associated with Maillard browning.

Free AA and small peptides are abundantly available as potential reactants in water-soluble Parmesan cheese extracts similar to the one used in our study (Hillmann and Hofmann, 2016). Strecker degradation of α -AA into aldehydes occurs during intermediate stages of the Maillard reaction and is accelerated by the presence of α -dicarbonyl compounds such as MG (Nursten, 2005). In this respect, formaldehyde and acetaldehyde can form from α -dicarbonyl-assisted oxidative decar-

boxylation of glycine and alanine, respectively. Therefore, aldehyde-amine condensation of acetaldehyde and tryptamine presents one possible explanation for the observed formation of the Harman-like compound in our study. Similarly, condensation of formaldehyde or acetaldehyde with tryptophan can derive TH β CA and 1-methyl-3,4-dihydro-2H- β -carboline-3-carboxylic acid, respectively.

This mechanism is evidenced by a study by Masuda et al. (2005), which incubated the Maillard reaction products MG, glyoxal, 3-deoxy-D-glucosonone, or furfural with tryptophan in buffered solution at 37°C for 4 wk and detected the formation of several β -carboline derivatives. Similarly, Nemet and Verga-Defterdarović (2008) demonstrated the formation of 1-acetyl- β -carboline from the reaction of MG with tryptophan, its methyl ester, or tryptamine. Together, these findings corroborate that MG can lead to the formation of β -carbolines by nonenzymatic conversion of indole compounds. Indeed, we analyzed PCE containing the exogenous addition of commercially available MG and found the formation of a single differential compound from the control. Both the biological production of MG and its exogenous addition to PCE leads to the formation of TH β CA; however, the exogenous addition of MG does not account for all the compounds detected herein. After exogenously adding MG to PCE we did not detect the formation of compounds that would otherwise resemble the ones found from expressing *mgsA* in *L. casei*. This discrepancy between exogenously added and bacterially produced MG could be explained by the different abundances of hydrated MG species in solution, reflecting a variation in preferential reactions. Methylglyoxal persists as a mono- or dihydrate through proton addition of either carbonyl group in aqueous solution. Both aldehydic and ketonic carbonyl groups form a geminal diol, in which the former group is thermodynamically more favorably hydrated (Nemet et al., 2004; Krizner et al., 2009; Axson et al., 2010). In neutral pH solution, the distribution of monohydrate, dihydrate, and unhydrated species is reported to be 56%, 44%, and trace amounts, respectively (McLellan and Thornalley, 1992).

Aldehyde-amine condensations are known to give rise to polymeric, high molecular mass, colored products consisting of heterocyclic ring systems. The observed compounds with chemical compositions of C₁₃H₈N₄O₄, C₁₇H₁₈N₂O₅, C₉H₁₀N₄O₃S, and C₈H₁₀N₄OS are likely also nitrogenous heterocycles, as inferred from their carbon to hydrogen ratio; however, further analysis is required for structural determination of pyrazine, pyridine, pyrrole, and imidazole sub-groups. Aldehydes can also react with each other by aldol condensation to derive cyclic compounds that are nitrogen-free, such as

the observed pyrone or quinone with chemical composition $C_6H_6O_3$.

CONCLUSIONS

We demonstrate that overexpressing MG synthase in *L. casei* readily contributes toward browning of a Parmesan cheese extract and leads to the formation of heterocyclic amines, including some β -carbolines. These findings pose greater significance on the quality of fermented foods because MG is a microbial metabolite able to accelerate the formation of Maillard reaction products in the absence of high temperatures and reducing sugars. Moreover, affected cheeses exhibiting similar reaction products to the ones determined herein merit closer examination for atypical microbiota that might contribute MG synthase activity. The use of *mgsA* as a genetic biomarker for the detection and control of offending microbiota could prove useful for improving the quality control of affected cheeses. Culture-independent methods for the detection of the gene *mgsA* in cheese might serve as an indication of MG-based browning in this manner. However, further studies are required to determine the frequency of association between *mgsA*-containing microbiota and affected cheeses as well as the extent contributed toward MG-mediated browning.

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