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

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Running title

- D-Alanine production from rat gut microbial strains

Abstract

D-alanine (D-Ala) and several other D-amino acids (D-AAs), unusual amino acids present in mammals, 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 secretion *in vitro*, focusing on D-Ala. Serial dilutions of intestinal content 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 secretion, combining a D-amino acid oxidase-based enzymatic assay for rapid assessment of overall D-AA amount, followed by 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 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 enzymatic assay. We observed large inter- and intra-species variation of D-AA secretion 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: D-alanine, amino acid, rat, microbiome, Bacillus, host-microbe, chiral analyses, mass spectrometry

Introduction

Microbial metabolites from gut microbiota have been reported to modulate the host's immune, endocrine, and nervous systems as well as metabolic homeostasis (1-3). For example, gut microbiota-derived short-chain fatty acids can regulate gut immunity, host metabolism, and enteric nervous systems (2). Other examples include aromatic amino acids (AAs) (1) and neurotransmitters such as gamma-aminobutyric acid (4). 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 (5). 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 (6, 7). 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 (8). 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 D-Asp is also related to testosterone synthesis (9-11). 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 (12, 13). In addition, D-Ala release from rat islets of Langerhans upon glucose stimulation has been demonstrated (14).

With the physiological relevance of many D-AAs being revealed, it is important to understand their source. 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 (6, 15, 16). Microbiota have been recognized as another important source 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 (17). By comparing germ-free (GF) and normal mice, D-Ala is believed to be mainly obtained from intestinal microorganisms (18). 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 (19), 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 (20), as well as to suppress sinonasal innate immune responses through sweet taste receptors (21).

The composition of gut microbiota changes in response to environmental factors, such as diet, leads to changes in microbial metabolite profiles (22). 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 (23). As strain-specific diversity in the gut microbiota is revealed using high-throughput approaches in microbial cultivation and functional characterization (24, 25), 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 secrete 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 neutral 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. These data suggest that different gut microbiota compositions may impact specific D-AA levels, especially for D-Ala, which cannot be synthesized by the host.

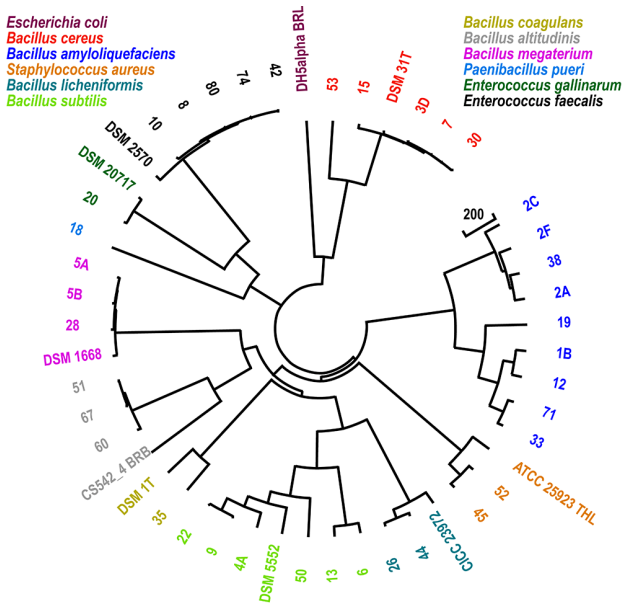

Figure 1. Class dendrogram of MALDI-TOF mass spectra of 38 microbial isolates from the rat gut. Microbial strains in the Bruker Taxonomy database were included, based on their similarities to rat isolates in spectrum pattern matching using the Bruker MALDI Biotyper 4.0 software; *E. coli* DH5alpha was included as an outgroup.

Results

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 (26) by supplementing the Schaedler 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.

D-AA levels in microbial secretes by DAAO assay

To analyze the overall production of neutral 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, quantification was calibrated using an external standard curve based on D-Ala and therefore reported as D-Ala equivalents (**Figure 2**). A 50:50 methanol:ddH_2O solution was utilized as a mild extraction method for *in vitro* agar cultures so that extracellular metabolites were primarily analyzed. 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 secretion. No clear correlation was observed between 16S rRNA gene sequences and D-AA production (**Figure 2**),

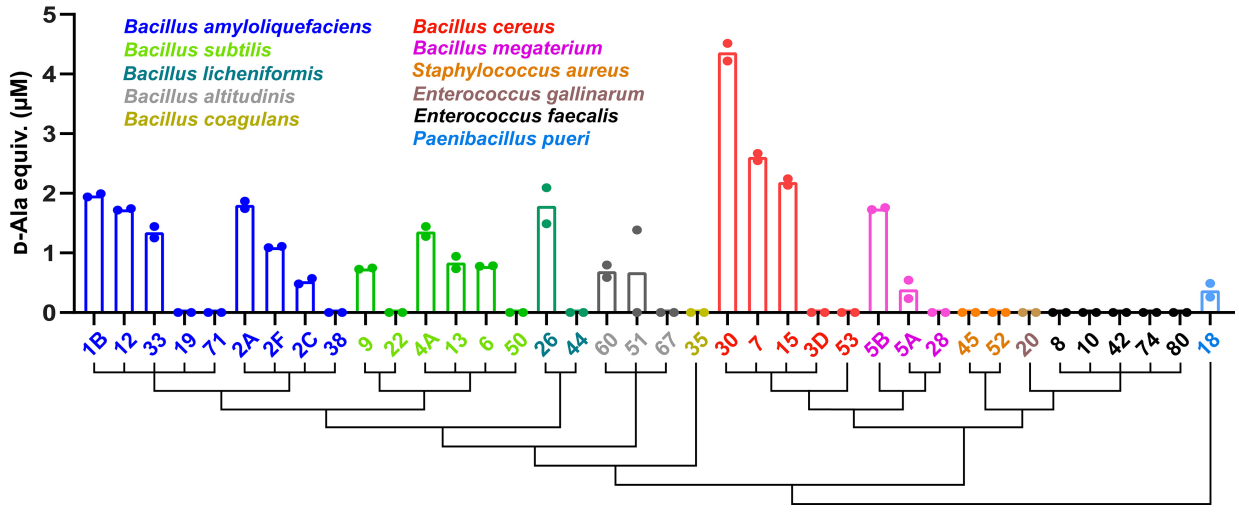

Figure 2. Secreted D-AA concentrations by rat gut isolates using the DAAO enzyme assay. Strains were grouped according to 16S rRNA gene sequences (Table S1). Average and individual values of replicates are shown in bars and dots, respectively (n = 2).

suggesting strain-level phenotypic variations cannot be solely deduced from 16S rRNA-based microbiome sequencing analysis.

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 rate was acceptable.

Table 1. The overall recovery rate of selected AAs using a SCX-SPE.

Amino Acids		Isoelectric Point	% Recovery (n=3)
Asp	D	2.77	31.7 ± 2.9
	L		33.4 ± 0.7
Glu	D	3.22	55.5 ± 2.3
	L		63.6 ± 1.0
Ser	D	5.68	53.4 ± 1.9
	L		55.7 ± 3.3
Ala	D	6.00	≈100 ± 6.5
	L		95.5 ± 1.6
Pro	D	6.30	≈100 ± 4.0
	L		≈100 ± 11.3

An LC-MS/MS approach was used to identify and quantitate 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 and standard spiking. Representative chromatograms of D/L-AAs in a sample before and after standard spiking are shown in **Figure 3**.

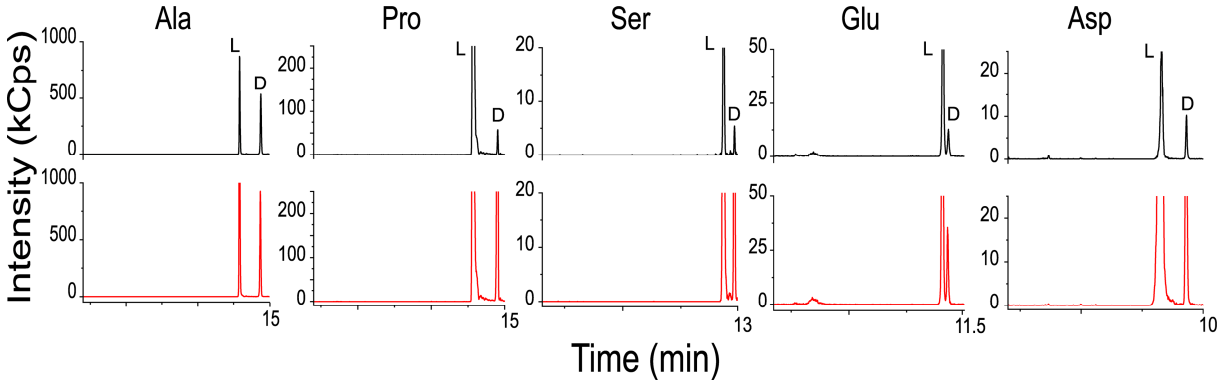

Figure 3. Representative chromatograms for D/L-Ala, Pro, Ser, Glu, and Asp. (Top) sample; (bottom) sample spiked with D/L-standards.

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. Whereas D-Ala and D-Glu are essential and universal components of microbial peptidoglycans, D-Asp is a less common metabolite in bacteria (27).

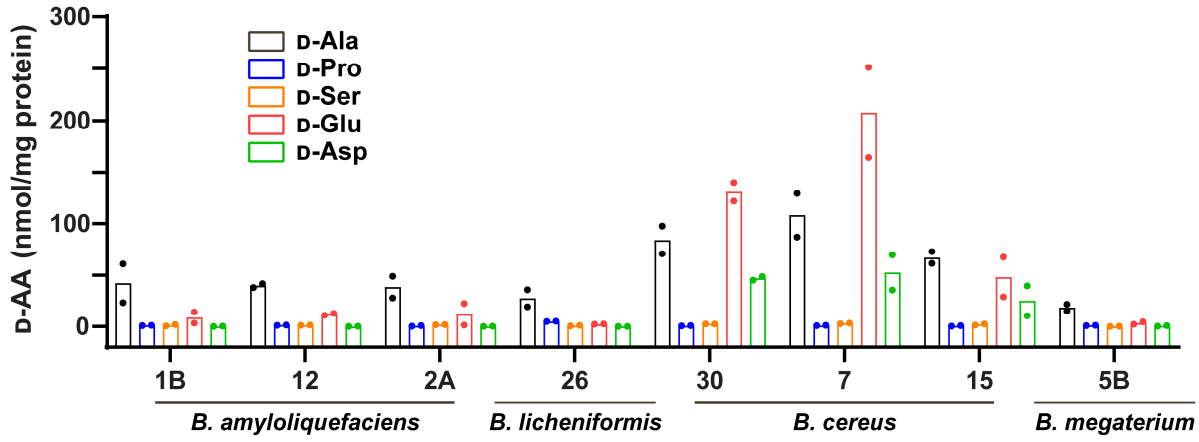

Figure 4. Free D-AAs in different gut bacterial strains (nmol/mg protein). Average and individual values of replicates are shown in bars and dots, respectively (n = 2).

Table 2. Free D-AA concentrations in different gut bacterial strains (nmol/mg protein) and the D-AA ratios (%D = D/(D+ L)).

Sample No.	Alanine		Proline		Serine		Glutamate		Aspartate	
	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 (0.7-1.9)	14.3 (12.9-15.7)	8.8 (3.4-14.3)	7.7 (6.4-9.1)	0.2 (0.1-0.3)	6.7 (3.1-10.4)
12	39.9 (38.0-41.8)	45.9 (43.6-48.1)	1.4 (1.3-1.5)	4.8 (4.2-5.4)	1.3 (1.2-1.4)	13.0 (12.0-13.9)	12.0 (11.1-13.0)	10.3 (10.1-10.5)	0.2 (0.2-0.3)	6.0 (5.9-6.2)
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)	1.9 (1.7-2.1)	32.1 (29.9-34.3)	12.0 (1.5-22.5)	12.3 (10.4-14.3)	0.2 (0.1-0.4)	7.9 (7.3-8.5)
26	27.7 (19.4-35.9)	67.1 (66.1-68.0)	5.0 (4.8-5.2)	0.7 (0.6-0.8)	1.0 (0.6-1.3)	10.4 (8.9-11.9)	2.3 (2.2-2.4)	3.9 (3.2-4.6)	0.10 (0.06-0.14)	3.4 (2.0-4.9)
30	84.2 (70.7-97.7)	44.5 (44.1-44.8)	0.8 (0.7-0.8)	1.9 (1.8-2.0)	2.47 (2.46-2.49)	12.9 (11.7-14.1)	131.1 (121.9-140.2)	49.4 (49.2-49.6)	47.0 (45.3-48.6)	66.5 (62.0-71.0)
7	108.3 (87.1-129.5)	46.5 (43.5-49.5)	1.1 (0.9-1.2)	2.2 (2.2-2.3)	3.0 (2.7-3.3)	17.3 (15.7-18.8)	207.8 (164.5-251.0)	49.5 (48.8-50.1)	52.7 (35.6-69.8)	73.5 (71.7-75.4)
15	67.2 (61.4-72.9)	42.6 (42.2-43.1)	0.6 (0.5-0.8)	1.7 (1.7-1.8)	2.0 (1.5-2.6)	14.8 (12.8-16.8)	48.3 (28.8-67.7)	39.6 (35.3-43.9)	25.1 (10.6-39.6)	71.6 (64.1-79.1)
5B	18.5 (15.3-21.6)	45.7 (43.6-47.8)	1.1 (1.0-1.2)	0.8 (0.7-0.8)	0.2 (0.2-0.3)	9.3 (8.7-9.9)	3.5 (2.3-4.6)	17.7 (17.6-17.9)	0.7 (0.4-0.9)	34.9 (23.5-46.3)

* Values are shown in the format of average (range) (n = 2).

Discussion

In this study, we isolated 38 bacterial strains from rat intestine and profiled *in vitro* production of free D-AAs, with a focus on D-Ala. The main sources of D-Ala in mammals are currently recognized as diet and gut microbiota (28). 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 (20, 29). More importantly, the bioinformatic analysis suggested D-Ala production was associated with certain taxa in intestinal microbiota (29), 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 (30). Also, previous studies found that the relative abundance of *Firmicutes* showed the highest correlation with free D-AA levels in colonic lumen among commensal microbial taxa in mice (29). Indeed, 19 out of 38 isolated strains were confirmed as D-AA producers via the DAAO assay under our experimental conditions (**Figure 2**). Substantial inter- and intra-species variations in the free D-AA types and levels were observed (**Figure 4** and **Table 2**), which is otherwise challenging to discover using culture-free methods such as metagenomic 16S rRNA sequencing.

A range of analytical techniques have been used to detect D-Ala in biological samples (31). To quantify secreted D-Ala in monoclonal cultures derived from rat gut microbiota, we first utilized an enzymatic assay that performs oxidative deamination of D-Ala followed by spectrophotometric detection of hydrogen peroxide as a reaction product (32). The absolute stereoselectivity of DAAO allowed high-throughput profiling of D-Ala content in complex matrices, such as microbial crude extracts using microtiter plates. However, due to the broad substrate range of DAAO towards neutral D-AAs, analytical modalities with improved molecular selectivity are necessary to separate and detect different D-AAs. For the top D-Ala producers determined by the DAAO assay, we applied chiral LC-MS/MS to quantify D-Ala, D-Pro, D-Ser, D-Glu, and D-Asp in their extracellular extracts. When compared with enzymatic assays, the analytical throughput of LC-MS/MS was much lower because of the use of SPE cleanup, derivatization, and chromatographic separation, but new insights were revealed due to the enhanced molecular resolution. The amount of D-Ala correlated well between the DAAO and LC-MS/MS results, while D-Ser and D-Pro were low but non-negligible (**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 DAAOs with complementary substrate scopes, such as D-Asp oxidase, which reacts with D-Glu and D-Asp, to improve the molecular coverage of enzymatic assays.

Both enzymatic measurement and LC-MS/MS quantification on D-AA levels (**Figure 2**, **Table 2**, and **Figure 4**) showed the inter- and intra-species variations of the amount and composition of D-AAs secreted by bacteria. While Ala and Glu racemases are ubiquitous in bacterial species for cell wall peptidoglycan synthesis, other AA racemases have been found in different bacterial species (33). 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 (23, 34). Our results further show that such variation in D-AA secretion 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 (35), 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 *B. cereus* and *B. amyloliquefaciens*, 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, as previous work has reported constitutive expression of these two racemases in *Mycobacterium* (36, 37). These different levels of secreted D-AAs may also be related to a variety of bacterial metabolite secretion mechanisms (38).

Among all five D-AAs being measured, our results showed that D-Ala and D-Glu were among the highest levels (**Figure 4**). This is not surprising, as D-Ala is an essential component of the peptidoglycans in bacterial cell wall structures. D-Ala also plays role in the assembly of teichoic acids in the cell walls of Gram-positive bacteria (39). The D-alanylation of teichoic acids was found to be strongly related to the pathogenicity of Gram-

positive bacteria and host immune responses (40, 41). For some *Bacillus* species, D-Ala and Ala racemase activities were found to suppress germination during spore development (42, 43). The eight highest D-AA producers we measured using LC-MS/MS are all *Bacillus* species and secrete noticeable amounts of D-Ala. This seems to contradict the results from Lam et al. (23) where no D-Ala was detected in the supernatant of a *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 addition to D-Ala, numerous Gram-positive and Gram-negative bacteria, including *B. subtilis*, produce D-Glu via Glu racemase (17, 23, 44). In our study, in contrast to D-Ala, which had a more consistent ratio throughout all *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 *B. cereus* compared to other *Bacillus* species (**Table 2** and **Figure 4**). The separate studies of Fox et al. (45), Leoff et al. (46), and Choudhury et al. (47) reported potential differences in bacterial cell wall architecture in different *Bacillus* species. It is thus possible that *B. cereus* putatively has a different cell wall composition of D-Glu than other *Bacillus* species. The differences in D-Glu composition among *Bacillus* species could be the result of a species-specific Glu racemase. For example, *B. subtilis* has RacE and YrpC as the two Glu racemases, while *B. anthracis* contains RacE1 and RacE2 (48). Since the steady-state kinetic parameters of these racemases differ (48), 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. In *B. cereus*, both high absolute concentrations and ratios of D-Asp were observed, while the D-Asp amount was relatively lower in other species (**Table 2** and **Figure 4**). The recent study by Kajitani et al. (49) reported enzymatic screening for D-Asp-producing lactic acid bacteria from food sources. Interestingly, their findings aligned with our current study in that the amounts of D-Asp varied among different strains of the same species. Our results further show that the gut microbiome's ability to produce and secrete D-AAs is strain-dependent. 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**). Unique to bacteria, the Ser/Ala racemase activity of VanT exchanges D-Ala with D-Ser for resistance against bactericidal agents like vancomycin (50). Some bacteria include the minor D-AAs for their peptidoglycan composition (51-54). Although Pro racemase was identified in *Clostridium difficile* and *Clostridium sticklandii*, the incorporation of D-Pro in peptidoglycan remains unknown (17).

The secretion of D-AAs from bacterial species is implicated in regulating microbial communities and in modifying host responses. In microbial communities, exogenous D-AAs secreted by D-AA producers may be modulators of microbial adaptation to the environment and microbial diversity (23, 55). D-AAs can induce host responses via interaction with mucosal or airway surfaces (20, 21, 34). The gut microbiota-produced free D-AAs can also be absorbed through the gut and potentially regulate host physiology. Unlike D-Ser and D-Asp, which originate from endogenous biosynthesis in addition to microbiota and diet, the main source of intestinal D-Ala, D-Glu, and D-Pro in mammals is the gut microbiota (18, 20). Though the mechanisms of transport and

metabolism of free D-AAs in hosts are poorly understood, results of previous studies, including our own, indicated that D-Ala may be involved in animal behavior and glucose metabolism (14, 56). A more detailed summary of the implications of D-Ala in hosts can be found in the recent review by Lee et al. (28). While D-Glu and D-Pro are produced by the gut microbiota strains, the roles of these two D-AAs in animals remain obscure, although recent studies suggest protective roles for D-Glu against Alzheimer's Disease (57-61), and sedative and hypnotic effects of D-Pro in neonatal chicks (62). We also found low but non-negligible amounts of D-Ser and D-Asp (except for *B. cereus*) in the gut microbial strains. The physiological roles of D-Ser and D-Asp have been extensively studied in the central nervous system (CNS) (61, 63) and endocrine system (63, 64). Although the biological roles of gut microbiota-derived D-Ser and D-Asp in the host are equivocal, putative functions of gut microbiota- or diet-derived D-Ser in the CNS (65, 66) and acute kidney injury (67) have been reported. An increase in D-Ser, as well as D-Asp, in some brain regions of GF mice compared to normal mice also reflects the effect of gut microbiota on the metabolism of D-AAs in the host (68). In general, the physiological and pathophysiological implications of these microbiota-associated D-AAs in hosts remain obscure and warrant further study.

Conclusions

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 various signal 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 metabolite production, which is otherwise challenging to discover using microbiome sequencing methods. As different strains of bacterial species can induce different host responses (69), 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.

Experimental procedures

Materials and chemicals

Materials and chemicals were purchased from Sigma-Aldrich or Fisher Scientific, unless noted otherwise.

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) were decapitated by a sharp guillotine. Fresh intestines, 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 (10^0–10^{-6}) were plated on R-medium agar plates (28.4 mg/mL BD BBL^{TM} 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 analyses, cells were spread on the R-medium agar as lawns, collected using a cotton swab, and transferred to solutions by soaking. Strains were stored as frozen stocks at –80 °C in R medium supplemented with 15% (*v*/*v*) glycerol until analysis.

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 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):ddH_2O: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 ddH_2O 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: 2,000 to 20,000 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 (70) to redraw the Class dendrogram using the MEGA-X software (71) (**Figure 1**).

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 proteinase 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) following the manufacturer's instructions. The 16S RNA gene sequences were

PCR amplified from genomic DNA by PrimeSTAR Max DNA polymerase (Takara) 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 (**Figure 2**).

Sample collection of D-AA secretion 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 ddH_2O 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 N_2 gas and stored at –80 °C until analysis.

Enzyme assay for D-AA quantification

The D-AA content was determined using a DAAO from porcine kidney (Sigma) coupled with a fluorometric hydrogen peroxide detection kit (Sigma, Catalog# MAK165). 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 of DAAO, and fluorescence readings with 540 nm excitation and 590 emission were taken at indicated time intervals.

LC-MS/MS for DAA 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:ddH_2O (pH was adjusted to 2~2.5 using 10 mM FA) before being loaded onto Discovery 60 mg DSC-SCX SPE columns (Waters). Columns were conditioned and washed using 2 mL of 200 mM FA in 50:50 methanol:ddH_2O (pH 2~2.5) before and after sample loading, respectively. AA content was eluted using 2 mL of 3 M NH_4OH in 50:50 methanol:ddH_2O and dried in a SpeedVac (ThermoFisher). Each elute was then reconstituted in 500 µL of ddH_2O 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 Nα-(2,4-Dinitro-5-fluorophenyl)-L-valinamide (1 mg/mL in ACN), a modified Marfey'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) – 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 was established from the corresponding derivatized standard. The resulting chromatograms were analyzed by Data Reviewer 8 (Bruker Corp.). 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.

Data availability

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.

Supporting information–This article contains supporting information.

Author contributions– **Cindy J. Lee**: Writing-Original Draft, Investigation, Formal analysis, **Tian A. Qiu**: Writing-Original Draft, Investigation, **Zhilai Hong**: Writing-Original Draft, Investigation, **Zhenkun Zhang**: Investigation, **Yuhao Min**: Investigation, **Linzixuan Zhang**: Investigation, **Lei Dai**: Formal analysis, **Huimin Zhao**: Supervision, **Tong Si**: Writing-Original Draft, Investigation, Conceptualization, Writing–Review & Editing, **Jonathan V. Sweedler**: Supervision, Conceptualization. Writing–Review & Editing

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Abbreviations– The abbreviations used are: AAs, amino acids; ACN, acetonitrile; Ala, alanine; Asp, aspartate; CNS, central nervous system; D-AA, D-amino acid; DAAO, D-amino acid oxidase; D-Ala, D-alanine; D-Ala, D-

alanine; D-Asp, D-aspartate; D-Glu, D-glutamate; D-Pro, D-proline; D-Ser, D-serine; 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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