Profiling of d-alanine production by the microbial isolates of rat gut microbiota

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Abstract
D-alanine (D-Ala) and several other D-amino acids (D-AAs) act as hormones and neuromodulators in nervous and endocrine systems. Unlike the endogenously synthesized D-serine in animals, D-Ala may be from exogenous sources, e.g., diet and intestinal microorganisms. However, it is unclear if the capability to produce D-Ala and other D-AAs varies among different microbial strains in the gut. We isolated individual microorganisms of rat gut microbiota and profiled their D-AA production in vitro, focusing on D-Ala. Serial dilutions of intestinal contents from adult male rats were plated on agar to obtain clonal cultures. Using MALDI-TOF MS for rapid strain typing, we identified 38 unique isolates, grouped into 11 species based on 16S rRNA gene sequences. We then used two-tier screening to profile bacterial D-AA production, combining a D-amino acid oxidase-based enzymatic assay for rapid assessment of non-acidic D-AA amount and chiral LC–MS/MS to quantify individual D-AAs, revealing 19 out of the 38 isolated strains as D-AA producers. LC–MS/MS analysis of the eight top D-AA producers showed high levels of D-Ala in all strains tested, with substantial inter- and intra-species variations. Though results from the enzymatic assay and LC–MS/MS analysis aligned well, LC–MS/MS further revealed the existence of D-glutamate and D-aspartate, which are poor substrates for this enzymatic assay. We observed large inter- and intra-species variation of D-AA production profiles from rat gut microbiome species, demonstrating the importance of chemical profiling of gut microbiota in addition to sequencing, furthering the idea that microbial metabolites modulate host physiology.

KEYWORDS
amino acid, gastrointestinal microbiome, host microbial interactions, mass spectrometry, rats

Abbreviations: AAs, amino acids; ACN, acetonitrile; Ala, alanine; Asp, aspartate; CNS, central nervous system; D-AA, D-amino acid; D-Ala, D-alanine; D-Asp, D-aspartate; D-Glu, D-glutamate; D-Pro, D-proline; D-Ser, D-serine; DAAO, D-amino acid oxidase; FA, formic acid; Glu, glutamate; HCCA, α-cyano-4-hydroxycinnamic acid; L-AAs, L-amino acids; NMDAR, N-methyl-D-aspartate receptor; Pro, proline; SCX, strong cation exchange; Ser, serine; SPE, solid phase extraction; T2D, type 2 diabetes.

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INTRODUCTION

Microbial metabolites from gut microbiota have been reported to modulate the host’s immune, endocrine, and nervous systems as well as metabolic homeostasis. For example, gut microbiota-derived short-chain fatty acids can regulate gut immunity, host metabolism, and enteric nervous systems. Other examples include aromatic amino acids (AAs) and neurotransmitters such as gamma-aminobutyric acid. The study of host-associated microbial metabolites is therefore key to understanding host–microbe interactions and opens new avenues for future biomedicines and therapeutics.

One enigmatic set of molecules from both host and microbial metabolism is the D-amino acids (D-AAs). Unlike the prevalence of L-amino acids (L-AAs), which are the building blocks of proteins, D-AAs exist in trace amounts in the biofluids, tissues, and organs of mammals. The biological roles and biosynthetic pathways of D-AAs are less understood than many other metabolites. The most studied D-AA in mammals, D-serine (D-Ser), is a neurotransmitter and a potential endocrine modulator produced by serine (Ser) racemases in the brain and pancreas. D-Ser is a potent co-agonist of the N-methyl-D-aspartate receptor (NMDAR), which is an ionotropic glutamate receptor important for the long-term potentiation of synaptic transmissions. Besides D-Ser, D-aspartate (D-Asp) can also activate NMDAR as an agonist and has been found in nervous and endocrine tissues. Some evidence shows that D-Asp is also related to testosterone synthesis. D-alanine (D-Ala), another potent NMDAR co-agonist, has been found in many parts of the animal body, including the insulin-secreting beta-cells and adrenocorticotropic hormone-secreting cells in the pituitary. In addition, D-Ala release from rat islets of Langerhans upon glucose stimulation has been demonstrated.

With the physiological relevance of many D-AAs being revealed, it is important to understand their sources. Endogenous enzymes, such as Ser and aspartate (Asp) racemases, have been found in animals; these enzymes convert L-serine and L-aspartate to their corresponding D-forms. Microbiota, along with diet, have been recognized as additional sources of D-AAs in animals. Microorganisms encode a large variety of AA racemases, such as those for alanine (Ala), Ser, glutamate (Glu), Asp, and proline (Pro). These racemases are responsible for producing D-AAs that regulate microbial processes such as cell wall synthesis and spore germination. By comparing germ-free (GF) and normal mice, D-Ala is believed to be mainly obtained from intestinal microorganisms. A metagenome-wide association study on the gut microbiota in type 2 diabetes (T2D) patients showed that several enzymes related to D-AA biosynthesis and metabolism were associated with T2D, indicating a potential role for microbiota-derived D-AAs in regulating glucose homeostasis. Microbiota-originated D-AAs are also shown to modify murine mucosal defense by inducing D-amino acid oxidase (DAAO) expression in the gut tissue, as well as to suppress sinonasal innate immune responses through sweet taste receptors.

The composition of gut microbiota changes in response to environmental factors, such as diet, leads to changes in microbial metabolite profiles. It is critical to identify major microbial producers of specific metabolites that mediate host-microbiota interaction, information that supports mechanistic studies and the development of microbial engineering strategies for microbiome-targeting therapeutics. Here, we developed a high-throughput analytical method to isolate microbial strains from the animal gut for profiling D-AA production. Species-level variations in the amount and composition of microbial D-AA production have been noted. As strain-specific diversity in the gut microbiota is revealed using high-throughput approaches in microbial cultivation and functional characterization, it is interesting to examine whether different strains within the same species may exhibit distinct D-AA profiles. Identification of the major D-AA producers in animal gut microbiota will help to elucidate the regulation and function of D-AA biosynthesis in the context of host-microbe interplay, to obtain culturable isolates to design mechanistic experiments, and to understand the genetic basis of strain diversity.

In this work, we identified and isolated individual microorganisms that produce high levels of D-Ala, along with four other D-AAs from rat gut microbiota, D-proline (D-Pro), D-Ser, D-glutamate (D-Glu), and D-Asp. Serial dilutions of colon contents from adult male Sprague-Dawley rats were plated on agar media to obtain clonal cultures, which were subjected to rapid strain typing using MALDI-TOF MS. Followed by 16S rRNA sequencing analysis, 38 unique strain isolates belonging to 11 bacterial species were confirmed. A two-tier screening workflow was devised to profile D-AA production. First, a 96-well microtiter assay using a broad-spectrum DAAO coupled with hydrogen peroxide detection was established for rapid assessment of non-acidic D-AA production. Then, chiral LC–MS/MS was utilized to quantify the levels of D-Ala, D-Pro, D-Ser, D-Glu, and D-Asp. Substantial species- and strain-specific production of various D-AA differences were observed among microbial isolates from the rat gut content. These data suggest that different gut microbiota compositions may impact specific D-AA levels, especially for D-Ala, which is suggested to be mainly from the microbiota.
2 | MATERIALS AND METHODS

2.1 | Materials and chemicals

Materials and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Hampton, NH, USA), unless noted otherwise.

2.2 | Microbial isolation from rat intestine

Vertebrate animal use was approved by the Institutional Animal Care and Use Committee, University of Illinois at Urbana-Champaign, and in full compliance with federal guidelines for the humane care and treatment of animals. Three adult male (3–4 months old) Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN, USA, RRID: RGD_737903) were decapitated by a sharp guillotine. Fresh cecum, surgically removed and containing digesta samples, were homogenized to a final concentration of 0.1 g/ml in iced PBS-R buffer (1 mg/ml ascorbic acid, 60 μg/ml uric acid, 100 μg/ml L-glutathione, 20% glycerol in PBS). Cell suspensions in ten-fold serial dilutions (10^6) were plated on R-medium agar plates (28.4 mg/ml BD BBL™ Schaedler Broth, 1 mg/ml ascorbic acid, 400 μg/ml uric acid, 100 μg/ml L-glutathione, 15.0 g/L agar) and incubated at 37°C for 24–48 h. Colonies were re-streaked three times for clonal purification. For subsequent analysis.

2.3 | Strain typing using MALDI-TOF MS

For strain typing, a small portion of a single colony was smeared as a thin film onto a Bruker (Billerica, MA, USA) MALDI Biotarget 384 sample spot by pipet tips. Each deposit was immediately overlaid with 1 μl of matrix solution (10 mg/ml α-Cyano-4-hydroxycinnamic acid (HCCA) in 50:47.5:2.5 acetonitrile (ACN):ddH2O:TFA) and air dried. To add the MALDI mass spectra of newly isolated microbes to the Bruker Taxonomy database, about 10 mg of biomass recovered from the frozen stocks were first washed using 300 μl of ddH2O followed by 900 μl of ethanol. Samples were then lysed with 80 μl of 70% formic acid (FA) followed by 80 μl of ACN. After centrifugation, 1 μl of supernatant was spotted onto a Bruker MALDI Biotarget 384 sample spot, immediately overlaid with 1 μl of HCCA matrix solution, and air dried. Mass spectra were collected using a Bruker autoflex maX LRF mass spectrometer, following recommended instrumental settings by the Bruker MALDI Biotyper Main Spectrum Peak identification standard method (mass range: 2000 to 20000 Da; linear positive method; laser frequency at 60 Hz). Strain typing was performed using the Bruker MALDI Biotyper software, version 4.0, by pattern matching between collected spectra and the reference spectra in the Bruker Taxonomy database. A spectra clustering graph of select 38 rat gut microbes was generated using the Biotyper software, and the spectra of closely related species and E. coli DH5alpha in the Bruker Taxonomy database were included as references and as an outgroup, respectively. From the graphic output, the correlation distances measured were obtained using the TreeSnatcher Plus software to redraw the Class dendrogram using the MEGA-X software (Figure 1).

2.4 | Strain typing using 16S rRNA gene sequencing

To isolate genomic DNA from microbial cultures, fresh agar cultures were resuspended in 100 μl of digestion buffer (10 mM Tris–HCl, 50 mM EDTA, pH 7.0 at 25°C), supplemented with 5 μl of 10 mg/ml lysozyme (10 mM Tris–HCl, pH 7.0, at 25°C, 50% glycerol) and 5 μl of 20 mg/ml protease K (10 mM Tris–HCl, pH 7.0 at 25°C, 50% glycerol), and incubated at 37°C for 1 h. Genomic DNA was then isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer’s instructions. The 16S rRNA gene sequences were PCR amplified from genomic DNA by PrimeSTAR Max DNA polymerase (Takara, Kusatsu, Shiga, Japan) using the 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGTTACGACTT-3’) primers. The 16S rRNA gene sequences were obtained by Sanger sequencing, processed using the Geneious Primer software (https://www.geneious.com), and analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A sequence identity cutoff of >98.7% was adopted as an acceptable probability for identification at the species level. The phylogeny of isolates was determined by calculating evolutionary distances to close neighbors using the MEGA-X software and shown together in Figure 2 with the DAAO enzymatic assay data.

2.5 | Amino acid extraction from microbial isolates

For each strain, biomass collected on Day 3 was scraped from the agar and resuspended in 500 μl methanol supplemented with 0.1% TFA. The organic phase was
collected after centrifugation at 16,300 g for 1 min, and then 500 μl of ddH2O supplemented with 0.1% TFA was added to the remaining biomass. Aqueous supernatant was collected after centrifugation at 16,300 g for 1 min and combined with the organic phase. Aliquots were dried under a constant flow of N2 gas and stored at −80°C until analysis.

2.6 | Enzyme assay for D-AA quantification

The D-AA content was determined using a DAAO from porcine kidney (Catalog# A5222, Sigma-Aldrich, St. Louis, MO, USA) coupled with a fluorometric hydrogen peroxide detection kit (Catalog# MAK165, Sigma-Aldrich,
St. Louis, MO, USA). D-Ala standard solutions at final concentrations of 300, 100, 30, 10, 3, and 1 μM were prepared from a 100 mM stock solution. A series of hydrogen peroxide standard solutions with the same concentrations as the D-Ala standards were prepared from 3% stock solution. Dried microbial secretes were thawed and reconstituted in 400 μl of the provided assay buffer. A 10× dilution of each sample was also prepared. Measurement was carried out in technical duplicates. The reactions were initiated by adding 5 μl (0.25 U, equally 167 μg) of DAAO, and fluorescence readings with 540 nm excitation and 590 nm emission were taken at indicated time intervals. Sodium benzoate was used to validate the specificity of DAAO assay.

2.7 | LC–MS/MS for D-AA quantification

For chiral LC–MS/MS analysis, microbial samples were first processed using SCX-SPE. One milliliter of crude, extracellular microbial extracts was reconstituted in 50:50 methanol:ddH₂O (pH was adjusted to 2.5 using 10 mM FA) before being loaded onto Discovery 50 mg DSC-SCX SPE columns (Waters, Milford, MA, USA). Columns were conditioned and washed using 2 ml of 200 mM FA in 50:50 methanol:ddH₂O (pH 2–2.5) before and after sample loading, respectively. AA content was eluted using 2 ml of 3 M NH₄OH in 50:50 methanol:ddH₂O and dried in a SpeedVac (ThermoFisher, Waltham, MA, USA). Each elute was then reconstituted in 500 μl of ddH₂O and processed using a 0.22 μM centrifugal filter. Each 20 μl sample aliquot was dried in a SpeedVac and reconstituted in 0.5 M sodium bicarbonate solution. The sample or standard was mixed with NaOH(2,4-Dinitro-5-fluorophenyl)-l-valinamide (1 mg/ml in ACN), a modified Marrey's reagent, in a 1:1 ratio. The reaction mixture was incubated in an oven at 60°C for 3 h. Following derivatization, the sample reaction mixture was diluted 20-fold in 95% LC mobile phase A/ACN and analyzed by LC–MS/MS. Chiral separations were performed using a Bruker EVOQ Elite Triple Quadrupole mass spectrometer coupled with an Elute UHPLC module. A reversed-phase Kinetex phenyl-hexyl HPLC column—2.6 μm particle size, 100 Å pore size, 100 mm (length)×2.1 mm inner diameter (Phenomenex, Torrance, CA, USA)—was used for the separation of enantiomers. A gradient method was developed using mobile phase A, 25 mM ammonium formate; mobile phase B, methanol; flow rate, 300 μl/min. The mass spectrometer equipped with an electrospray ionization source was operated under the following conditions: spray voltage for negative mode, −3500 V; cone temperature, 250°C; cone gas flow, 20; heated probe temperature, 400°C; probe gas flow, 45; nebulizer gas flow, 50. Each analyte retention time and fragmentation parameter were established from the corresponding derivatized standard. The resulting chromatograms were analyzed by Data Reviewer 8 (Bruker Corp., Billerica, MA, USA). Protein quantification was performed using the Pierce BCA Protein Assay Kit. The protein levels were used to adjust samples for different sizes (protein amounts) to allow D-AA level comparison between the strains.

3 | RESULTS

3.1 | Bacterial isolates from the rat intestine

We utilized R medium to isolate microbial strains from the rat gut. R medium permits cultivation of anaerobic bacteria in aerobic atmosphere by supplemeting the Schaeuler agar with reducing agents, including ascorbic acid, glutathione, and uric acid. From the fresh intestinal digesta of the male adult rats, around 600 microbial isolates were obtained following serial dilutions, agar cultivation, and clone-purifying streaking. Based on MALDI-TOF MS strain typing, 38 isolates exhibiting representative, distinct spectrum patterns (Figure 1) were selected for 16S rRNA gene sequencing, which revealed 15 full-length 16S rRNA sequences grouped into 11 bacterial species, four genres (Bacillus, Staphylococcus, Enterococcus, and Paenibacillus), and one phylum (Firmicutes) (Table S1). Interestingly, the strains with different 16S rRNA genes of the same species (B. amyloliquefaciens, B. subtilis, B. cereus) (Table S1) also tended to be grouped differently when subjected to MALDI mass spectra clustering (Figure 1). Moreover, strain typing results using MALDI-TOF MS and 16S rRNA sequencing were consistent for most rat gut isolates, except for B. amyloliquefaciens and Paenibacillus pueri, whose MALDI mass spectra did not exhibit significant similarities with those included in the Bruker Taxonomy database, version 4.0.

3.2 | D-AA levels in microbial biomass by DAAO assay

To analyze the overall production of non-acidic D-AAs, including D-Ala, an enzyme assay was first performed on the agar cultures of gut-sourced microbes. Because DAAO (EC 1.4.3.3) displays a wide spectrum of substrate specificity for neutral and basic D-AAs, quantification was performed using an external standard curve based on D-Ala and therefore reported as D-Ala equivalents (Figures S1 and 2). The linear range was 0–30 μM with a limit of detection (LOD) of 0.24 μM (determined using 3 times the standard deviation of blank divided by calibration curve
slopes). Methanol and H₂O were sequentially applied as a mild extraction method for in vitro agar cultures; without sonication and other lysis procedures, extracellular metabolites were primarily extracted while a fraction of intracellular metabolites may also be included. With the stated conditions and detectability, only the Bacillus and Paenibacillus strains showed observable D-AA production (Figure 2). Substantial species- and strain-level variations were also observed. For example, three of nine B. amyloliquefaciens, two of six B. subtilis, and two of five B. cereus strains did not show detectable D-AA production. No clear correlation was observed between 16S rRNA gene sequences and D-AA production (Figure 2), suggesting strain-level phenotypic variations cannot be solely deduced from 16S rRNA-based microbiome sequencing analysis.

### 3.3 | LC–MS/MS analysis of Ala, Pro, Ser, Glu, and Asp in L- and D-configurations

Because DAAO mainly catalyzes the oxidative deamination of neutral and basic D-AAs, the corresponding enzymatic assay estimated the overall sum of those D-AAs. Chiral LC–MS/MS measurements are necessary to analyze the levels of different D-AAs with improved chemical specificity. However, because of the generally low abundance of D-enantiomers of AAs, as well as the high sensitivity and limited dynamic range of the MS modality, carefully designed sample preparation steps are required to remove interfering compounds and enrich AA content before chiral LC–MS/MS analysis.

Here, we utilized strong cation exchange (SCX)-solid phase extraction (SPE) to prepare samples for LC–MS/MS. Table 1 shows the overall recovery of SCX-SPE, calculated from the average values of peak areas obtained for D-AA standards before and after SPE. The efficiency of this SPE treatment is similar for both enantiomers of a particular AA. Depending on the isoelectric point of each AA, the recovery rate varied from 30 to 100%. Since this SPE treatment was performed on samples positive for the DAAO activity that focuses on D-Ala, D-Pro, and D-Ser, the recovery rates varied from 30 to 100%. Since this SPE treatment was performed on samples positive for the DAAO activity that focuses on D-Ala, D-Pro, and D-Ser, the recovery rate was acceptable.

An LC–MS/MS approach was used to identify and quantify D/L-Ala, -Pro, -Ser, -Glu, and -Asp in the gut strain samples. Each analyte peak was identified by both retention time comparison to AA standards using the multiple reaction monitoring mode as shown in Figure S2 and standard spiking. Representative chromatograms of D/L-AAs in a sample before and after standard spiking are shown in Figure 3.

Using the abovementioned workflow combining SCX-SPE with LC–MS/MS, we selected eight strains exhibiting the highest DAAO assay results and analyzed their production levels of D-Ala, D-Pro, D-Ser, D-Glu, and D-Asp in the extracellular crude extracts. Figure 4 and Table 2 summarize the concentrations of D-Ala, D-Pro, D-Ser, D-Glu, and D-Asp, normalized by protein amount, in each sample, in addition to each D-AA by percentage, i.e., the amount of D-AA per the total corresponding AA (Table 2). The LC–MS/MS results confirmed D-Ala secretion by the select strains and revealed relatively low levels of D-Pro and D-Ser, which are also DAAO substrates. On the other hand, notable production of acidic D-Glu and D-Asp was observed for B. cereus (Figure 4 and Table 2). Because DAAO exhibits no or minimal activity towards acidic D-AAs, it may not be possible to find B. cereus isolates as D-Glu and D-Asp producers solely based on the DAAO assay.

### 4 | DISCUSSION

Using R-medium, we were able to isolate 38 bacterial strains from rat intestine contents, while limited taxonomic diversity was observed. The R-medium allows cultivation of strict anaerobes under aerobic conditions. While culturomics prefer to use multiple choices of media to expand diversity of microbes being cultured, a single, quasi-universal medium as R-medium was sufficient to serve the purpose of our study. Following isolation of the 38 bacterial strains, we profiled in vitro production of free D-AAs. The main sources of D-Ala in mammals are currently recognized as diet and gut microbiota. Significantly higher levels of D-Ala, together with D-Asp, D-Glu, and D-Pro, were detected in the rodent intestinal content with a normal gut microbiota relative to GF animals. The bioinformatic analysis suggested that D-Ala production was associated with certain taxa in intestinal microbiota, but to the best of our knowledge,
no experimental evidence has been ever reported. The advance in strain typing by MALDI-TOF MS has made it straightforward to rapidly distinguish different microbial species and strains based on distinct proteomic signatures in mass spectra. From more than 600 microbial colonies, 38 bacterial strains belonging to 11 species and four genres were rapidly isolated from rat intestine (Figure 1). All isolates were assigned to the *Firmicutes* phylum. Such limited taxonomic diversity was likely due to the use of only one medium and may be improved by screening more cultivation conditions. Nonetheless, *Firmicutes* comprises the majority (>78%) of microbial taxa in the rat digestive tract.\(^3\)\(^3\) Also, previous studies found that the relative abundance of *Firmicutes* showed the highest correlation with free D-AAs levels in colonic lumen among commensal microbial taxa in mice.\(^3\)\(^2\)

To quantify D-AAs in monoclonal cultures derived from rat gut microbiota, we first utilized an enzymatic assay that performs oxidative deamination of non-acidic D-AAs followed by spectrophotometric detection of hydrogen peroxide as a reaction product.\(^3\)\(^4\) The absolute stereoselectivity of DAAO allowed high-throughput profiling of D-Ala content in complex matrices, such as microbial crude extracts using microtiter plates. Interestingly, only 19 out of 38 isolated strains were confirmed as D-AA producers via the DAAO assay (Figure 2), despite that D-AAs are considered ubiquitous in bacterial species for cell wall peptidoglycan synthesis,\(^3\)\(^5\) with exceptions for atypical wall-less bacteria, like *Mollicutes*.\(^3\)\(^6\) With our sample preparation, primarily extracellular D-AAs were extracted from biomass for measurements; it may be that there are intracellular D-AA contents of these cultured microbes that can be subject to future studies. When compared with enzymatic assays, the analytical throughput of LC–MS/MS is much lower because of the use of SPE cleanup, derivatization, and chromatographic separation. However, due to the broad substrate range of DAAO towards D-AAs, analytical modalities with enhanced molecular resolution were necessary. The amount of D-Ala correlated well between the DAAO and LC–MS/MS results for the top D-Ala producers determined by the DAAO assay, while D-Ser and D-Pro were low but non-negligible in them (Table 2). In addition, poor DAAO substrates, including acidic D-Glu and D-Asp, were detected using LC–MS/MS. Future enzymatic screening experiments can benefit from including DAAO with complementary substrate scopes, such as D-Asp oxidase, which reacts with D-Glu and D-Asp, or employing engineered DAAO variants that have altered substrate specificity to improve the molecular coverage of enzymatic assays.\(^3\)\(^7\),\(^3\)\(^8\)

Both enzymatic measurement and LC–MS/MS quantification on D-AA levels (Figures 2, 4, and Table 2) showed
the inter- and intra-species variations of the amount and composition of d-AAs production by bacteria, which is otherwise challenging to discover using culture-free methods such as metagenomic 16S rRNA sequencing. Previous research showed that there is a large diversity in the phylogenetic distribution of AA racemases and in the composition and amount of D-AA released by bacteria, mostly on the genus level.\textsuperscript{25,39} Our results further show that such variation in D-AA production exists within the same genus and even the same species. Intra-species diversity in the form of haplotypes within bacterial strains of the same species in the human microbiome has been reported,\textsuperscript{40} and such genetic variations can affect the phenotype of microbial strains, including metabolite production. Thus, chemical characterization of bacterial metabolites is necessary to predict the production of D-AAs as bacterial metabolites, as well as deeper analysis of the microbial genome. Interestingly, looking at \textit{B. cereus} and \textit{B. amylo liquefaciens}, we noticed that different strains of the same species can produce different total levels of D-AAs while keeping the ratios of D-Ala and D-Glu similar within the same species (Table 2 and Figure 4). The similar ratios of D-Ala and D-Glu may imply a synergistic and constitutive expression of Ala and Glu racemases in the species investigated. Previous work has reported constitutive expression of these two racemases in \textit{Mycobacterium}.\textsuperscript{41,42} The different levels of secreted d-AAs may also be related to a variety of bacterial metabolite secretion mechanisms.\textsuperscript{43}

The eight highest D-AA producers we measured using LC–MS/MS are all \textit{Bacillus} species and secreted noticeable amounts of d-Ala and d-Glu (Figure 4). D-Ala is an essential component of the bacterial peptidoglycans and the teichoic acids in the cell walls of Gram-positive bacteria.\textsuperscript{44} For some \textit{Bacillus} species, D-Ala and Ala racemase activities were also found to suppress spore germination.\textsuperscript{45,46} Our results seem to contradict those from Lam et al.\textsuperscript{25} where no D-Ala was detected in the supernatant of a \textit{B. subtilis} culture. However, we also noticed that even within the same species, some strains do not secrete d-AAs at all, whereas others are high D-AA producers (Figure 2). This again shows the importance of chemical phenotyping to capture bacterial intraspecies variation within the microbiota.

In contrast to D-Ala, which had a more consistent ratio throughout all \textit{Bacillus} species, the amounts of D-Glu varied in both terms of percentage and absolute concentration. More specifically, high levels of D-Glu were secreted from \textit{B. cereus} compared to other \textit{Bacillus} species (Table 2 and Figure 4). This may be due to the different bacterial cell wall architectures that vary in different \textit{Bacillus} species.\textsuperscript{47–49} The differences in D-Glu level among \textit{Bacillus} species could also be the result of a species-specific Glu racemase. For example, \textit{B. subtilis} has RacE and YrpC as the two Glu racemases, while \textit{B. anthracis} contains RacE1

| Table 2 | Free d-AA concentrations in different gut bacterial strains (nmol/mg protein) and the d-AA ratios (%d = d/[d+1]x100) |
|---------|----------------------------------------------------------|
| Sample  | Alanine | Proline | Serine | Glutamate | Aspartate |
| No. | d | %d | d | %d | d | %d | d | %d | d | %d |
| 1B | 46.3 (36.7–56.0) | 44.7 (43.5–46.0) | 1.1 (0.9–1.3) | 3.7 (3.5–3.8) | 1.3 (0.9–1.3) | 14.3 (12.9–15.7) | 9.7 (6.4–10.4) | 7.7 (6.4–11.0) | 0.2 (0.1–0.3) | 6.7 (5.9–6.2) |
| 12 | 39.9 (34.0–41.8) | 45.9 (43.6–48.1) | 1.4 (1.2–1.5) | 4.2 (4.0–4.3) | 1.3 (1.2–1.4) | 13.0 (12.2–13.0) | 12.0 (11.1–13.0) | 12.0 (11.5–13.5) | 0.2 (0.1–0.4) | 7.9 (7.3–8.5) |
| 2A | 38.4 (27.9–49.0) | 56.8 (56.0–57.7) | 0.6 (0.3–0.8) | 1.0 (0.8–1.3) | 1.3 (1.1–1.7) | 21.5 (18.9–24.3) | 12.0 (10.3–14.3) | 12.0 (10.4–13.5) | 0.2 (0.1–0.4) | 9.9 (7.5–11.0) |
| 2B | 54.2 (40.5–60.7) | 67.1 (66.1–68.0) | 5.0 (4.8–5.2) | 0.7 (0.6–0.8) | 1.0 (0.9–1.1) | 27.7 (15.9–42.4) | 13.1 (11.3–14.2) | 13.1 (11.5–14.2) | 0.4 (0.3–0.5) | 32.7 (26.5–47.0) |
| 15 | 67.2 (61.4–72.9) | 42.6 (42.2–43.1) | 0.6 (0.5–0.7) | 1.7 (1.6–1.8) | 2.0 (1.5–2.0) | 14.5 (13.5–15.5) | 11.0 (10.0–12.0) | 11.0 (10.0–12.0) | 0.2 (0.2–0.3) | 3.5 (2.6–3.6) |
| 7B | 83.5 (71.3–97.7) | 45.7 (43.6–47.8) | 1.1 (1.0–1.2) | 0.8 (0.7–0.9) | 0.2 (0.4–0.6) | 17.7 (16.7–18.7) | 17.7 (16.6–18.7) | 17.7 (16.6–18.7) | 0.2 (0.2–0.3) | 9.3 (7.7–9.9) |

Note: Values are shown in the format of average (range) (n = 2).
and RacE2. Since the steady-state kinetic parameters of these racemases differ, the formation of d-Glu will vary among the bacterial species as a result.

The pattern of elevated d-AAs in B. cereus also applied to d-Asp, another acidic d-AA, while the d-Asp amount was relatively lower in other Bacillus species (Table 2 and Figure 4). The recent study by Kajitani et al. reported enzymatic screening for d-Asp-producing lactic acid bacteria from food sources. Their findings aligned with our current study in that the amounts of d-Asp varied among different strains of the same species. The amounts of D-Ser and D-Pro, other DAAO substrates, remained relatively low but non-negligible regardless of species and strains (Table 2 and Figure 4). Some bacteria include these low-abundance d-AAs for their peptidoglycan composition. Unique to bacteria, the Ser/Ala racemase activity of VanT exchanges d-Ala with d-Ser for resistance against bactericidal agents like vancomycin. Thus, the observed heterogeneity in d-AA production could also be due to the microbial metabolic regulation of antibiotic resistance.

Gut microbiota produce a range of diverse compounds that affect host CNS function, immunity, and metabolism. A number of research studies highlight the importance of one of those gut-derived compounds, the d-AAs, in microbial communities and in various signaling pathways in hosts and thus, their therapeutic potentials. Here, we describe an analytical approach to isolate gut microbial species and determine their d-AA production via a combination of DAAO enzymatic assay and LC–MS/MS. We observed strain-specific d-AA production where the absolute concentrations of d-AAs varied, despite the percentage of some d-AAs remaining constant regardless of bacterial strain or species. Our work demonstrates that the chemical phenotyping of gut microbial strains is necessary to reveal the intra- and inter-species differences in metabolism, which is otherwise challenging to discover using microbiome sequencing methods. As different strains of bacterial species can induce different host responses, it is important to characterize the unique d-AA production profiles from the gut bacterial strains to better understand the physiological and pathophysiological implications of strain-specific d-AAs on the host.

AUTHOR CONTRIBUTIONS
Performed research, analyzed data, and wrote the paper: Cindy J. Lee; Performed research and wrote the paper: Tian A. Qiu; Performed research and wrote the paper: Zhilai Hong; Performed research: Zhenkun Zhang, Yuhao Min, and Linzixuan Zhang; Analyzed data: Lei Dai; Supervised research: Huimin Zhao; Performed research, wrote the paper and designed research: Tong Si; Designed research and supervised research: Jonathan V. Sweedler.

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DISCLOSURES
The authors declare that they have no conflicts of interest with the contents of this article.

DATA AVAILABILITY STATEMENT
All processed data supporting the conclusions are presented in the main text and Supporting Information. All original data are available and can be shared upon request by contacting the corresponding authors.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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