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Anaerobic Conversion of Proteinogenic Amino Acids When Methanogenesis Is Inhibited: Carboxylic Acid Production from Single Amino Acids

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Abstract: Proteins are an abundant biopolymer in organic waste feedstocks for biorefining. When degraded, amino acids are released, but their fate in non-methanogenic microbiomes is not well understood. The ability of a microbiome obtained from an anaerobic digester to produce volatile fatty acids from the twenty proteinogenic amino acids was tested using batch experiments. Batch tests were conducted using an initial concentration of each amino acid of 9000 mg COD L^{-1} along with 9000 mg COD L^{-1} acetate. Butyrate production was observed from lysine, glutamate, and serine fermentation. Lesser amounts of propionate, iso-butyrate, and iso-valerate were also observed from individual amino acids. Based on 16S rRNA gene amplicon sequencing, *Anaerostignum*, *Intestimonas*, *Aminipila*, and *Oscillibacter* all likely play a role in the conversion of amino acids to butyrate. The specific roles of other abundant taxa, including *Coprothermobacter*, *Fervidobacterium*, *Desulfovibrio*, and *Wolinella*, remain unknown, but these genera should be studied for their role in fermentation of amino acids and proteins to VFAs.

Keywords: carboxylic acids; amino acids; proteins; anaerobic digestion; chain elongation

Citation: Conrado, L.; McCoy, J.; Rabinovich, L.; Davoudimehr, M.; Stamatopoulou, P.; Scarborough, M. Anaerobic Conversion of Proteinogenic Amino Acids When Methanogenesis Is Inhibited: Carboxylic Acid Production from Single Amino Acids. *Fermentation* **2024**, *10*, 237. https://doi.org/10.3390/fermentation10050237

Academic Editors: Thaddeus Ezeji, Jose Luis García-Morales and Francisco Jesús Fernández Morales

Received: 18 March 2024
Revised: 15 April 2024
Accepted: 25 April 2024
Published: 29 April 2024

1. Introduction

Organic wastes have been proposed as a renewable source of carbon to produce beneficial chemicals, including methane and volatile fatty acids (VFAs) [1]. While highly variable, these wastes consist of mixtures of biopolymers encompassing carbohydrates, proteins, lipids, and other trace organic and inorganic molecules [2]. Carbohydrates and proteins are abundant in many common organic wastes, including food wastes. The degradation of complex carbohydrates is well-studied, but biological protein degradation in anaerobic systems needs to be better understood. While it is known that proteins and amino acids can be converted to biogas through conventional anaerobic digestion [3–5], little is known about the fermentation of amino acids when methanogenesis is inhibited. To unlock the full potential of proteins as a feedstock for biorefining, more needs to be known about the biotransformation of amino acids under anaerobic conditions.

Volatile fatty acids have been targeted as end products and key intermediates in advanced organic waste fermentation processes, such as the carboxylate platform and chain elongation [6,7]. VFAs, such as acetate, propionate, butyrate, iso-butyrate, valerate, and iso-valerate, serve as fundamental chemical components extensively utilized across various industries, including the food, textile, and pharmaceutical sectors [8]. Amino acids can be fermented into organic acids, including VFAs, through three classes of metabolic pathways: the Stickland reaction, oxidative deamination of individual amino acids, and reductive deamination of individual amino acids [9–11]. Generally, Stickland reactions are common and couple the oxidation of an amino acid with the reduction of another. Oxidation of amino acids typically yields hydrogen as a product, while many amino acid reduction pathways rely on hydrogen as an electron donor. For over five decades, the ability of pure

cultures to produce a variety of VFAs from amino acids has been known [12,13], but the fate and role of amino acids in anaerobic microbiome-based processes, either in conventional anaerobic digestion or in alternative fermentations, is largely unexplored.

Only a few studies have reported results on amino acid bioconversion under anaerobic conditions when methanogenesis is inhibited. Recent work by Wang et al. provided a detailed analysis of the fermentation of eight amino acids (alanine, glutamate, glutamine, leucine, lysine, methionine, threonine, and valine) using metagenomic and metaproteomic approaches and found that L-isomers of amino acids promoted VFA production more than D-isomers [14]. Additionally, Regueira et al. performed an extensive modeling analysis for the conversion of amino acids to carboxylic acids [15]. While these studies have provided valuable modeling and experimental insights into the potential to convert amino acids to beneficial products, the fate of amino acids and the microbial communities involved in amino acid fermentations are only beginning to emerge.

To further understand the potential role of amino acids and proteins in biorefining, the anaerobic biotransformation of all twenty proteinogenic amino acids was tested while chemically inhibiting methanogenesis. In addition to measuring the consumption of amino acids, the production of VFAs was assessed. Further, 16S rRNA gene amplicon sequencing was used to assess how microbial communities changed during incubations. In total, this work provides a comprehensive analysis of amino conversion to VFAs and a preliminary assessment of the microbial communities responsible for these conversions. These results should inform future work with bioreactors to convert single or mixed amino acids to VFAs.

2. Materials and Methods

2.1. Materials

Sludge was acquired from a full-scale anaerobic digester at a wastewater treatment facility in South Burlington, VT, USA, and utilized as an inoculum. Sludge was collected from the same location for all experiments. A synthetic medium containing 10 g COD L^{-1} of acetate and 10 g COD L^{-1} of twenty individual L-isomer amino acids was prepared for all experiments. The chemical composition of the synthetic medium was as follows (g/L): one amino acid (Table 1), sodium acetate anhydrous ($NaC_2H_3O_2$) 12.82, potassium phosphate dibasic (K_2HPO_4) 2.0, sodium phosphate monobasic (NaH_2PO_4) 0.4, magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) 0.1, calcium chloride ($CaCl_2$) 0.05, and 2-bromoethanesulfanoate (BRES) 3.3. Acetate was added as a supplemental electron acceptor and to provide a substrate that is commonly produced by anaerobic microbiomes. BRES was added to inhibit methanogenesis [16]. Further, 1 mL of trace-mineral supplement (ATCC, Manassas, VA, USA) was added to 1 L of media to provide trace metals. Potassium phosphate dibasic (K_2HPO_4) and sodium phosphate monobasic were added to the media to help maintain a pH greater than 5.5 during fermentation. No additional pH control mechanism was used. The initial pH of the media is provided (Table 1) and varied according to the amino acid added with basic amino acids, resulting in an increased pH and acidic amino acids, which resulted in a decreased pH. Arginine resulted in a high pH of 9.68 due to its highly basic characteristics [17].

Table 1. Concentrations of amino acids used in media preparation.

Amino Acid	Chemical Name (As Added)	Concentration Added (g L^{-1})	Media pH
Alanine	L-alanine	9.34	6.69
Arginine	L-arginine	9.90	9.68
Asparagine	L-asparagine monohydrate	15.63	7.10
Aspartate	L-aspartic acid potassium salt	17.83	6.88
Cysteine	L-cysteine	15.14	6.00
Glutamine	L-glutamine	10.15	6.78
Glutamate	Sodium L-glutamate monohydrate	12.98	6.98
Glycine	Glycine	15.64	6.20

Table 1. *Cont.*

Amino Acid	Chemical Name (As Added)	Concentration Added (g L^{-1})	Media pH
Histidine	L-histidine	9.70	7.36
Isoleucine	L-isoleucine	5.46	6.28
Leucine	L-leucine	5.47	6.34
Lysine	L-lysine monohydrochloride	8.15	6.73
Methionine	L-methionine	8.48	7.07
Phenylalanine	L-phenylalanine	5.15	6.96
Proline	L-proline	6.54	7.34
Serine	L-serine	13.14	6.88
Threonine	L-threonine	9.31	6.90
Tryptophan	L-tryptophan	5.67	5.67
Tyrosine	L-tyrosine	5.96	5.96
Valine	L-valine	6.10	6.39

2.2. Experimental Setup

Biological triplicates were used for each amino acid along with control experiments without any amino acid provided to the media. The medium for the control experiments included acetate as the supplemental carbon source and BRES to inhibit methanogenesis, but no amino acids were added. Serum bottles with a 125 mL total volume were inoculated with 10 mL of sludge and 90 mL of medium, meaning that the inoculum sludge biomass and chemical constituent concentration were diluted 1:10 with the synthetic medium. After combining sludge and media, nitrogen gas was sparged into the bottles for at least 1 min, and the bottles were sealed with butyl rubber stoppers to maintain anaerobic conditions. The serum bottles were incubated in an incubator shaker at 35 °C while being agitated at a setting of 100 rpm. The bottles were incubated for a total of 14 days. In total, six rounds of batch experiments were performed with different batches of inoculum sludge with 2 to 4 amino acids being tested for each round.

2.3. Analytical Methods

The inoculum sludge was tested for pH, total solids (TS), volatile solids (VS), soluble chemical oxygen demand (COD), and soluble ammoniacal nitrogen before the start of experiments. These tests were performed according to Standard Methods for the Examination of Water and Wastewater [18]. TS and VS tests were conducted according to standard methods 2540B and 2540E [18]. For soluble COD and ammoniacal nitrogen analyses, samples were filtered through a 0.20 µm syringe filter prior to testing. COD testing was performed according to standard method 5220 with Hach test kits for high-range COD (Hach Method 8000, Hach, Loveland, CO, USA). Ammoniacal nitrogen analyses were performed according to standard method 4500 using Hach test kits (Hach Method 8155, Hach, Loveland, CO, USA).

On sampling days, headspace gas was collected from the serum bottles and analyzed with a gas chromatogram with a thermal conductivity detector (GC-TCD). An SGE 25 mL gas-tight fitted syringe was used to collect headspace samples. The samples were directly injected into a GC-TCD Nexis GC-2030 system (Shimadzu, Kyoto, Japan) to quantify the methane. After de-capping the serum bottles, liquid samples were centrifuged at 9600 rcf for 10 min, and the supernatant and the biomass pellet were stored separately for further analyses. Liquid samples were stored in the −20 °C freezer and used to measure amino acids, VFAs, and ammoniacal nitrogen. Samples for amino acid analyses were delivered to the Dana Farber Cancer Institute at Harvard University for analysis by high-performance liquid chromatography (HPLC) with derivatization of amino acids with fluorescent tags. Cysteine and tryptophan were excluded from the amino acid analyses because they could not be measured using the methods employed. Cysteine is challenging to quantify, given its instability in air [19], and tryptophan degrades substantially during sample preparation to derivatize amino acids before analyzing with chromatography [20]. VFAs, including acetic acid, propionic acid, iso-propionic acid, butyric acid, iso-butyric acid, valeric acid,

iso-valeric acid, caproic acid, heptanoic acid, and caprylic acid, were analyzed using gas chromatography–mass spectrometry (GC-MS) with a GC-MS TQ8040NX system (Shimadzu, Kyoto, Japan). For the GC-MS method, a 2.5 mL smart syringe at 150 °C was used to collect 1 mL of the headspace gas in 10 mL vials. The incubation temperature was 95 °C, the incubation time was 40 min, and the agitator's speed was 250 rpm. The column used was the DB-FATWAX UI (Agilent Technologies, CA, USA) with 30 m length, 0.25 µm thickness, and 0.23 mm diameter. The column temperature started at 80 °C for 1 min and increased by 20 °C/minute until it reached 240 °C for 1.5 min. The MS acquisition mode was a Q3 scan, with the ion source temperature and the interface temperature at 280 °C and 250 °C, respectively.

2.4. Microbial Community Analyses

The biomass pellets were stored at −80 °C for DNA extraction using the Qiagen DNAEasy PowerSoil extraction kit (Qiagen, Hilden, Germany). After extraction, DNA quality and quantity were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were diluted to provide a DNA concentration of 1 ng ml^{-1} for library preparation. The Vermont Integrative Genomics Resource performed 16S rRNA gene amplification using the standard V3/V4 primer set [21] and sequenced the resulting amplified libraries on the Illumina MiSeq platform to generate 2 × 300 bp reads. The reads were analyzed using the Qiime2 [22] pipeline with the following steps: (1) raw reads and sample metadata were imported as a Qiime artifact; (2) reads were denoised and quality trimmed with dada2 [23]; (3) taxonomy was assigned using the Silva database [24]; and (4) abundance tables were created using default Qiime commands. Relative abundance data was visualized using the superheat package in R [25]. Raw DNA-sequencing data are available from NCBI under Bioproject number PRJNA1087599.

3. Results and Discussion

3.1. Characterization of Inoculum Sludge

In total, six batches of sludge were used for seeding the batch experiments (Table 2). The sludge TS was 2.3 ± 0.6% with 69 ± 14% of the TS being vs. Further, the soluble COD was 809 ± 192 mg COD L^{-1}, and the soluble ammonia was 451 ± 125. The pH varied from 6.95 to 7.56 with an average of 7.36 ± 0.26. In total, there was some fluctuation between sludge characteristics, but the overall characteristics align with what is expected in a methanogenic anaerobic digester consuming waste-activated sludge at a municipal wastewater treatment facility. In total, the results also suggest that, when diluted 1:10 with the synthetic media, the initial ammoniacal nitrogen concentration should be low, between 25 and 57 mg N L^{-1}. Further, when mixed with the media, the soluble COD remaining in the sludge would be only a minor fraction (e.g., <1 percent) of the soluble COD provided to the incubations.

Table 2. Characteristics of inoculum sludge.

	TS (%)	VS (% of TS)	Soluble COD (mg L^{-1})	Soluble NH_3-N (mg L^{-1})	pH
Sludge 1	2.7	61	972	254	7.56
Sludge 2	1.6	97	755	573	7.22
Sludge 3	1.9	70	534	361	7.64
Sludge 4	3.2	61	755	447	7.51
Sludge 5	2.1	62	755	567	6.95
Sludge 6	2.1	64	1080	505	7.25
Average	2.3	69	809	451	7.36
Stdev	0.6	14	192	125	0.26

3.2. Amino Acid Degradation

All of the amino acids measured showed a decrease in concentration from Day 0 to Day 14 (Figure 1). Asparagine, aspartate, glutamate, glycine, histidine, lysine, serine, and tyrosine showed almost complete degradation (>90%). All these amino acids have pathways described for their degradation without Stickland reactions. Asparagine can serve as the sole carbon and nitrogen source for multiple organisms via a two-step pathway that produces fumarate [26,27]. Aspartate can be degraded by Campylobacter, which produces succinate and formate [12]. Glutamate fermentation via multiple routes, including the methylaspartate and hydroxyglutarate pathways, have been described and produce acetic and butyric acids [28]. Histidine can be fermented to glutamate, which is fermented via the pathways described previously but also produces formamide with unknown fates in anaerobic environments [29]. Lysine fermentation to acetate and butyrate via multiple metabolic routes has been described, and serine fermentation to acetate and propionate has been shown [12,26,29]. Fermentation of tyrosine has only been shown via Stickland-type reactions previously, but our results suggest that it may be degraded without additional amino acids. Given the high electron density of tyrosine (1.68 g COD g^{-1} tyrosine), it may be using the acetate provided in the media as an electron acceptor.

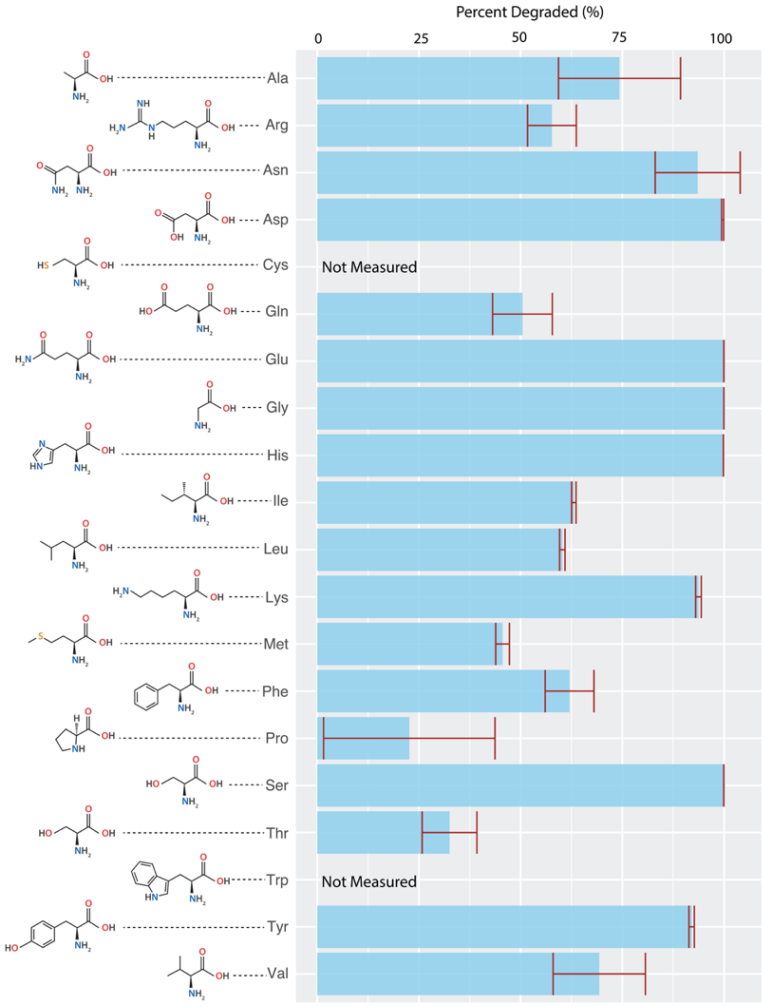

Figure 1. Extent of amino acid degradation during batch experiments with individual amino acids after 14 days of incubation. Error bars represent the standard deviation of biological replicates (n = 3). Abbreviations are standard amino acid three-letter abbreviations as follows: alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), cysteine (Cys), glutamine (Gln), glutamate (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Try), and valine (Val).

To assess amino acid consumption byproducts, methane, pH, and ammoniacal nitrogen were analyzed (Figure 2A–C). The methane composition of the headspace after 14 days was always less than 5%, while most incubations resulted in a composition of less than 1% (Figure 2A). This suggests that BRES was dosed at an appropriate level to inhibit methanogenesis. The pH typically dropped during incubations (Figure 2B) and the pH was similar between the biological replicates. The lowest pH was 5.9, suggesting that the pH was maintained at appropriate values for mixed VFA production, which typically occurs above a pH of 5 [30]. Ammoniacal nitrogen concentrations increased during the degradation of all amino acids (Figure 2C). During anaerobic degradation, amino acids undergo deamination, releasing ammonia that may participate in the formation of other compounds, such as urea [31]. Asparagine, aspartate, glycine, histidine, lysine, and serine had the highest increase in ammonia concentrations. Asparagine is utilized in some organisms as the sole carbon and nitrogen source through the action of two enzymes. The first one catalyzes the breakdown of asparagine into aspartate and ammonium. The second enzyme, aspartase ammonia-lyase, facilitates the reversible deamination of aspartate, resulting in the production of fumarate and ammonium [27]. In all incubations, acetic acid was consumed (Figure 2D) and decreased from the initial concentration of 9000 mg COD L^{-1}, but residual acetic acid remained in all incubations.

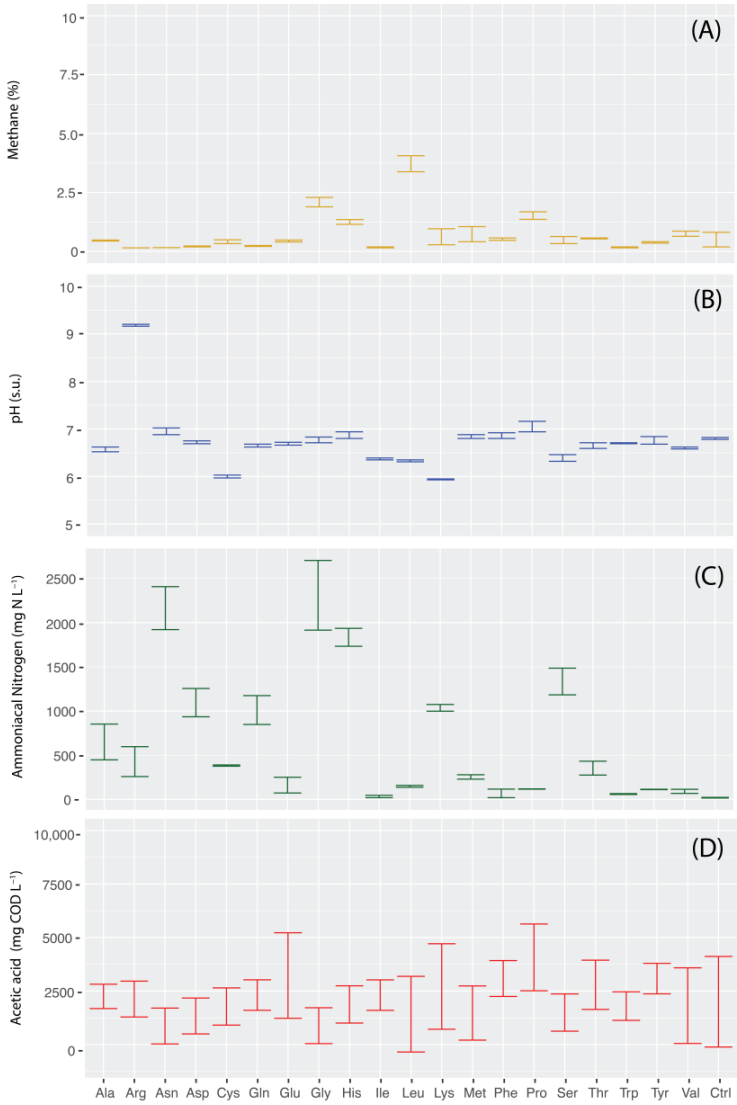

Figure 2. The measured headspace methane composition (**A**), pH (**B**), ammoniacal nitrogen concentration (**C**), and acetic acid concentration (**D**) after 14 days of incubation. Error bars represent the standard

deviation of biological replicates (n = 3). Abbreviations are standard amino acid three-letter abbreviations as follows: alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), cysteine (Cys), glutamine (Gln), glutamate (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Try), and valine (Val).

3.3. Production of Volatile Fatty Acids

Of the 20 amino acids tested (Figure 3), 12 produced a significantly higher amount of VFAs ($p < 0.05$) compared to the controls (Figure 4) without amino acids added, but the quantity and type of VFAs produced varied between the amino acids. The overall COD conversion to VFAs was low, with a maximum of 8% of the COD added as acetic acid and amino acids being converted to VFAs in incubations with lysine. Among VFAs other than acetic acid, butyric acid was the most commonly produced VFA, followed by propionic, iso-butyric, and iso-valeric acids (Figure 3). Only trace amounts of other analyzed VFAs (e.g., valeric, hexanoic, and octanoic acids) were found.

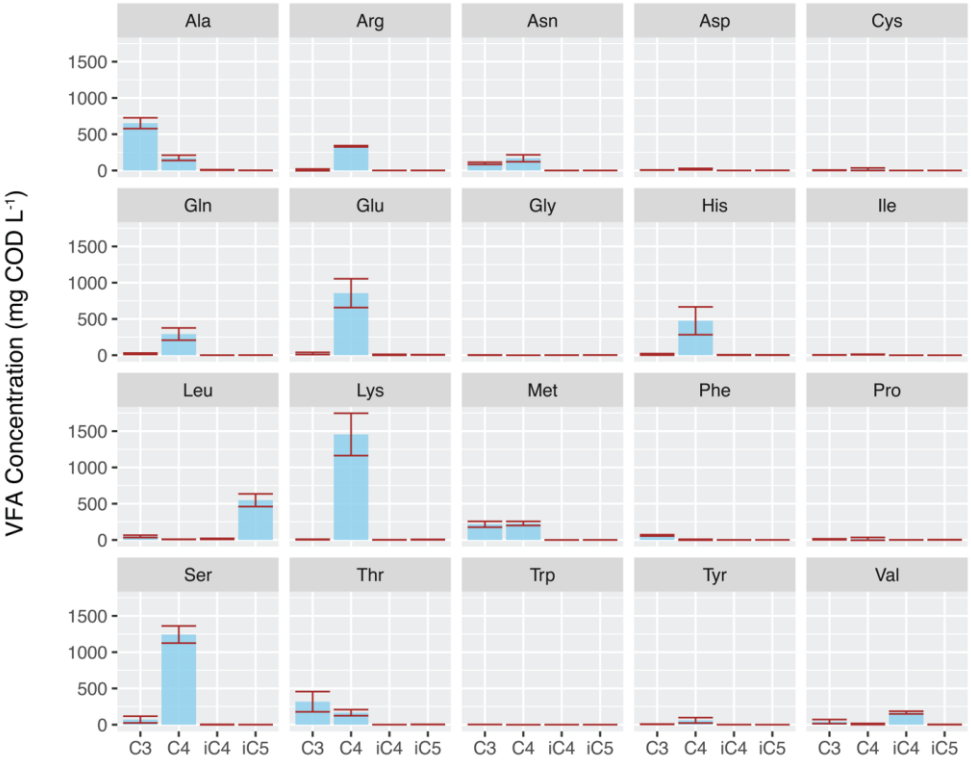

Figure 3. Propionic (C3), butyric, (C4), iso-butyric (iC4), and iso-valeric (iC5) acid concentrations after 14 days of incubation. Error bars represent the standard deviation of biological replicates (n = 3). Abbreviations are standard amino acid three-letter abbreviations as follows: alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), cysteine (Cys), glutamine (Gln), glutamate (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Try), and valine (Val).

Lysine resulted in the highest butyrate production of 1455 ± 292 mg COD L^{-1} followed by serine and glutamate. A pathway for lysine conversion to butyrate was described for *Clostridium sticklandii* in 1954 [32] and more recently has been proposed to be fermented to butyric acid by human gut microbes [33]. Serine conversion to butyrate is less well described but has been found to be performed by *Cloacibacillus porcorum* isolated from the swine intestinal tract [34]. Serine is likely to be initially converted to pyruvate as an intermediate via a single enzymatic step with L-serine ammonia lyase [35] prior to being directed to butyric acid production via reverse β-oxidation. Histidine incubations also showed the production of butyric acid. Histidine has several proposed degradation

routes with glutamate as an intermediate [36], and glutamate has previously been shown to produce butyric acid as a fermentation end product utilizing electron-bifurcating enzymes to conserve energy in anaerobic bacteria [28,37].

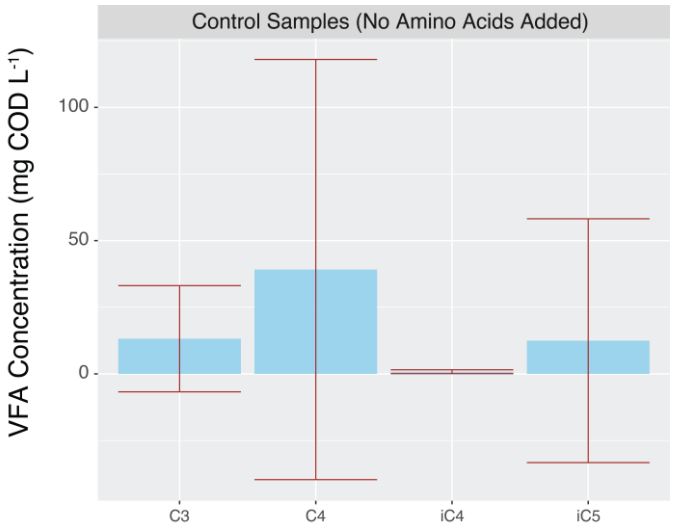

Figure 4. Propionic (C3), butyric, (C4), iso-butyric (iC4), and iso-valeric (iC5) acid concentrations after 14 days of incubation with controls that had sludge and media without amino acids. Error bars represent the standard deviation of biological replicates (n = 6). Abbreviations are standard amino acid three-letter abbreviations as follows: alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), cysteine (Cys), glutamine (Gln), glutamate (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Try), and valine (Val).

While butyric acid was the most commonly produced VFA, propionic, iso-butyric, and iso-valeric acids were also produced in some incubations (Table 3, Figure 3). Alanine had the highest propionate production of 652 ± 74.5 mg COD L^{-1}. Others have shown that propionate can be produced from alanine [38], but the metabolic routes of alanine conversion to propionate remain unclear. Threonine and methionine incubations also produced propionate, although at lower concentrations. Valine had the highest iso-butyric acid production of 167 ± 18.6 mg COD L^{-1} and was the only amino acid to produce significantly higher amounts of iso-butyric acid than the controls in experiments with multiple strains of *Clostridium* species [12] and goat ruminal fluid [39]. Likewise, anaerobic degradation of L-leucine produced the highest amount of iso-valeric acid, and this has also been demonstrated with multiple strains of *Clostridium* species [12] and goat ruminal fluid [39].

In total, the batch experiments demonstrated that VFAs are produced to varying extents from multiple amino acids. Alanine and threonine produced mostly propionic acid, while butyric acid was the primary VFA product of arginine, glutamine, glutamate, lysine, and serine incubations. Lastly, when incubated with the branched-chain amino acids valine and leucine, the predominate products were the branched-chain VFAs iso-butyric and iso-valeric acids, respectively. While only a small portion of the COD was converted to VFA end products, these results demonstrate that amino acids can be fermented by anaerobic microbiomes.

Table 3. Electron balance as COD during amino acid degradation.

Amino Acid	Final Amino Acid Concentration (mg COD L^{-1})	Percent Conversion (%) [1]			
		Propionic Acid	Butyric Acid	Iso-Butyric Acid	Iso-Valeric Acid
Alanine	2280 ± 1230	7.2 ± 0.83	1.9 ±0.40	0.08 ± 0.05	0.02 ± 0.01
Arginine	3900 ± 822	0.10 ± 0.13	3.7 ± 0.10	0.01 ± 0.00	0.01 ± 0.01
Asparagine	571 ± 920	1.1 ± 0.17	1.9 ± 0.53	0.00 ± 0.00	0.01 ± 0.00
Aspartate	30.7 ± 19.4	0.06 ± 0.02	0.21 ± 0.11	0.01 ± 0.00	0.02 ± 0.01
Cysteine	Not Measured	0.03 ± 0.03	0.20 ± 0.20	0.00 ± 0.00	0.00 ± 0.00
Glutamine	4660 ± 545	0.23 ± 0.11	3.3 ± 0.94	0.01 ± 0.01	0.02 ± 0.01
Glutamate	1.46 ± 2.53	0.27 ± 0.17	9.5 ± 2.2	0.06 ± 0.07	0.06 ± 0.03
Glycine	3.93 ± 0.83	0.02 ± 0.02	BDL [2]	0.01 ± 0.01	0.03 ± 0.01
Histidine	14.0 ± 1.67	0.13 ± 0.12	5.3 ± 2.1	0.04 ± 0.03	0.03 ± 0.02
Isoleucine	3340 ± 97.5	0.04 ± 0.02	0.11 ± 0.05	BDL [2]	BDL [2]
Leucine	3570 ± 97.2	0.55 ± 0.18	0.09 ± 0.02	0.16 ± 0.08	6.1 ± 0.97
Lysine	559 ± 67.6	0.07 ± 0.04	16 ± 3.2	0.02 ± 0.01	0.05 ± 0.04
Methionine	4870 ± 63.3	2.4 ± 0.45	2.5 ± 0.32	0.00 ± 0.00	0.01 ± 0.01
Phenylalanine	3340 ± 492	0.70 ± 0.10	0.04 ± 0.07	0.01 ± 0.01	0.01 ± 0.01
Proline	6954 ± 1800	0.10 ± 0.07	0.17 ± 0.21	0.01 ± 0.01	0.03 ± 0.02
Serine	5.18 ± 1.53	0.79 ± 0.51	14 ± 1.3	0.02 ± 0.03	0.01 ± 0.01
Threonine	5980 ± 598	3.5 ± 1.6	1.8 ± 0.47	0.01 ± 0.00	0.04 ± 0.02
Tryptophan	Not Measured	0.03 ± 0.00	0.0 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Tyrosine	744 ± 91.5	0.08 ± 0.03	0.67 ± 0.14	0.01 ± 0.00	0.01 ± 0.00
Valine	2740 ± 2370	0.48 ± 0.31	0.10 ± 0.09	1.9 ± 0.21	0.03 ± 0.02

[1] Percent conversion is based on COD of amino acid added. [2] BDL indicates that the final VFA concentration was below the detection limit.

3.4. Microbial Community

To further understand the conversion of amino acids to VFAs during the incubation experiments, an analysis of the microbial community was performed using 16S rRNA gene amplicon sequencing. This analysis resulted in 49 distinct taxa being identified at the genus level with at least 3% relative abundance in at least one sample (Figure 5). While 49 genera were identified, 10 were unclassified at the genus level but could be classified at a higher level of taxonomy. Organisms related to *Coprothermobacter*, *Fervidobacterium*, Uncultured *Spirochaetaceae*, *Lentimicrobium*, *Acetomicrobium*, *Aminicenantales*, *Comamonadaceae* (Unclassified), and *Bacteroides* (Unclassified) were the predominant bacterial genera during all batch experiments. All these taxa were also abundant in the seed sludge samples.

Coprothermobacter species were abundant in all incubations and are known for their ability to consume a wide variety of proteins and amino acids and produce hydrogen gas [40]. A metaproteomic analysis suggested that *Coprothermobacter* produces a variety of fermentation end products, including formate, pyruvate, acetate, and butanol [41]. *Coprothermobacter* genera were abundant in the following amino acid batch tests: aspartate, phenylalanine, isoleucine, threonine, and valine. Except for threonine, all the other amino acids showed a degradation percentage higher than 60% in the batch tests. *Fervidobacterium* species have been found to be abundant in anaerobic digesters [42]. *Fervidobacterium* abundance has been found to correlate positively with VFA production from protein-rich feedstocks [43]. This genus was abundant during the fermentation of the following amino acids in our batch experiments: phenylalanine, proline, and tryptophan. All these amino did not produce significant amounts of VFAs.

Alanine incubations, which produced an increase in butyrate (Figure 3), experienced an enrichment in *Anaerostignum* and *Desulfovibrio*. *Desulfovibrio* species were also enriched during incubations with leucine, tryptophan, and tyrosine. *Anaerostignum* (aka *Anaerotignum*) species have been shown to produce acetate, propionate, and butyrate using alanine, serine, and threonine, and the type species of this genus, *Anaerostignum aminivornas*, was isolated from an anaerobic digester treating food-processing wastewater [44]. Further, co-cultures of

Anaerotignum neopropionicum and *Clostirum kluyveri* have been used to produce odd-chain carboxylic acids from dilute ethanol [45]. *Desulfovibrio* species are canonical sulfur-reducing bacteria, but *Desulfovibrio* aminophilus, isolated from an anaerobic dairy-waste lagoon, has been shown to degrade alanine, aspartate, leucine, isoleucine, valine, and methionine [46].

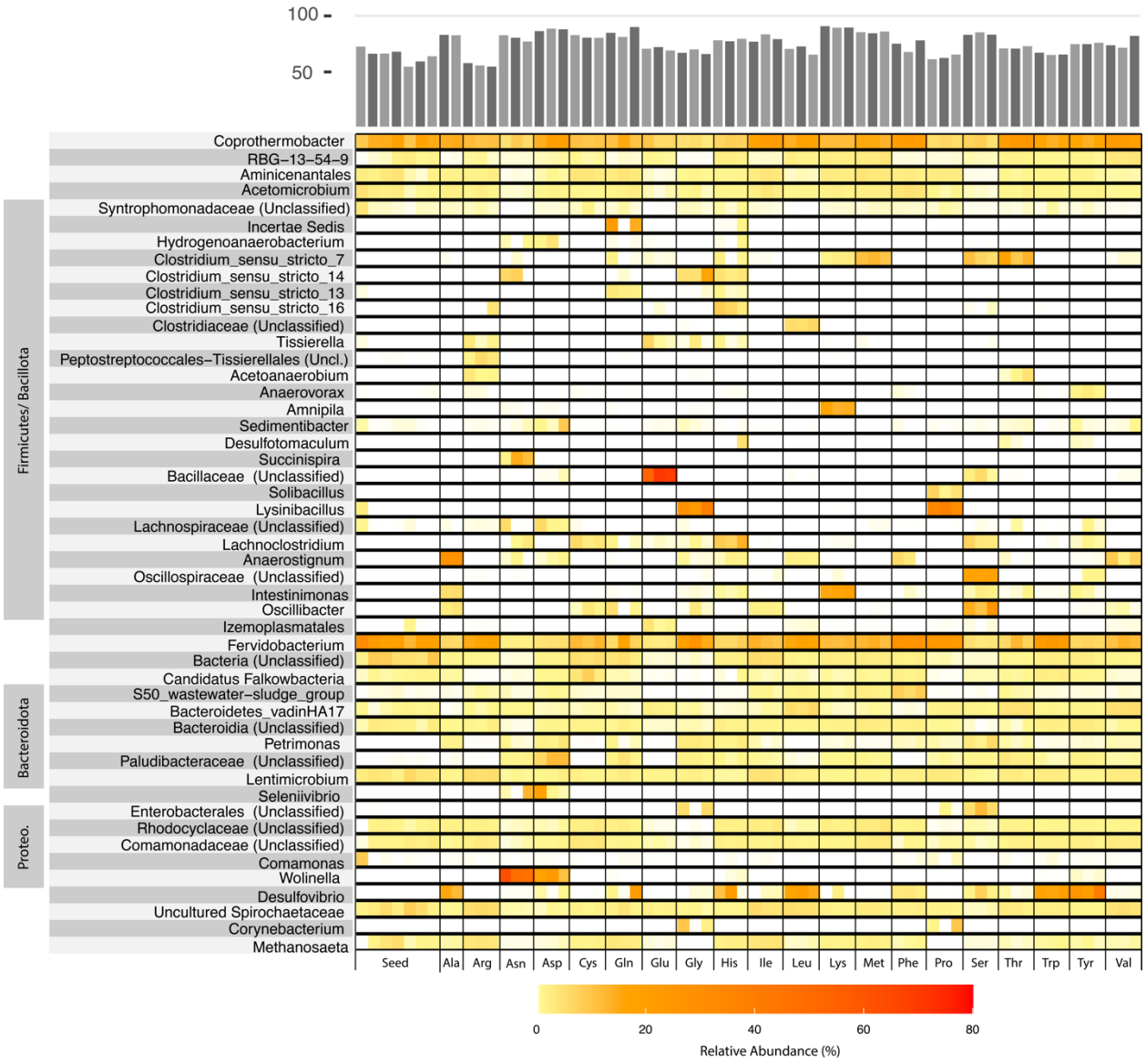

Figure 5. Relative abundance of genera based on 16S rRNA gene amplicon sequencing. Red intensity indicated relative abundance. Triplicates are provided for all twenty amino acids except alanine, which has only two sequencing replicates. The inoculum sludge was sequenced for each round of batch experiments and is shown as "seed". Abbreviations are standard amino acid three-letter abbreviations as follows: alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), cysteine (Cys), glutamine (Gln), glutamate (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Try), and valine (Val).

Lysine incubations resulted in an increased abundance of *Intestinomonas* species and *Amnipila* species. *Intestimonas* species, including *Intestimonas butyricproducens*, are well-studied for their ability to produce butyrate in the human gut and have been shown to produce butyrate from both amino acids and sugars [47]. This genera has also been proposed to consume lysine in infant and adult humans, as it was highly enriched from

stool when incubated with lysine [48]. A metabolic pathway for lysine conversion to butyrate has been proposed that relies on the conversion of 5-amino-3-heaxanoate and acetyl-CoA to L-3-amino-butyryl-CoA and then deamination of L-3-amino-butyryl-CoA to crotonyl-CoA, a key intermediate of reverse β-oxidation [33]. *Amnipila* (aka, *Aminipila*) is a genus within the Peptostreptococcales–Tissierellales order, and species such as *Aminipila luticellari* have been shown to produce butyrate and consume a variety of amino acids, including L-lysine [49].

In addition to lysine, both glutamate and serine incubations resulted in the accumulation of butyrate. Glutamate incubations resulted in a sharp increase in the abundance of unclassified Bacillaceae. The Bacillaceae family falls within the Firmicutes phylum and encompasses a wide variety of organisms isolated from vastly different ecosystems (e.g., soils, hypersaline lakes, and hydrothermal vents) [50]. *Lysinibacillus* is a genus within this family that was enriched during incubations with glycine. This genus is known to oxidize several amino acids [51], but there is no known characterization of its fermentation end products. Serine incubations resulted in an enrichment of an Unclassified Oscillospiraceae as well as a genus within this family, *Oscillibacter*. The type species of *Oscillibacter*, *Oscillibacter valericigenes*, is known to produce valerate (C5) as the primary product of glucose fermentation [52]. However, this genus has been identified in other microbial communities producing butyrate enriched from the human colon microbiome [53].

Although asparagine and aspartate incubations did not result in high VFA production, *Wolinella* species were highly enriched during incubations (Figures 3 and 5). This genus has been shown to oxidize formate and reduce fumarate to succinate, and the species, *Wolinella succinogenes*, was isolated from the cow rumen [54]. The genus does not degrade carbohydrates, but there are no known studies on its ability to ferment amino acids. Wolinella species have been identified in anaerobic digesters previously [55,56], but their functional role and degradation capabilities are largely unknown.

4. Conclusions

This work demonstrates that, when methanogenesis is inhibited, amino acids can be converted to VFAs. Although the conversions represented only a small fraction of the total COD (up to 8%), no attempt was made to optimize the production of VFAs other than inhibiting methanogenesis and providing a buffer to keep the pH above 5.0 during the 14-day incubations. Future work with chemostats is expected to further increase the production of VFAs from amino acids. The microbial community analyses revealed several taxa already described to convert amino acids to VFAs including *Anaerostignum*, *Intestimonas*, *Aminipila*, and *Oscillibacter*; all have the potential to play a role in the conversion of amino acids to butyrate, which could subsequently be elongated to even higher value medium-chain carboxylic acids. The specific roles of abundant *Coprothermobacter*, *Fervidobacterium*, *Desulfovibrio*, and *Wolinella* remain unknown, and these genera should be studied more for their potential role in the fermentation of amino acids and proteins to VFAs.

Author Contributions: Conceptualization, L.C. and M.S.; Data curation, L.C.; Formal analysis, L.C.; Funding acquisition, M.S.; Investigation, L.C., J.M., L.R., M.D. and P.S.; Methodology, L.C., P.S. and M.S.; Project administration, M.S.; Resources, M.S.; Software, P.S.; Supervision, M.S.; Validation, M.S.; Visualization, L.C.; Writing—original draft, L.C. and M.S.; Writing—review and editing, L.C., J.M., M.D., P.S. and M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the United States National Science Foundation, grant number 2221828.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

Acknowledgments: The authors thank Peyton Lienhart and Kennedy Brown for their assistance in sample collection.

Conflicts of Interest: The authors declare no conflicts of interest.

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