

**Mousy Off-Flavor of Wine: Precursors and Biosynthesis of the Causative N-Heterocycles 2-Ethyltetrahydropyridine, 2-Acetyltetrahydropyridine, and 2-Acetyl-1-pyrroline by *Lactobacillus hilgardii* DSM 20176**

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The N-heterocyclic bases, 2-ethyltetrahydropyridine (**1**), 2-acetyl-1-pyrroline (**2**), and 2-acetyltetrahydropyridine (**3**) are associated with the occurrence of mousy off-flavor in wine. The biosynthesis of these N-heterocycles by the wine lactic acid bacterium, *Lactobacillus hilgardii* DSM 20176, was studied by high-cell-density incubation in combination with a minimal chemically defined N-heterocycle assay medium. The key components of the defined N-heterocycle assay medium included D-fructose, ethanol, L-lysine, L-ornithine, and mineral salts. N-heterocycle formation was quantitatively determined by gas chromatography–mass spectrometry. The formation of **2** and **3** required the concomitant availability of a fermentable carbohydrate (D-fructose), ethanol, and iron (Fe<sup>2+</sup>). In addition, L-ornithine stimulated the formation of **2** and repressed **3** formation, whereas L-lysine stimulated the formation of **3** and repressed **2** formation. Incorporation of *d*<sub>6</sub>-ethanol into the acetyl side chain of **2** and **3**, and of *d*<sub>4</sub>-acetaldehyde into the acetyl side chain of **3**, confirmed that ethanol and acetaldehyde could serve as major side chain precursors. A pathway for the formation of **2** and **3** by heterofermentative lactic acid bacteria is proposed involving the interaction of accumulated C-2 intermediates from the heterolactic pathway and N-heterocyclic intermediates derived from the metabolism of L-ornithine and L-lysine.

**KEYWORDS:** Mousy off-flavor; 2-acetyltetrahydropyridine; 2-acetyl-1-pyrroline; 2-ethyltetrahydropyridine; N-heterocycles; *Lactobacillus*; wine; lactic acid bacteria; L-ornithine; L-lysine

**INTRODUCTION**

Throughout the winemaking process, wine is continuously exposed to the risk of contamination by several species of bacteria and other microorganisms which may grow and produce a diverse range of metabolic end-products. The final concentrations and sensory properties of these metabolites may have desirable or undesirable effects on wine quality. Mousy off-flavor represents a potentially serious form of microbiologically induced spoilage of wine (1–3). This type of spoilage has long been recognized to occur infrequently in wines and other alcoholic beverages, and is characterized by the development of an offensive “mousy-like” off-flavor. In severe cases, this spoilage can render the wine unpalatable and, since there is no known satisfactory method for its removal, its occurrence can cause serious economic loss to the wine producer (4, 5).

The compounds reported to cause mousy off-flavor are the sensorially potent N-heterocyclic bases 2-ethyltetrahydropyridine (**1**) (6), 2-acetyltetrahydropyridine (**3**) (7), and, more recently

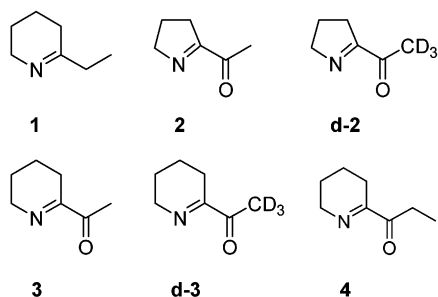
reported by our laboratory, 2-acetyl-1-pyrroline (**2**) (8) (Figure 1). Of these, **3** and **2** are the most potent, having odor thresholds in water of 1.6 μg/L (9) and 0.1 μg/L (10), respectively. Wines affected by mousy off-flavor have been found to contain one or more of **1** (2.7–18.7 μg/L), **2** (up to 7.8 μg/L), and **3** (4.8–106 μg/L) (5). In addition to wine, it is interesting to note that **2** has also been associated with mousy aroma in wetted ground pearl millet (11). However, **2** and **3** are also important desirable odorants of a variety of roasted foods including popcorn (12, 13) and wheat bread crust (12, 14, 15). Such deviation in the sensory properties reported for **2** has been considered to stem from differences in relative concentration, matrix effects in different foods and beverages, or variability among individuals (8).

Further to earlier work demonstrating that certain species and strains of the spoilage yeast *Brettanomyces/Dekkera* and wine lactobacilli were capable of producing mousy off-flavor and mousy N-heterocycles (1, 6), it is now recognized that this capability is extended to all known type strains of *Brettanomyces/Dekkera* and is particularly widespread among heterofermentative wine lactic acid bacteria (LAB) (4, 16, 17). In a survey of 34 strains of wine LAB, most *Lactobacillus* spp.,

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**Figure 1.** Chemical structures of substituted N-heterocycles: 2-ethyltetrahydropyridine (**1**), 2-acetyl-1-pyrroline (**2**), d<sub>3</sub>-2-acetyl-1-pyrroline (**d-2**), 2-acetyltetrahydropyridine (**3**), d<sub>3</sub>-2-acetyltetrahydropyridine (**d-3**), and 2-propionyltetrahydropyridine (**4**)

*Oenococcus oeni*, and a strain of *Leuconostoc mesenteroides* were found capable of producing mousy off-flavor in an ethanolic grape juice medium, although *Pediococcus* spp. were generally lacking in this ability (17). Similarly, further quantitative study of mousy N-heterocycle production by representative wine LAB in a synthetic medium revealed that the highest concentration of **3** was produced by the heterofermentative *Lactobacillus* spp., including strains of *L. hilgardii* (404–580 µg/L) and *L. brevis* (328 µg/L), whereas the homofermentative *L. plantarum* L11a and *Pediococcus* spp. produced little or no detectable mousy N-heterocycles (17). This study also showed that most LAB strains produced a moderate concentration of **2** ( $\leq 57$  µg/L), and three of five strains of the important wine production malolactic bacterium, *O. oeni*, produced a relatively high concentration of **1** (87–162 µg/L). In addition to LAB and *Brettanomyces/Dekkera* yeasts, an acetic acid bacterium (*Gluconobacter* sp.) has been reported capable of producing **1**, **2**, and **3** (5), and *Bacillus cereus* strains isolated from cocoa fermentation have been shown to produce **2** (18).

Despite recent advances in the elucidation of the microorganisms and compounds associated with mousy off-flavor, there is little knowledge of the substrates and precursors governing its formation, particularly by LAB. Ethanol is known to be a necessary substrate for mousy off-flavor formation (1, 6) and biosynthesis of **3** (1). The key role of alcohol in this metabolism was further established from the novel formation of the propionyl homologue of **3**, 2-propionyltetrahydropyridine (**4** in Figure 1), by both *Brettanomyces intermedius* and *L. brevis* when ethanol in the fermentation medium was replaced with *n*-propanol (1). Furthermore, the amino acid L-lysine was demonstrated as another substrate in the formation of mousy off-flavor (6) and of **3** (1) by *Brettanomyces* yeast.

The deficiency of knowledge concerning N-heterocycle metabolism by LAB has been exacerbated by the lack of an appropriate chemically defined test medium. Compared with complex media, chemically defined media have performed poorly in supporting the formation of both mousy off-flavor (6) and mousy N-heterocycles (19) by LAB. This shortcoming was recently overcome, however, by the development of a high-cell-density incubation technique (19). The use of a high cell inoculum allowed us to formulate a simple assay medium of defined composition (chemically defined N-heterocycle assay medium) which supported the formation of both mousy off-flavor and mousy N-heterocycles without the requirement for cell growth. Very few substrates were required in the chemically defined N-heterocycle assay medium to induce this reaction by LAB, and included a carbohydrate source (D-fructose), ethanol, acetaldehyde, L-ornithine, L-lysine, and various mineral salts. Furthermore, screening of wine LAB for mousy N-heterocycle

**Table 1.** Composition of Chemically Defined N-Heterocycle Assay Medium<sup>a</sup> Used for Testing the Formation of Mousy N-Heterocycles by High-Cell-Density Incubation of Lactic Acid Bacteria

component	concentration (per liter)
D-fructose	50 g
L-ornithine	5 g
L-lysine	5 g
citric acid	2 g
L-malic acid	5 g
KH <sub>2</sub> PO <sub>4</sub>	5.5 g
KCl	4.25 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	25 mg
MnSO <sub>4</sub> ·H <sub>2</sub> O	25 mg
FeSO <sub>4</sub> ·7H <sub>2</sub> O	43 mg
CaCl <sub>2</sub> ·2H <sub>2</sub> O	1.66 g
ethanol (redistilled, 96% v/v)	52 mL (5.0% v/v ethanol)
acetaldehyde	100 mg

<sup>a</sup> Final pH was adjusted to 4.5

formation by this method (17) provided evidence to indicate the involvement of the heterolactic pathway in this biosynthesis. In the present work, the high-cell-density incubation technique is further utilized to investigate the major substrates and precursors of the biosynthesis of mousy N-heterocycles in the LAB strain *L. hilgardii* DSM 20176.

**Definition of Terms.** The term mousy off-flavor is used in this paper to refer to the sensory perception of this attribute in microbiological media or wine. The term mousy N-heterocycles is used to collectively refer to the three compounds, **1**, **2** and **3**, which are associated with the occurrence of mousy off-flavor in wine.

## MATERIALS AND METHODS

**Bacterial Strain.** The type strain *L. hilgardii* DSM 20176 was used as a test organism in this study and was obtained from the German Collection of Microorganisms and Cell Cultures (DSM), Braunschweig, Germany. This strain was selected as it was previously found to produce the highest concentration of **3** compared to 10 other strains of representative wine LAB (17). The strain was maintained in MRSA medium [de Man, Rogosa, and Sharpe (MRS) medium (Amyl Media, Australia) supplemented with 20% v/v preservative-free apple juice (20)], stored at 4 °C, and further subcultured into fresh MRSA medium at approximately 2-week intervals.

**Cell Preculture.** Cells were precultured for 7 days at 27 °C in 10-mL volumes of MRSA medium, after which time they were inoculated (2% v/v) into a second preculture medium (MRS-CT) and incubated at 25 °C until stationary phase. The MRS-CT medium was a modification of de Man, Rogosa, and Sharpe medium and consisted of (per liter) yeast extract (Oxoid) (5 g), casamino acids (Difco) (5 g), trypticase peptone (BBL) (5 g), D-glucose (20 g), D-fructose (10 g), L-malic acid (3 g), sodium acetate·3H<sub>2</sub>O (5.0 g), triammonium citrate (2 g), K<sub>2</sub>HPO<sub>4</sub> (2 g), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.2 g), MnSO<sub>4</sub>·4H<sub>2</sub>O (0.05 g), and Tween 80 (1 mL), and the pH was adjusted to 4.5.

**Chemically Defined Medium for Testing Mousy N-Heterocycle Formation.** The substrates and precursors of mousy N-heterocycle formation by *L. hilgardii* DSM 20176 were studied using the chemically defined N-heterocycle assay medium described in Table 1. This medium was a modification of that previously described for the screening of wine LAB for mousy N-heterocycle formation (17) by inclusion of acetaldehyde (100 mg/L). Prior to experimentation, a stock solution (2.0× or 2.5×) of chemically defined N-heterocycle assay medium (pH 4.5) was prepared and sterilized by filtration with a 0.22-µm pore size sterile membrane. Acetaldehyde (from a 100× stock solution) was added to the stock solution of the chemically defined N-heterocycle assay medium prior to the addition of LAB cells.

**High-Cell-Density Incubation.** Stationary phase cells were harvested from approximately 2 L of MRS-CT medium by centrifugation (approximately 9000g, 15 min, 20 °C) and washed twice with sterile (0.22- $\mu$ m pore size membrane filtered) phosphate–KCl buffer (KH<sub>2</sub>PO<sub>4</sub>, 5.5 g/L; KCl, 4.25 g/L; pH 4.5). The cell pellet was resuspended in 20–25 mL of phosphate–KCl buffer. The absorbance (650 nm, multiwell plate reader method, see below) of this suspension was then measured, and the volume required to achieve a final cell density of 10.0 absorbance units in 10–25 mL of chemically defined N-heterocycle assay medium was calculated. The high-cell-density incubation was undertaken in 30-mL screw-capped glass bottles and initiated by addition of the cell suspension to appropriate volumes of chemically defined N-heterocycle assay medium stock solution and phosphate–KCl buffer. High-cell-density incubation was conducted statically and aerobically at 25 °C for 16 h, after which time cells from inoculated assays were removed by centrifugation (approximately 9000g, 15 min, 4 °C). Uninoculated chemically defined N-heterocycle assay medium served as a control to determine if mousy N-heterocycles were produced after incubation in the absence of cells, and was prepared by substituting the washed cell suspension with an equivalent volume of phosphate–KCl buffer. A second control experiment was performed to determine whether mousy N-heterocycles could be released by high-cell-density incubation of *L. hilgardii* DSM 20176 in phosphate–KCl buffer (pH 4.5). Supernatants and uninoculated control assay media from high-cell-density incubation experiments were stored frozen (–20 °C) for subsequent extraction and GC–MS quantification of mousy N-heterocycles.

**Reference N-Heterocycles.** Reference **3** was synthesized from the thermal reaction of proline, dihydroxyacetone, and sodium bisulfite using previously described methods (21, 22), and was further purified by a second vacuum distillation process (0.5–1 mm hg; 0–66 °C) in which fractions were collected in glass traps cooled with liquid nitrogen. These fractions were made up to 50 mL with ethanol and stored at –20 °C. Material from the second fraction (bp 45 °C) of this second distillation yielded the highest recovery (85% w/w, data not shown) and was used as the source of reference **3**. Authentic samples of **1** and **2** were kindly provided by, respectively, Paul Grbin from our laboratory, and Dr. Ron Buttery of the Agricultural Research Service, Western Regional Center, U.S. Department of Agriculture, Albany, CA. The identities of **1**, **2**, and **3** were confirmed by comparison of mass spectral data of the reference sample to published data.

**Extraction and Quantification of Mousy N-Heterocycles.** The mousy N-heterocycles **1**, **2**, and **3** were quantified from 10–22 mL of clarified culture supernatants using continuous liquid/liquid (Freon 11) extraction and gas chromatography/mass spectrometry (GC–MS) procedures similar to those described for the analysis of **2** in mousy wines (8). Pre-extraction of acidic and neutral compounds (pH 2.5) from assay media was not required as the high-cell-density assay produced little background interference for GC–MS analysis of basic compounds. Clarified samples were saturated with NaCl, and the first internal standard (4-acetylpyridine) was added. Samples were then adjusted to pH 8.0 with 5 N NaOH, and basic compounds were continuously extracted overnight with Freon 11 (200 mL). After the extracts were dried over Na<sub>2</sub>SO<sub>4</sub>, a second internal standard (3-acetylpyridine) was added to the organic phase, which was then concentrated by careful distillation (bath temperature 37 °C) using a water-jacketed Vigreux column (1 × 15 cm). During the concentration process, Freon 11 was sequentially replaced with approximately 0.5 mL of dichloromethane. Prior to GC–MS analysis, the extract was further concentrated (10-fold) into 10  $\mu$ L of *iso*-octane under a gentle stream of nitrogen. Freon 11 collected from concentration was recycled by distillation for subsequent use. Mousy N-heterocycles were detected in sample extracts by GC–MS using scan mode and the selected ion chromatograms for **1** (*m/z* 111, 110), **2** (*m/z* 111, 83), and **3** (*m/z* 125, 82), and their identities were further confirmed by comparison of retention times and mass spectral data with those of synthetic reference compounds and published data. In addition to the tautomeric forms of **3** (**3** tautomer I, **3** tautomer II), the chromatographic conditions used in this study also yielded two isomers of both **2** (**2** tautomer I, **2** tautomer II) and **1** (**1** tautomer I, **1** tautomer II). The presumed tautomeric peaks

**2** (tautomer II) and **1** (tautomer II), however, contributed only a small proportion of the total amount for each compound.

The concentrations of **1**, **2**, and **3** were calculated from respective molecular ion responses (peak areas) of **1** (*m/z* 111), **2** (*m/z* 111), **3** (*m/z* 125) and the internal standard, 4-acetylpyridine (*m/z* 121), and predetermined relative response factors (RRF). Relative response factors were determined from replicate GC–MS analyses of reference solutions containing similar concentrations of **2**, **3**, and 4-acetylpyridine, and calculated from the ratio of the reconstructed ion chromatogram (RIC) responses of target compound and internal standard to that of respective molecular ion responses of target compound and internal standard. The calculated RRF values for **2** (tautomer I), **3** (tautomer I), and **3** (tautomer II) were 3.99, 1.60, and 1.08, respectively. Relative response factors for **2** (tautomer II) and **1** were not determined and were assumed as unity. The final concentration of mousy N-heterocycles was calculated by summation of respective presumed tautomers.

The relative proportions of **1**, **2**, and **3** produced by *L. hilgardii* DSM 20176 in chemically defined N-heterocycle assay medium between experiments was highly consistent. The average ratio of **1**:**2**:**3** (expressed as a percentage of the sum concentration of the 3 N-heterocycles in each experiment) over 7 separate assays was 0.6:8.3:91.1, with respective standard deviations of 0.4, 2.2, and 2.0%.

**Determination of Cell Biomass.** Cell biomass was measured by absorbance (650 nm) using a Molecular Devices kinetic microplate reader system and multiwell plates (96-well) dispensed with duplicate 0.3-mL aliquots of culture suspension.

#### Substrates and Precursors of Mousy N-Heterocycle Formation.

In experiments investigating the dose–response relationship between N-heterocycle formation and the substrates D-fructose and the amino acids L-ornithine and L-lysine, chemically defined N-heterocycle assay medium was initially prepared by respective omission of D-fructose, and of amino acids. The dose–response of these components was investigated in the final concentration ranges of 0–100 g/L for D-fructose, and 0–5 g/L for L-ornithine and L-lysine. Other substrates including ethanol, acetaldehyde, and metal ions were studied by respective omission from chemically defined N-heterocycle assay medium. Acetaldehyde was further studied at the higher concentration of 500 mg/L. The effects of different alcohols (*n*-propanol, 2-propanol, and *n*-butanol) and aldehydes (butyraldehyde and propionaldehyde) on mousy compound formation were also investigated by respective substitution of ethanol or acetaldehyde. In addition, some of the precursors of mousy compounds were studied using chemically defined N-heterocycle assay medium in which ethanol, acetaldehyde, and fructose were replaced by respective deuterated isotopes, that is, *d*<sub>6</sub>-ethanol (99%); *d*<sub>4</sub>-acetaldehyde (99%) and 2-*d*-D-glucose (99%) (Cambridge Isotope Laboratories, Andover, MA).

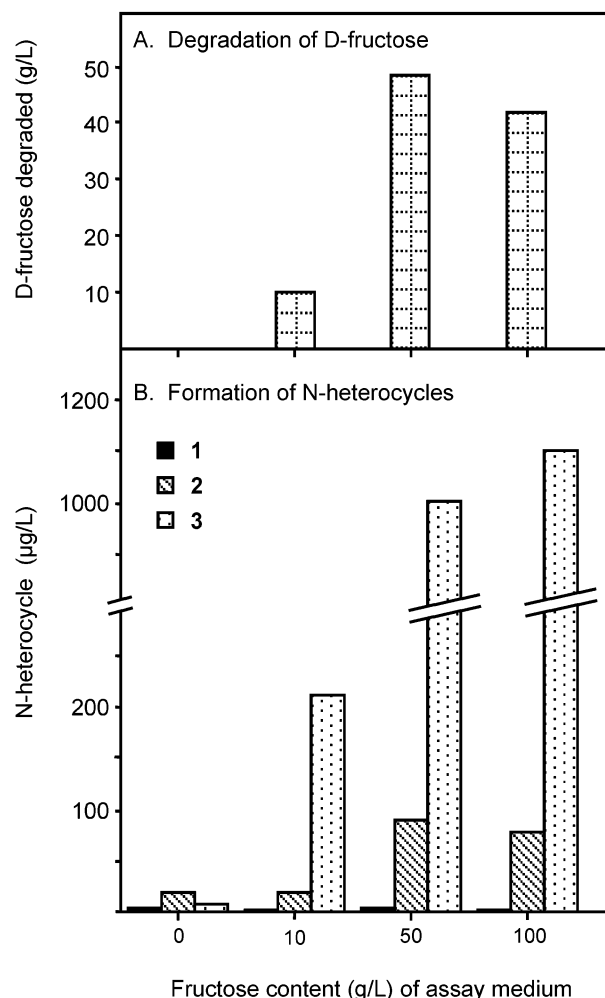
**Concentration of D-Fructose.** The concentration of D-fructose was determined enzymatically using the kit reagents of Boehringer Mannheim (D-glucose/D-fructose kit, no. 139 106) in combination with an automated analyzer (Roche Cobas FARA).

## RESULTS

### Factors Affecting the Production of Mousy N-Heterocycles

**by *Lactobacillus hilgardii* DSM 20176.** Under the conditions of this study, mousy N-heterocycles were not detected in the uninoculated control high-cell-density incubation assay. Further, with the exception of a relatively minor quantity of **3** (2.1  $\mu$ g/L), no other mousy N-heterocycles were released by cells of *L. hilgardii* DSM 20176 after high-cell-density incubation in phosphate–KCl buffer (data not shown). In contrast, mousy N-heterocycle formation by *L. hilgardii* DSM 20176, particularly that of **2** and **3**, readily occurred after high-cell-density incubation in mousy N-heterocycle assay medium, and this metabolism was affected by a number of nutritional factors.

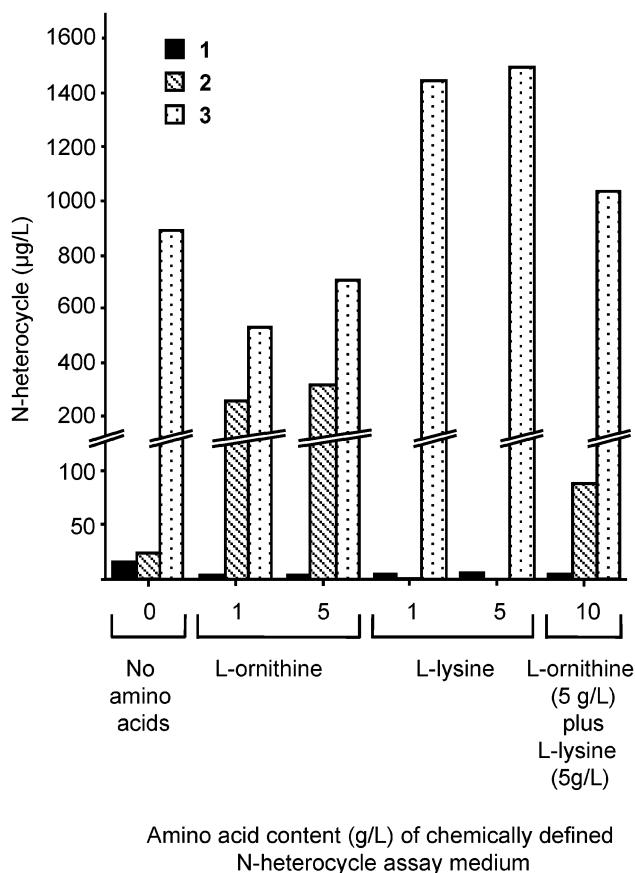
**Concentration of D-Fructose.** The production of **2** and **3** by *L. hilgardii* DSM 20176 was approximately proportional to the concentration of D-fructose consumed; however, the production of **1** was generally unaffected by the concentration of D-fructose



**Figure 2.** Effect of D-fructose content of chemically defined N-heterocycle assay medium on the formation of 2-ethyltetrahydropyridine (1), 2-acetyl-1-pyrroline (2), and 2-acetyltetrahydropyridine (3) by *L. hilgardii* DSM 20176 cells (B). (A) shows the degradation of D-fructose in each assay. Data presented are mean concentrations of duplicate assays.

consumed and remained in the range 2–5 μg/L (Figure 2). In the absence of D-fructose, a comparatively low concentration of 2 (20 μg/L) and 3 (8 μg/L) was produced. A concentration of 10 g/L D-fructose stimulated the production of 3 by 27-fold to 212 μg/L, whereas 50 g/L D-fructose stimulated, relative to the control (0 g/L D-fructose), 2 by 4.5-fold (89 μg/L) and 3 by 140-fold (1041 μg/L). A higher concentration of D-fructose (100 g/L) had little effect on either sugar consumption or N-heterocycle production. The increased production of 2 and 3 by *L. hilgardii* DSM 20176 was also associated with greater utilization of D-fructose: at 10 g/L, 50 g/L, and 100 g/L fructose, 100% (10 g/L), 97% (48 g/L), and 47% (47.2 g/L) of the carbohydrate was utilized, respectively (Figure 2).

**Concentration of L-Ornithine and L-Lysine.** The concentration of L-ornithine and L-lysine in the chemically defined N-heterocycle assay medium had a considerable effect on the production of the three N-heterocycles by *L. hilgardii* DSM 20176 (Figure 3). In the complete absence of these amino acids, the concentrations of 1, 2, and 3 produced were, respectively, 16 μg/L, 25 μg/L, and 895 μg/L. The response in the production of 2 and 3 to the presence of 1 g/L or 5 g/L of either L-ornithine or L-lysine in the assay medium was similar. L-Ornithine increased the concentration of 2 by 10.3–12.8-fold (258–320 μg/L), whereas 3 was decreased by 21–40% (537–708 μg/L).

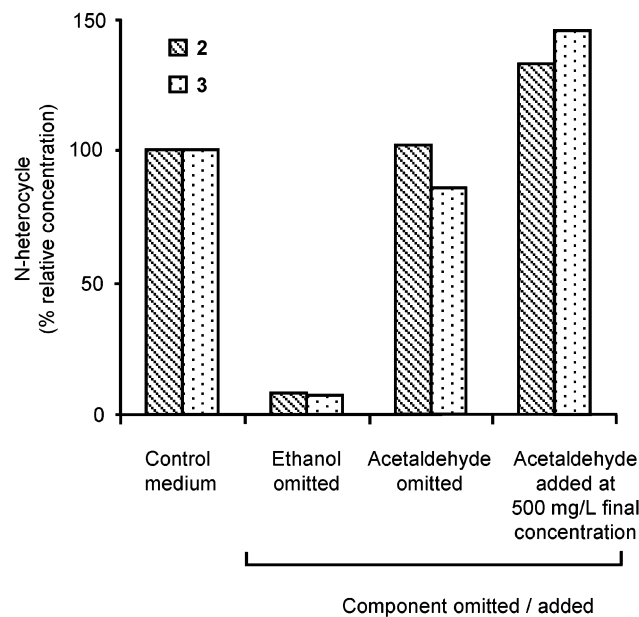


**Figure 3.** Effect of L-ornithine and L-lysine content of chemically defined N-heterocycle assay medium on the formation of 2-ethyltetrahydropyridine (1), 2-acetyl-1-pyrroline (2), and 2-acetyltetrahydropyridine (3) by *L. hilgardii* DSM 20176 cells. Data presented are mean concentrations of duplicate assays.

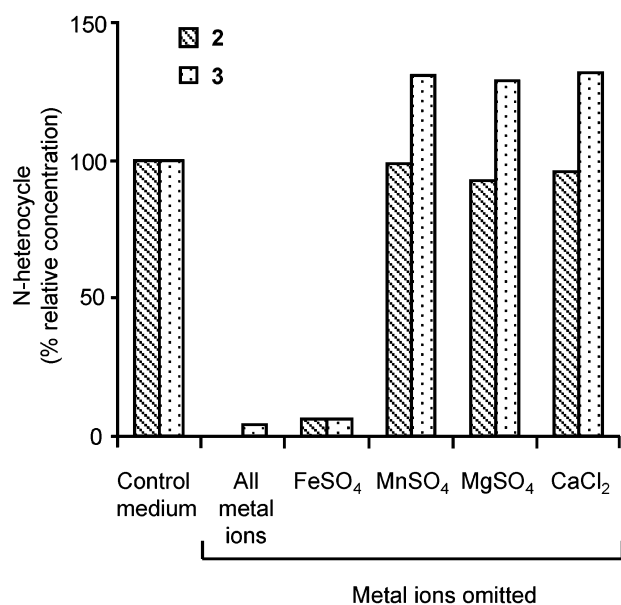
In contrast, L-lysine increased the concentration of 3 1.6–1.7-fold (1451–1497 μg/L), but essentially repressed the formation of 2. The production of 1, on the other hand, was consistently reduced by 60–81% after the addition of L-ornithine or L-lysine. When the two amino acids were added to the assay medium in combination (each at a final concentration of 5 g/L), the production of both 2 and 3 was stimulated relative to that of the nil addition control, by 356% (+64 μg/L) and 116% (+146 μg/L), respectively.

**Ethanol and Acetaldehyde.** The effects of omitting ethanol and acetaldehyde from the chemically defined N-heterocycle assay medium on the formation of the mousy N-heterocycle compounds by *L. hilgardii* DSM 20176 is shown in Figure 4. Omission of ethanol dramatically reduced the production of 2 (88% reduction) and 3 (89% reduction) compared to that of the control medium which contained ethanol at a concentration of 5% v/v. In contrast, the omission of acetaldehyde had much less influence on the production of either 2 (2% increase) or 3 (14% decrease). On the other hand, increasing the concentration of acetaldehyde 5-fold to 500 mg/L caused a relatively large increase in the formation of both 2 (33% increase) and 3 (45% increase). The formation of 1 in these experiments was low (<6 μg/L) and inconsistent, hence no general conclusions could be drawn regarding the influence of ethanol and acetaldehyde on the formation of this N-heterocycle (data not shown).

**Metal Ions.** The formation of the acetylated N-heterocycles, 2 and 3, by *L. hilgardii* DSM 20176 was greatly affected by the omission of metal cations from the control chemically



**Figure 4.** Effects of ethanol and acetaldehyde on the relative concentration of 2-acetyl-1-pyrroline (**2**) and 2-acetyl-1-pyrroline (**3**) produced by high-cell-density incubation of *L. hilgardii* DSM 20176 in chemically defined N-heterocycle assay medium. Relative concentrations were calculated as the percentage ratio of each compound relative to the control assay medium. Average data from duplicate assays are shown.



**Figure 5.** Effects of metal ions on the relative concentrations of 2-acetyl-1-pyrroline (**2**) and 2-acetyl-1-pyrroline (**3**) produced by high-cell-density incubation of *L. hilgardii* DSM 20176 in chemically defined N-heterocycle assay medium. Relative concentrations were calculated as the percentage ratio of each compound relative to the control. Average data from duplicate assays are shown.

defined N-heterocycle assay medium (**Figure 5**). Compared to the control assay medium which contained metal cations, the omission of all metal cations tested ( $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Ca}^{2+}$ ) completely prevented the formation of **2** and reduced the formation of **3** by 96%. Similarly, omission of  $\text{FeSO}_4$  from the chemically defined N-heterocycle assay medium also gave a large reduction in the formation of both **2** and **3** (both 94% reduction). In contrast, omission of either  $\text{MnSO}_4$ ,  $\text{MgSO}_4$ , or  $\text{CaCl}_2$  caused only a small reduction in the production of **2**

**Table 2.** Mass Spectra of 2-Acetyl-1-pyrroline (**2**),  $d_3$ -2-Acetylpyrroline (**d-2**), 2-Acetyl-1-pyrroline (**3**),  $d_3$ -2-Acetyl-1-pyrroline (**d-3**), and 2-Propionyl-1-pyrroline (**4**) Detected in Extracts of N-Heterocycle Assay Medium after Incubation with *L. hilgardii* DSM 20176

compound	mass spectrum (EI) $m/z$ (%)
<b>2</b>	
tautomer 1	111 (45), 83 (45), 69 (20), 68 (25), 43 (100), 42 (25), 41 (60)
tautomer 2	111 (55), 83 (25), 55 (100), 54 (10), 42 (60), 41 (20)
<b>d-2<sup>a</sup></b>	
tautomer 1	114 (27), 113 (23), 86 (41), 85 (39), 71 (16), 70 (18), 57 (62), 46 (100), 45 (67), 42 (32)
tautomer 2	114 (43), 86 (33), 58 (100), 57 (23), 45 (36), 42 (41)
<b>3</b>	
tautomer 1	125 (80), 83 (65), 82 (70), 55 (75), 54 (68), 43 (100)
tautomer 2	125 (100), 83 (9), 82 (88), 55 (28), 54 (55), 43 (40)
<b>4<sup>b</sup></b>	
tautomer 1	139 (80), 124 (30), 111 (13), 110 (18), 83 (67), 82 (54), 57 (43), 55 (45), 54 (97), 42 (100), 41 (72)
tautomer 2	139 (92), 124 (34), 111 (18), 110 (20), 83 (76), 82 (63), 57 (62), 55 (63), 54 (100), 41 (62)
<b>d-3<sup>a</sup></b>	
tautomer 1	128 (90), 127 (50), 100 (26), 84 (72), 83 (31), 82 (86), 56 (31), 55 (100), 54 (39), 46 (94), 45 (41)
tautomer 2	128 (100), 127 (54), 83 (32), 82 (77), 55 (23), 54 (50), 46 (37)

<sup>a</sup> **d-2** and **d-3** were detected when ethanol was replaced with  $d_6$ -ethanol in the N-heterocycle assay medium. **d-2** was also detected when acetaldehyde was replaced with  $d_4$ -acetaldehyde. <sup>b</sup> **4** was detected when ethanol was replaced with  $n$ -propanol in the N-heterocycle assay medium.

(reductions of 1%, 7%, and 5%, respectively), and increased the production of **3** by approximately 30%. Again, the production of **1** was low ( $<7 \mu\text{g/L}$ ) in this series of experiments, and only trends could be observed. In comparison with **2** and **3**, omission of all metal cations from the chemically defined N-heterocycle assay medium reduced the formation of **1** from  $7 \mu\text{g/L}$  to  $3 \mu\text{g/L}$ . The omission of either  $\text{MnSO}_4$  or  $\text{MgSO}_4$  did not significantly affect production of **1**. As for **2** and **3**, the omission of  $\text{FeSO}_4$  reduced production of **1** by 90%, however, the omission of  $\text{CaCl}_2$  doubled the production of **1** (data not shown).

**Substrates and Precursors of Mousy N-Heterocycles Produced by *Lactobacillus hilgardii* DSM 20176. Nonlabeled Substrates.** Various alcohols ( $n$ -propanol, 2-propanol, and butan-1-ol) and aldehydes (propionaldehyde and butyraldehyde) were separately incorporated into the chemically defined N-heterocycle assay medium, replacing ethanol or acetaldehyde, to observe the ability of *L. hilgardii* DSM 20176 to produce respective C-3 and C-4 substituted homologues of the mousy N-heterocycles.

**Alcohols.** When ethanol in the chemically defined N-heterocycle assay medium was replaced with  $n$ -propanol, *L. hilgardii* DSM 20176 produced two unique compounds featuring molecular ions  $M^+$   $m/z$  139, similar mass spectra, and which eluted from the GC column shortly after (1.7 min) each of the two tautomers of **3**. The mass spectral data of these two compounds (**Table 2**) were consistent with those reported for

**Table 3.** Production of Deuterated Mousy N-Heterocycles<sup>a</sup> ( $\mu\text{g/L}$ ) by High-Cell-Density Incubation of *Lactobacillus hilgardii* DSM 20176 in Chemically Defined N-Heterocycle Assay Medium Containing  $d_6$ -Ethanol or  $d_4$ -Acetaldehyde<sup>b</sup>

N-heterocycle	<i>m/z</i>	concentration of N-heterocycle ( $\mu\text{g/L}$ )		
		labeled substrate		
		no labeled substrate (control)	$d_6$ -ethanol	$d_4$ -acetaldehyde
<b>2</b>	111	45.3	10.6	52.1
<b>d-2</b>	114	nd <sup>c</sup>	10.4	nd
<b>3</b>	125	469.3	59.4	479.2
<b>d-3</b>	128	nd	244.7	0.6

<sup>a</sup> Deuterated isotopes of **1** were not detected in any of the treatments. <sup>b</sup> N-Heterocycle assay medium prepared by substituting ethanol with  $d_6$ -ethanol, or by substituting acetaldehyde with  $d_4$ -acetaldehyde. <sup>c</sup> nd = not detected.

the tautomers of **4** (*I*), and hence were tentatively assigned the structure shown in **Figure 1**. The total concentration of both tautomers of **4** in this sample ( $46 \mu\text{g/L}$ ) was more than double that of **3** ( $18 \mu\text{g/L}$ ). However, other C-3 substituted compounds including the propionyl homologue of **2**, that is, 2-propionyl-1-pyrroline ( $m/z$   $M^+$  125), and 2-propyltetrahydropyridine ( $m/z$   $M^+$  125), as well as **1** and **2**, were not detected in this sample.

In contrast, *L. hilgardii* DSM 20176 failed to produce **4** in the chemically defined N-heterocycle assay medium when ethanol was replaced with 2-propanol. Another compound, however, occurred only in this extract and which chromatographed shortly after **1**. The mass spectrum of this compound exhibited similarities to that of **1** and, having an  $M^+$   $m/z$  125, was tentatively identified as 2-propyltetrahydropyridine (data not shown). The concentration of the tentatively identified 2-propyltetrahydropyridine ( $1 \mu\text{g/L}$ ) was lower than that of **1** ( $3 \mu\text{g/L}$ ). Other C-3 substituted N-heterocycles were not detected in this sample. No substituted N-heterocycles were detected in the chemically defined N-heterocycle assay medium by *L. hilgardii* DSM 20176 when ethanol was substituted with *n*-butanol.

**Aldehydes.** Substitution of acetaldehyde with either propionaldehyde or butyraldehyde in the chemically defined N-heterocycle assay medium did not produce detectable amounts of any C-3 or C-4 substituted homologues of **1**, **2**, or **3** after high-cell-density incubation with *L. hilgardii* DSM 20176 (data not shown).

**Labeled Substrates.  $d_6$ -Ethanol.** In addition to the production of **2** and **3**, deuterated isotopes of both tautomers of **2** ( $d_3$ -2-acetyl-1-pyrroline (**d-2**),  $m/z$  114) and of **3** ( $d_3$ -2-acetyltetrahydropyridine (**d-3**),  $m/z$  118) were produced by *L. hilgardii* DSM 20176 when ethanol was substituted with  $d_6$ -ethanol (**Tables 2** and **3**). Further, the increase of 3 atomic mass units for the ion fragment  $m/z$  43  $\rightarrow$  46 of **3** (tautomers I & II) and **2** (tautomer I), and of  $m/z$  42  $\rightarrow$  45 for **2** (tautomer II), indicated that three deuterium atoms from  $d_6$ -ethanol were incorporated into the acetyl side chain of each isotope. These data suggest the structures shown in **Figure 1** for the isotopes **d-2** and **d-3**. Moreover, the ratio of the  $d_3$ -isotopomer **d-3** to the unlabeled **3** (approximately 4:1) and of the  $d_3$ -isotopomer **d-2** to the unlabeled **2** (1:1) indicated similar proportions of both labeled and unlabeled N-heterocycles were produced. In contrast, labeled isotopic peaks for **1** ( $m/z$  112–116) were not detected in the  $d_6$ -ethanol assays.

**$d_4$ -Acetaldehyde.** Minor quantities of  $d_3$ -isotopomers of both tautomers of **3** ( $m/z$  128) (total concentration  $0.6 \mu\text{g/L}$ ) were produced by *L. hilgardii* DSM 20176 when the acetaldehyde component of chemically defined N-heterocycle assay medium

**Table 4.** Increase in  $\{M+1 (m/z 126)/\text{Molecular}\}$  Ions of 2-Acetyltetrahydropyridine (**3**) Produced by High-Cell-Density Incubation of *Lactobacillus hilgardii* DSM 20176 in Chemically Defined N-Heterocycle Assay Medium Containing 2-*d*-D-Glucose<sup>a</sup>

tautomer of <b>3</b>	ratio (%) of ( $m/z$ 126)/( $m/z$ 125) ions of <b>3</b> <sup>a</sup>		increase (%) in ratio of ( $m/z$ 126)/( $m/z$ 125) ions of <b>3</b> produced from 2- <i>d</i> -D-glucose
	carbohydrate source		
	D-fructose (unlabeled control)	2- <i>d</i> -D-glucose	
I	8.75	9.94	13.6
II	8.40	12.95	54.2

<sup>a</sup> Calculated from peak areas of respective ions.

was replaced with  $d_4$ -acetaldehyde (**Tables 2** and **3**). However, labeled isotopes of **1** or **2** were not detected in these assays (**Table 3**).

**2-*d*-D-Glucose.** Distinct chromatographic peaks of  $d_1$ -2-acetyltetrahydropyridine or other deuterated mousy compounds were not observed after high-cell-density incubation of *L. hilgardii* DSM 20176 when D-fructose was replaced with the deuterated substrate 2-*d*-D-glucose. In this latter sample, however, the ratio of  $M+1 (m/z 126)$ :  $M^+ (m/z 125)$  ions for **3** (tautomer I) and **1** (tautomer II) increased by 14% and 54%, respectively (**Table 4**). This increase in the abundance of  $M+1 (m/z 126)$  ions suggests that 2-*d*-D-glucose has contributed to the formation of deuterated  $d_1$ -2-acetyltetrahydropyridine tautomers.

## DISCUSSION

In this paper, the metabolism of mousy N-heterocycle formation by *L. hilgardii* DSM 20176 was investigated using a high-cell-density incubation technique which exploited the biocatalytic properties of a high concentration of bacterial cells. These studies demonstrated that the formation of the acetylated N-heterocycles, **2** and **3**, by this lactic acid bacterium involved the co-metabolism of several key substrates, including (i) a fermentable carbohydrate (D-fructose), (ii) ethanol, (iii) L-ornithine and L-lysine, and (iv) the presence of  $\text{Fe}^{2+}$  ions. Significantly, these results suggest that the biosynthesis of acetylated N-heterocycles is concomitantly dependent upon the metabolic pathways involved in the lactic fermentation of sugars, the metabolism of ethanol, and of L-ornithine and L-lysine.

Further insight into the formation of mousy N-heterocycles, particularly that of the acetyl side chain, was obtained from experiments using different unlabeled and deuterated substrates. The demonstration that the propionyl analogues of the tautomers of **3** were produced by *L. hilgardii* DSM 20176 when only *n*-propanol, and not 2-propanol, was substituted for ethanol extends previous findings (*I*) with *Brettanomyces* spp. to suggest that the formation of acetylated N-heterocycles requires a primary alcohol, and not a secondary alcohol. Such a trend may be restricted to alcohols containing a maximum of three carbons, as no N-heterocycles were detected when *n*-butanol was used as an alcohol source. Moreover, the absence of propionyl-substituted pyrroline compounds, which were anticipated in high-cell-density incubation assays using *n*-propanol, was unexpected. It cannot be ruled out, however, that the efficiency of formation of the latter analogues was very low, and the assay method was not sufficiently sensitive to detect these trace compounds.

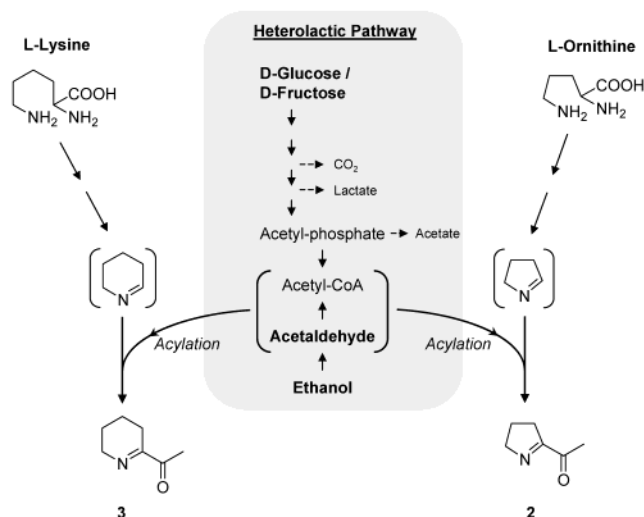
Clear evidence, for the first time, that ethanol is a direct precursor of acetylated N-heterocycles was obtained from demonstration that *L. hilgardii* DSM 20176 incorporated three

deuterium atoms from  $d_6$ -ethanol as the acetyl side chain of both **2** and **3**. Similar conclusions regarding the role of acetaldehyde in side chain formation by *L. hilgardii* DSM 20176 were made after demonstration of, first, the stimulation of **2** and **3** formation afforded by 500 mg/L acetaldehyde, and, second, the incorporation of three deuterium atoms from  $d_4$ -acetaldehyde into the acetyl side chain of **3**, yielding the isotopomer **d-3**. Reasons for the absence of deuterium labeling of **2** in the  $d_4$ -acetaldehyde assays are, however, uncertain. Nevertheless, it is possible that very low proportions of the isotopomer **d-2** may have been produced from  $d_4$ -acetaldehyde, as in the case of the isotopomer **d-3** in which only 0.1% was produced from  $d_4$ -acetaldehyde. The final concentration of **d-3** produced from  $d_4$ -acetaldehyde, however, may have been below the detection limits of the present assay. Moreover, the absence of labeled **1** from assays using  $d_6$ -ethanol and  $d_4$ -acetaldehyde suggests that the biosynthesis of alkyl- and acyl-substituted N-heterocycles involves the activities of separate pathways, but again the possibility exists that the concentration of these compounds was too low to be detected. Further experimentation is required to establish the role of acetaldehyde in the formation of **1** and **2**.

The dose–response relationship between D-fructose consumption and the formation of both **2** and **3** by *L. hilgardii* DSM 20176 clearly demonstrated that a fermentable carbohydrate was another key substrate required for the formation of acetylated N-heterocycles. This observation was further strengthened by the indication that 2-*d*-D-glucose was incorporated into **3**, and also by the considerable proportions of nonsubstituted and nonlabeled **3**, respectively, produced in the *n*-propanol and  $d_6$ -ethanol assays. Similarly, others (18) have previously shown that *B. cereus* utilized  $^{13}\text{C}$ -labeled D-glucose in the formation of the side chain of **2**. Significantly, in the current study, the identification of a carbohydrate source, ethanol, and acetaldehyde as three of the key substrates collectively involved in the biosynthesis of acetylated N-heterocycles by *L. hilgardii* DSM 20176 substantiates our previous assumptions (17) that the production of these compounds by LAB is linked with the heterolactic fermentation of sugars.

In the heterolactic pathway, hexoses are catabolized via the phosphoketolase (PK) pathway (or 6-phosphogluconate pathway), the main end-products of which are lactate,  $\text{CO}_2$ , and ethanol and/or acetate (23, 24). Under the conditions of the high-cell-density incubation assay, however, both the sugar substrate and C-2 end-products ethanol and acetaldehyde are supplied in excess, thus facilitating possible accumulation of certain C-2 intermediates in the ethanol branch for other acetylation reactions. Although the identity of the acylating C-2 intermediate(s) is not known, a likely candidate is the acylating cofactor, acetyl-CoA. The production of propionyl-tetrahydropyridine from *n*-propanol fits into the proposed scheme whereby respective (reversed) alcohol and aldehyde dehydrogenase activities may generate the C-3 carrier, propionyl-CoA. Moreover, because only primary alcohols can generate corresponding aldehydes, this also explains why propionyl-tetrahydropyridine was not generated from 2-propanol. However, even though acetyl-CoA seems a likely acylating cofactor, other possible C-2 reactants from the heterolactic pathway, such as acetaldehyde, cannot be ignored.

It is anticipated that only very low quantities of reactive C-2 intermediates would be required to yield the part-per-billion yields of N-heterocycle end-products observed with *L. hilgardii*. However, although we assume that some reversal of “normal” metabolic flux could occur in the ethanol branch under the



**Figure 6.** Proposed pathway for formation of the acetylated mousy N-heterocycles, 2-acetyl-1-pyrroline (**2**) and 2-acetyl-1-piperidine (**3**), by the heterofermentative lactic acid bacterium, *L. hilgardii* DSM 20176. Compounds in bold type (D-glucose, D-fructose, ethanol, acetaldehyde, L-lysine, and L-ornithine) are considered key substrates. The reaction was also dependent upon the presence of ferrous ions.

conditions of ethanol excess, the ability of *L. hilgardii* to oxidize ethanol to acetaldehyde under the assay conditions of this study requires verification. Nonetheless, it is worthy to note that certain lactobacilli and bifidobacteria have been reported to exhibit a limited capacity to oxidize ethanol to acetaldehyde, and that a probiotic strain *Lactobacillus* GG ATCC 53103 exhibited the highest acetaldehyde-metabolizing capacity in the presence or absence of ethanol (25).

The association of L-ornithine and L-lysine with the formation of **2** and **3** suggests that these amino acids may be respective sources of the N-heterocyclic intermediates, 1-pyrroline and 1-piperidine. The formation of 1-piperidine from lysine has been demonstrated in certain *Pseudomonas* (26) and *Streptomyces* spp. (27) and proceeds via cadaverine. An analogous pathway from ornithine, via putrescine, leading to the formation of 1-pyrroline has also been indicated (26). Such routes closely resemble the initial steps of the metabolism of piperidine and pyrrolidine based alkaloids (28, 29) in which decarboxylation and deamination of the parent amino acids proceeds via the intermediary of a lysine- or ornithine-pyridoxal phosphate complex. The alkaloid precursors, 4-amino-butanal and 5-amino-pentanal, exist in equilibrium with the cyclic imines, 1-pyrroline and 1-piperidine. Further generation of alkaloids concerns the enzyme-mediated entry of a side chain, which can involve acyl-CoA derivatives, at C-2 of the 1-piperidine and 1-pyrroline rings (29, 30).

From the information presented, a mechanism can now be postulated for the biosynthesis of **2** and **3** by heterofermentative LAB. This proposed scheme involves the interaction of intermediates from two disparate pathways, that is (i) N-heterocyclic intermediates, 1-piperidine and 1-pyrroline, derived from the catabolism of L-lysine and L-ornithine, and (ii) acylating agents such as acetyl-CoA accumulated from the heterolactic fermentation (**Figure 6**). In the amino acid branches of this pathway, the intermediates 1-piperidine and 1-pyrroline could accumulate from the metabolism of L-lysine and L-ornithine via the cadaverine and putrescine pathways, respectively. Concomitantly, when the cell is in the presence of both a carbohydrate source, such as D-fructose, and ethanol, an acyl-carrier such as acetyl-CoA may also accumulate in the ethanol branch of the

heterolactic fermentation of sugars. The 1-pyrroline and 1-piperidine intermediates could then be subject to acylation at the C-2 position by the accumulated acyl-CoA (or similar) derivatives, thus yielding the acylated mousy N-heterocycles, **2** and **3**. In this proposed 1-pyrroline/1-piperidine pathway, the resultant acetylated N-heterocycles are secondary metabolites of the basic amino acids, L-ornithine and L-lysine. Other known pathways of lysine metabolism, such as the conversion of lysine to the cyclic imino acid pipercolate in aerobic bacteria (*Pseudomonas*) (26, 31, 32), do not fit the proposed model. Furthermore, under the conditions of the high-cell-density assay, D,L-pipercolate repressed the formation of **2** and **3** by *L. hilgardii* DSM 20176 (data not shown). To our knowledge, the proposed 1-pyrroline/1-piperidine pathway represents the first report of a biological mechanism by which **2** and **3** may be synthesized by LAB from ornithine and lysine, respectively. The scheme presented in this study also explains why homofermentative LAB, that is the pediococci and homofermentative lactobacilli, may fail to produce significant quantities of acetylated mousy N-heterocycles. In contrast to the heterolactic fermentation, the homolactic fermentation (glycolysis) lacks an ethanol branch, thus precluding homofermentative LAB from the proposed accumulation of higher levels of acylating cofactors such as acetyl-CoA from the reverse metabolism of ethanol. Nevertheless, there are several alternative pathways for the dissimilation of pyruvate in homofermentative LAB (such as the pyruvate dehydrogenase pathway) which, under certain conditions, can produce C-2 intermediates including acetyl-CoA (33). It is possible, therefore, that such alternative pathways of pyruvate catabolism could explain the formation of, albeit minor, quantities of acetylated mousy N-heterocycles previously demonstrated in homofermentative LAB (17).

Another major factor affecting the production of mousy N-heterocycles in the high-cell-density incubation assay was the presence of metal ions, particularly  $\text{Fe}^{2+}$ . The dependency of mousy compound formation on the presence of  $\text{Fe}^{2+}$  ions, and not on  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ , or  $\text{Ca}^{2+}$ , supports earlier observations of Unguryan and colleagues (cited by (6)) that a sufficient "active" iron content was one of the major physicochemical factors necessary for mousy off-flavor development in wine. However, the exact role of  $\text{Fe}^{2+}$  in the biosynthesis of mousy N-heterocycles by LAB remains unclear, particularly because many lactobacilli, pediococci, and leuconostocs contain and require little or no iron (34). Further studies would be required to ascertain whether the role of  $\text{Fe}^{2+}$  in N-heterocycle biosynthesis by LAB is chemical or biological, such as serving a redox function or as a cofactor to a hitherto unknown critical enzyme. Moreover, from the current study, it remains unknown as to why **3**, and not **2**, formation was greater in the absence of either  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ , or  $\text{Ca}^{2+}$  ions. Further studies are thus also required to elucidate the role(s) of these trace elements in the biosynthesis of mousy N-heterocycles.

Although the mechanism of **2** and **3** formation postulated in this paper appears to fit the available evidence, the existence of other biosynthetic mechanisms cannot be discounted. By analogy, the Maillard-like generation of roasty, cracker-like N-heterocycle odorants including **2** and **3** involves thermal reactions between amino acids, including proline and ornithine, as well as amino acid derived cyclic intermediates such as 1-pyrroline, with reducing sugars and other carbonyls such as 2-oxopropanal (reviewed by (35)). It is thus possible that the generation of mousy N-heterocycles by *L. hilgardii* may otherwise involve a combination of similar, enzyme-mediated and chemical pathways, and in which reactive metabolic

intermediates such as diacetyl could arise from citrate and carbohydrate metabolism. Further research is thus required to establish the overall mechanism by which this and other wine LAB produce mousy N-heterocycles.

The lack of response in the production of **1** from various treatments including lack of incorporation of deuterium-labeled ethanol and acetaldehyde, suggests that the biosynthetic route for this compound by *L. hilgardii* is different from that for the acetylated N-heterocycles, in particular **3**. In this regard, it is relevant to note that other structurally related piperidine alkaloids including nigrifactin, produced by a strain of *Streptomyces*, and the hemlock alkaloids coniine (2-propylpiperidine) and coniceine (2-propyltetrahydropyridine) are predominantly derived from acetate, and not from lysine (29). Nevertheless, in the current study, coniceine (2-propyltetrahydropyridine) was tentatively identified as a minor product of *L. hilgardii* DSM 20176 in the presence of 2-propanol. Although the role of this alcohol in the latter metabolism is not understood, it would be advantageous for future metabolic studies of alkyl-substituted N-heterocycles to utilize other LAB as test strains, such as *O. oeni* strains previously observed to produce high concentrations of **1** (17).

Overall, the high-cell-density incubation procedure has provided a convenient quantitative method to study the metabolism of mousy N-heterocycles by LAB in a synthetic medium. By this technique, we have demonstrated that *L. hilgardii* can produce large concentrations of mousy off-flavor compounds in the presence of a combination of several essential substrates including ethanol, a carbohydrate source (particularly D-fructose), and  $\text{Fe}^{2+}$  ions. Other substrates stimulatory to this metabolism have also been identified including the amino acids L-ornithine and L-lysine, and acetaldehyde. Hence, it may be speculated that during the vinification process, the opportunistic growth of certain *Lactobacillus* spp., especially in wines containing residual sugar or in stuck fermentations, having high pH and also minimal sulfite and moderate temperature (20–30 °C), may provide ideal conditions for the rapid development of bacteria and consequential mousy off-flavor. The critical role of  $\text{Fe}^{2+}$  in mousy N-heterocycle formation further highlights that in order for wine producers to reduce the risk of this spoilage, contamination from iron during the vinification process should be minimized.

Because the formulation of the N-heterocycle assay medium was chosen to facilitate the study of N-heterocycles by LAB (19), the concentration of precursor compounds may or may not resemble those typically found in grape juice or wine. Further studies on mousy N-heterocycle formation are therefore required to determine the impact of precursor compounds at concentrations typically found in grape juice and wine, and to ascertain the effects of other wine conditions, including pH values less than 4 and ethanol concentration greater than approximately 11–12% v/v. It also remains to determine the mechanism by which other wine bacteria, in particular the homofermentative *Pediococci* and *Lactobacilli*, and also acetic acid bacteria, can generate mousy off-flavor compounds.

#### ABBREVIATIONS USED

LAB, lactic acid bacteria; MLF, malolactic fermentation; GC-MS, gas chromatography-mass spectrometry; MRSA medium, de Man, Rogosa and Sharpe medium supplemented with 20% v/v preservative-free apple juice; MRS-CT medium, de Man, Rogosa and Sharpe medium modified by the inclusion of casamino acids and trypticase peptone; *m/z*, mass-to-charge ratio.



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