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Changes in the free amino acid profile of pickling cucumber during lactic acid fermentation

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Abstract: Free amino acid (FAA) profiles of fresh, acidified, naturally fermented, and starter culture fermented cucumbers were analyzed by liquid chromatography triple quadrupole mass spectrometry. Fermented cucumbers contained more total FAA than acidified cucumbers (1,302 ± 102 mg/kg and 635 ± 35 mg/kg, respectively). Total FAA content of fermented cucumber was similar regardless of brine salt levels (2–6% NaCl) and starter culture addition. Glutamine (1491.4 ± 69.3 mg/kg), γ-aminobutyric acid (GABA, 269.6 ± 21.4 mg/kg), asparagine (113.0 ± 6.4 mg/kg), and citrulline (110.3 ± 8.5 mg/kg) were the most abundant FAA in fresh pickling cucumber, whereas GABA (181.3 ± 21.5 mg/kg), isoleucine (165.2 ± 11.2 mg/kg), leucine (129.8 ± 10.9 mg/kg), and lysine (110.9 ± 5.0 mg/kg) were the most abundant in fermented cucumber. GABA and ornithine were produced during fermentation, indicating glutamate decarboxylase and arginine deiminase activities. Notably, ornithine was significantly higher in natural (63.3 ± 31.5 mg/kg) versus starter culture fermented cucumbers (3.0 ± 0.7 mg/kg). This new information on FAA composition of fresh and fermented pickling cucumbers shows the impact of fermentation conditions on cucumber amino acid profiles while providing insight for manipulating fermentations for health promotion and consumer acceptance.

KEYWORDS
composition, health-promoting properties, natural cucumber fermentation, taste-active amino acids, vegetable preservation

Practical Application: This study reports changes in the free amino acid profiles of raw, fermented and acidified cucumbers, which may be valuable for understanding the impact of these foods on human health and nutrition. This information is useful for food microbiologists studying the metabolism of lactic acid bacteria during fermentation and/or designing starter cultures and could contribute to the development of novel fermented cucumber pickle products with enhanced nutritional value.

1 | INTRODUCTION

Pickled vegetable consumption in the United States is dominated by cucumber pickles, about 40% of which are fermented. Americans consume approximately 2.5 billion pounds of all pickle types annually, equating to ~7.5 pounds per person (Pickle Packers International, 2020). Cucumber pickles may be produced by one of two primary processes: direct acidification or fermentation. Acidified cucumbers are produced by brining fresh cucumbers in water, salt, and acetic acid and are either pasteurized to prevent fermentation or contain preservatives to inhibit microbial activity. Fermented cucumbers are produced by brining fresh cucumbers in a mildly acidic salt solution and allowing them to undergo fermentation by naturally occurring lactic acid bacteria (LAB). During fermentation, LAB consume fructose and glucose and produce primarily lactic acid and small amounts of carbon dioxide and acetic acid. A recent re-assessment of the LAB responsible for natural, commercial scale cucumber fermentation identified the following organisms as the primary LAB involved: *Lactobacillus pentosus (Lactiplantibacillus pentosus)*, *Lactobacillus plantarum (Lactiplantibacillus plantarum)*, *Lactobacillus brevis (Levilactobacillus brevis)*, *Weissella spp*., *Pediococcus ethanolidurans*, *Leuconostoc spp*., and *Lactococcus spp*. (Pérez-Díaz et al., 2017).

LAB are fastidious microorganisms with complex nutritional requirements. In addition to serving as a source of carbohydrates for energy production, cucumbers provide proteinaceous material for LAB survival, including free amino acids and proteins that can be enzymatically hydrolyzed to peptides and amino acids. Costilow and Fabian (1953) found that seven amino acids—cysteine (cys), glutamic acid (glu), isoleucine (ile), leucine (leu), threonine (thr), tryptophan (trp), and valine (val)—were essential for the growth of four *Lb. plantarum* strains isolated from commercial cucumber fermentations. They also determined that concentrations of cys, glu, ile, leu, and val were greater in fermented than acidified cucumbers after 24 hours of incubation, likely indicating proteolytic activity. Amino acid biosynthetic pathways in many lactobacilli are deficient; therefore, their genomes typically encode peptidases, amino acid permeases and transporters to recover and utilize amino acids from the environment (Klaenhammer et al., 2005). Amino acid catabolic pathways in LAB have been extensively reviewed, including the arginine deiminase pathway (ADI), glutamate decarboxylase (GAD) system, branched chain amino acid (BCAA) metabolism, and aromatic amino acid metabolism (Fernández & Zúñiga, 2006). Various amino acids involved in these pathways have been reported to provide added benefits for human health, including arginine (arg) and citrulline (cit) for nitric oxide metabolism and cardiovascular health (Schwedhelm et al., 2008), ornithine (orn) for ammonia metabolism and fatigue attenuation (Sugino et al., 2008), glutamine (gln) for gastrointestinal health (Klimberg & Souba, 1990), GABA for antihypertensive effects (Diana et al., 2014a), and leu, ile, and lysine (lys) for activation of muscle protein synthesis (Blomstrand et al., 2006) and soreness reduction (Rahimi et al., 2017). Therefore, the changes in free amino acids that occur during preservation of cucumber by lactic acid fermentation may have implications for health. Proteolysis has been demonstrated during cucumber fermentation by an increase in total peptides and generation of four bioactive di- and tripeptides (Fideler et al., 2019). Additionally, changes in gln content and conversion of free glu to γ-aminobutyric acid (GABA) during LAB fermentation of cucumber has been observed (Moore et al., 2021), demonstrating the need to further understand changes in free amino acids (FAA) during fermentation.

Much is known regarding the biochemical changes in organic acids and sugars during cucumber fermentation (Pérez-Díaz et al., 2013) and, as mentioned, recent publications have elucidated compositional changes related to peptides, gln, glu, and GABA in fresh, acidified, and fermented cucumbers. As of yet, comprehensive FAA profiles of these foods have not been reported. In this study, the FAA profile of fresh, acidified, and fermented cucumbers over time and under various brine salt concentrations was studied. These data will aid in further understanding the impact of commercial fermentation processes on cucumber composition as well as provide information on the nutritional implications for both humans and LAB.

2 | MATERIALS AND METHODS

2.1 | Chemicals and materials

Vinegar (acetic acid, 20%), pickling salt [sodium chloride (NaCl) ≥99%] and pickling cucumbers were provided by a local pickling company. LC-MS grade acetonitrile, calcium hydroxide [$Ca(OH)_2$, ≥95%], LC-MS grade water, and 10 kDa cutoff Amicon Ultra-0.5 filters were purchased from Thermo Fisher Scientific. Sodium benzoate (≥99%) and tyrosine (tyr, 98%) was purchased from Acros Organics (Waltham, MA, USA). Arginine (arg, ≥98%), aspartic acid (asp, 99%), isoleucine (ile, 99%), leucine (leu, 99%), lysine (lys, ≥98%), methionine (met, ≥98%), phenylalanine (phe, ≥98%), and proline (pro, 99%) were purchased from MP Biomedicals (Santa Ana, CA, USA). Aminoadipic acid (AAA, ≥ 98%), ammonium formate (≥99%), asparagine (asn, ≥98%), calcium chloride ($CaCl_2$, ≥93%), citrulline (cit, ≥98%), formic acid (≥98.5%), GABA (99%), glutamic

acid (glu, 99.5%), glutamine (gln, 98%) hydrochloric acid (HCl, ≥37%), lactic acid (≥85%), ornithine (orn, 99%), and sulfuric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). The stable isotope labeled internal standards 4-aminobutyric acid-2,2,3,3,4,4-d_6 (97 atom %), L-citrulline-4,4,5,5-d_4 (98 atom %), L-ornithine $^{13}C_5$ hydrochloride (99 atom %), and a universally labelled ^{13}C, ^{15}N mixture containing ala, arg, asn, asp, cys, glu, gln, gly, histidine (his), ile, leu, lys, met, phe, pro, ser, thr, trp, tyr, and val in water (98 atom %) were also purchased from Sigma-Aldrich.

2.2 | Experimental design

Three experiments were conducted to (1) identify differences in FAA between fresh, acidified, naturally fermented, and starter culture fermented cucumbers; (2) investigate changes in FAA over time; and 3) determine the influence of brining salt content on changes in FAA during cucumber fermentation. Pickling cucumbers (undesignated cultivar) were sourced from a commercial pickle producer. The initial experiment included one lot of cucumbers grown in the United States with each treatment independently prepared in triplicate (Figure S1). The time course experiment included two lots of cucumbers (sourced from Mexico and the United States) with each treatment independently prepared in duplicate for each lot at each sampling time point (Figure S2). For the brining salt study, three lots of cucumbers were obtained (sourced from Mexico and the United States), and treatments were independently replicated in duplicate for each lot (Figure S3). For all experiments, acidified cucumbers were prepared as a control to mimic the acid and salt content of fermented cucumbers, and fermentation was prevented by the addition of sodium benzoate. Fresh cucumbers were collected for analysis on the day of brining (day 0).

2.3 | Brining and fermentation of cucumbers

Acidified and fermented cucumbers were prepared as described by Fideler et al. (2019). Briefly, pickling cucumbers (size 2B, 3.5−3.8 cm diameter) were packed into 1.36 L (46 oz) glass jars, covered with brine (55:45 cucumber:brine ratio), and jars sealed with a lid fitted with a septum for aseptic brine sampling. Starter culture fermented cucumbers were brined and inoculated with 6.4×10^5 CFU/mL *Lb. pentosus* strain LA0445 (Food Science and Market Quality & Handling Research Unit Culture Collection; USDA-ARS, Raleigh, NC, USA), a culture originally isolated from commercial cucumber fermentations. Acidification and fermentation brines were formulated to achieve equilibrated concentrations in brined cucumbers of 0.684 M (4%) NaCl, 18 mM $Ca(OH)_2$, 12 mM $CaCl_2$, and 53 mM acetic acid. Lactic acid (110 mM, equilibrated) was added to acidified cucumbers to mimic fermented cucumber acid content along with sodium benzoate (8 mM, equilibrated) to inhibit fermentation. Brine pH was adjusted to 2.75 with HCl so that equilibrated pH would reach 3.25. Brined cucumbers were incubated for 6 weeks at 28°C with whole cucumbers sampled at the end of fermentation. For the time course experiment, fermented cucumber and acidified cucumber samples were prepared as described previously for naturally fermented and acidified treatments, except lids were not fitted with septa since whole jars were sampled at each time point. Brined cucumbers were incubated for 6 months (178 days) at 28°C and individual jars were sampled on days 1, 3, 7, 14, 21, 30, 48, 107, and 178. To study the effect of brining salt concentration, naturally fermented and acidified cucumbers were prepared with modified brine composition. Four brines were formulated to achieve equilibrated concentrations of 18 mM $Ca(OH)_2$, 12 mM $CaCl_2$, and 53 mM acetic acid and either 2%, 3%, 4%, or 6% NaCl. Jars were incubated for 27 days at 28°C. Brines were aseptically sampled at eight time points (days 1, 2, 3, 5, 7, 14, 20, and 27) and whole fermented cucumbers were analyzed at the end of fermentation (day 27).

2.4 | Preparation of cucumber slurries for analysis

On the indicated sampling days, whole cucumbers were separated from the brine and cellular debris, sectioned into 1-inch pieces, and blended with a Waring Model CB15V blender (Waring Commercial, Torrington, CT, USA). Cucumber pieces were blended for 1 min at speed 3 and 2 min at speed 6 to form a slurry. Slurries were stored immediately at −80°C until analysis. Fresh cucumbers were similarly prepared and stored on day 0. Cucumber slurries were used for analysis of organic acids, sugars, and amino acids.

2.5 | Fermentation biochemistry

Lactic acid, acetic acid, glucose, and fructose were quantified using a Shimadzu UFLC (Shimadzu Corporation, Kyoto, Japan) per McFeeters and Barish (2003) with minor modifications. Cucumber slurries were thawed, vortexed, and centrifuged at 12,000 × *g* for 10 min. Supernatants were injected onto an Aminex HPX-87H resin column

(300 × 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA) with temperature set to 60°C. Separation was performed with 0.01 N sulfuric acid mobile phase at 0.9 ml/min. A SPD-20A UV-visible light detector (Shimadzu Corporation) at 210 nm was used to detect acids and an RID-10A refractive index detector (Shimadzu Corporation) detected sugars. Detectors were connected in series. External standards were prepared to generate eight-point calibration curves for all analytes (0.5–100 mM).

2.6 | Amino acid quantification by LC-QQQ-MS

Samples were prepared for amino acid quantification per Moore et al. (2021). In summary, slurries were thawed and cryogenically ground to rupture intact plant cells and release amino acids using a Retsch mixer mill MM301 (Cole-Parmer, Vernon Hills, IL, USA). Fresh cucumber slurries were prepared in duplicate for each lot. Ground cucumber slurries were mixed 55:45 with mock brine to achieve the same acid and salt content as the acidified cucumber to control for potential matrix effects. Slurry (2.5 g) was added to 25 ml stainless steel capsules with three 9 mm stainless steel beads. Capsules were immersed in liquid nitrogen for 30 s prior to oscillation at 30 Hz for 4 min. Slurries were centrifuged at 11,000 × *g* for 5 min and supernatant filtered with 10 kDa centrifugal cutoff filters at 11,000 × *g* for 10 min. Filtrate (135 µl), internal standard mixture (15 µl), and mobile phase A (850 µl) were added to LC-MS vials for analysis. Modifications were made for the time course and brining salt experiments to optimize sample preparation. After cryogenic grinding, slurries were centrifuged at 12,000 × *g* for 5 min at 4°C. Supernatant (90 µl) was added to mobile phase A (565 µl) and stored for 1 h at 4°C to precipitate solvent-insoluble material. Samples were centrifuged at 12,000 × *g* for 8 min at 4°C. Supernatants were filtered using 10 kDa centrifugal cutoff filters at 14,000 × *g* for 10 min at 4°C. Filtrate (323 µl) and isotopically labeled internal standard mixture (10 µl) were transferred to LC-MS vials and samples analyzed within 36 h. Samples for all experiments were stored at 4°C in a refrigerator or autosampler tray until analysis.

Amino acid profiles were generated using a Shimadzu LCMS-8040 triple quadrupole LC-MS/MS (Shimadzu Corporation). HPLC separation was carried out as described by Qiu et al. (2020). Briefly, amino acids were separated with an Atlantis HILIC column (100 × 4.6 mm, 3.0 µm; Waters Corporation, Milford, MA, USA) at 37°C with a segmented gradient of mobile phase A (85% acetonitrile, 10 mM ammonium formate, 0.15% formic acid) and mobile phase B (10 mM ammonium formate with 0.15% formic acid). Flow was diverted to waste for the first 2 min to reduce salt accumulation on the ion source. Multiple reaction monitoring in positive ion mode was used for MS detection of all amino acids. Drying and nebulizing gases were both nitrogen with flow rates of 15 l/min and 3 l/min, respectively. The heat block was set to 400°C. Loop time was 1 s and first and third quadrupole resolutions were set to 0.7 and 1.0 Da, respectively. Transitions, reference ions, collision energies, and calibration curve ranges were optimized for each experiment (Tables 1 and 2). Ala was detected in fresh, acidified, and fermented cucumbers but quantities were not reported due to poor standard curves ($r^2 < 0.97$). Asp, thr, and trp were present at levels below quantification limits.

Eight-point standard curves were prepared using mixtures of amino acids. All amino acids except for asn and gln were prepared individually in 0.1 N HCl, mixed, then stored frozen (−19°C). Asn and gln were prepared in LC-MS grade water to prevent degradation and stored frozen (−19°C). Quantification standards were prepared from the concentrated stock solutions by combining internal standards, amino acid mixtures, asn, gln, and mobile phase A to a final volume of 1 ml in LC-MS vials and were used within 48 h of preparation. LabSolutions software ver. 5.8 (Shimadzu) was used for analysis and quantification by peak height. The analysis method was unable to detect cysteine, serine, and valine.

2.7 | Statistical analysis

Analysis of variance (ANOVA) with post-hoc Tukey tests ($\alpha = 0.05$) were performed using JMP Pro v. 15 (SAS Institute Inc., Cary, NC, USA). ANOVA was run to determine differences among treatments and differences between lots of fresh cucumbers.

3 | RESULTS AND DISCUSSION

3.1 | Fermentation biochemistry

Fermentation progressed normally in both natural and starter culture fermented cucumbers as indicated by decreases in sugars to <2 mM each of glucose and fructose and an increase in lactic acid to >100 mM. Between days 2 and 5, cucumbers fermented in 2% NaCl had significantly higher lactic acid and lower sugars than cucumbers fermented in 6% NaCl (Figure 1). No significant differences were observed in the lactic acid or sugar content of cucumber fermentations at varying salt levels between days 7 and 27 (Figure 1). Lactic acid increased slightly after day 7, reaching maximum lactic acid content by day 14. These

TABLE 1 Free amino acid composition of fresh, acidified, and fermented cucumbers

Amino acid	Transition (m/z)[a] $[M+H]^+$	Concentration (mg/kg)[b] Fresh	Acidified	Fermented	Starter culture fermented	Standard curve range (μM)
Aminoadipic Acid	161.90 > 98.80	33.7 ± 2.0a	9.2 ± 1.5b	9.9 ± 1.0b	8.8 ± 0.6b	0.15 – 45.0
Arginine	175.00 > 70.00	114.6 ± 4.7ab	71.8 ± 2.8bc	54.8 ± 42.8c	131.3 ± 10.7a	1.15 – 195
Asparagine	133.00 > 74.05	113.0 ± 6.4a	57.6 ± 4.3c	94.6 ± 9.4b	91.6 ± 4.0b	19 – 1173
Aspartate	134.25 > 88.05	BQL	BQL	BQL	BQL	0.4 – 23.0
Citrulline	176.00 > 70.05	110.3 ± 8.5a	61.1 ± 8.9b	69.5 ± 6.0b	104.8 ± 27.8a	0.71 – 68.5
GABA	104.00 > 87.10	269.6 ± 21.4a	92.9 ± 9.5c	181.3 ± 21.5b	166.8 ± 18.6b	0.40 – 339
Glutamate	147.95 > 84.05	67.4 ± 3.4a	7.2 ± 1.7c	33.2 ± 24.4b	15.6 ± 9.2bc	0.85 – 102
Glutamine	146.90 > 84.05	1491.4 ± 69.3a	66.3 ± 7.3b	106.6 ± 17.2b	104.1 ± 21.0b	0.86 – 205
Histidine	155.90 > 110.0	28.8 ± 0.4b	19.6 ± 2.2b	36.7 ± 4.7a	34.6 ± 4.7ab	0.61 – 96.7
Isoleucine	131.80 > 69.15	38.9 ± 2.6b	60.3 ± 5.1b	165.2 ± 11.2a	151.7 ± 22.6a	1.91 – 183
Leucine	132.00 > 86.15	38.9 ± 4.1c	44.8 ± 6.1c	129.8 ± 10.9a	112.4 ± 7.9b	2.44 – 228
Lysine	147.00 > 84.10	28.7 ± 0.6c	33.8 ± 4.3c	110.9 ± 5.0a	94.8 ± 6.0b	1.71 – 205
Methionine	149.95 > 56.05	24.4 ± 0.1 b	18.8 ± 1.9b	37.9 ± 3.5a	36.3 ± 6.2a	0.70 – 84.0
Ornithine	132.95 > 70.10	1.9 ± 0.1b	0.6 ± 0.2b	63.3 ± 31.5a	3.0 ± 0.7b	0.03 – 90.8
Phenylalanine	165.95 > 120.0	29.8 ± 2.8b	30.4 ± 3.5b	65.2 ± 5.5a	64.2 ± 7.2a	0.67 – 109
Proline	115.95 > 70.05	17.9 ± 0.4c	13.4 ± 1.0c	51.2 ± 3.5a	38.3 ± 2.0b	0.17 – 130
Tyrosine	182.00 > 136.05	33.0 ± 4.5b	47.4 ± 4.2b	92.6 ± 26.8a	91.7 ± 15.7a	0.90 – 69.0
Tryptophan	204.90 > 146.05	BQL	BQL	BQL	BQL	0.20 – 5.00
Total		2442 ± 39a	635 ± 35c	1302 ± 102b	1250 ± 108b	

[a]Mass-to-charge ratio of the optimized precursor and product ions using electrospray ionization in positive ion mode for quantification of each amino acid.
[b]Inline letters within a row indicate significant differences between treatments ($P < 0.05$) using a one-way analysis of variance with post-hoc Tukey HSD.

TABLE 2 Free amino acid composition of fermented cucumbers at varying brine salt levels

Amino acid	Transition (m/z)[a] $[M+H]^+$	Concentration (mg/kg)[b] 2% NaCl	3% NaCl	4% NaCl	6% NaCl	Standard curve range (μM)
Arginine	175.10 > 70.10	6.3 ± 1.5b	9.6 ± 2.9b	49.9 ± 21.4a	129.8 ± 65.7a	0.50 – 500
Asparagine	133.00 > 74.05	96.6 ± 53.1a	76.9 ± 76.0a	42.8 ± 20.3a	86.3 ± 95.4a	18.9 – 1174
Aspartate	133.45 > 88.05	8.1 ± 4.2b	10.0 ± 2.7b	10.7 ± 3.9b	20.1 ± 8.2a	0.40 – 25
Citrulline	176.00 > 70.05	78.6 ± 41.5a	81.1 ± 54.2a	166.3 ± 97.8a	146.4 ± 108.0a	0.50 – 500
GABA	104.00 > 87.10	170.68 ± 38.3a	125.8 ± 17.9b	90.4 ± 16.2b	107.4 ± 27.7b	0.40 – 350
Glutamate	148.25 > 83.95	22.9 ± 12.0a	18.2 ± 7.5a	12.0 ± 6.9a	21.6 ± 9.2a	0.50 – 60
Glutamine	147.20 > 84.05	140.3 ± 104.7a	110.8 ± 54.2a	102.6 ± 60.8a	121.1 ± 97.5a	1.5 – 1450
Histidine	156.00 > 110.0	49.1 ± 34.2a	38.1 ± 22.6a	41.1 ± 15.6a	48.6 ± 17.1a	1.5 – 200
Isoleucine	132.10 > 69.05	85.8 ± 17.4ab	102.2 ± 33.3a	50.7 ± 15.4b	92.8 ± 24.5a	1.5 – 200
Leucine	132.10 > 86.15	111.2 ± 26.3a	110.6 ± 25.8a	87.5 ± 27.4a	110.0 ± 37.9a	2.0 – 230
Lysine	147.00 > 84.00	128.0 ± 39.0a	92.1 ± 21.8a	90.0 ± 31.8a	120.2 ± 38.2a	1.5 – 200
Methionine	149.95 > 56.05	29.4 ± 8.9a	24.9 ± 5.9a	17.5 ± 5.5a	22.4 ± 11.0a	0.60 – 95
Ornithine	133.05 > 70.10	19.6 ± 7.3b	31.8 ± 17.5ab	64.9 ± 42.3a	62.3 ± 17.0a	0.50 – 500
Phenylalanine	166.05 > 120.10	59.8 ± 11.5a	53.3 ± 7.9a	47.3 ± 19.4a	57.8 ± 17.9a	0.60 – 110
Proline	116.00 > 70.15	64.3 ± 16.3a	40.7 ± 12.4b	32.9 ± 8.4b	39.2 ± 10.1b	0.20 – 140
Tyrosine	182.00 > 136.05	10.7 ± 8.9b	12.9 ± 10.3b	25.2 ± 14.1ab	35.0 ± 4.8a	0.80 – 50
Tryptophan	204.90 > 146.05	BQL	BQL	BQL	BQL	0.20 – 5.0
Total:		1081 ± 315a	939 ± 242a	932 ± 169a	1221 ± 409a	

[a]Mass-to-charge ratio of the optimized precursor and product ions using electrospray ionization in positive ion mode for quantification of each amino acid.
[b]Inline letters within a row indicate significant differences between treatments ($P < 0.05$) using a one-way analysis of variance with post-hoc Tukey HSD.

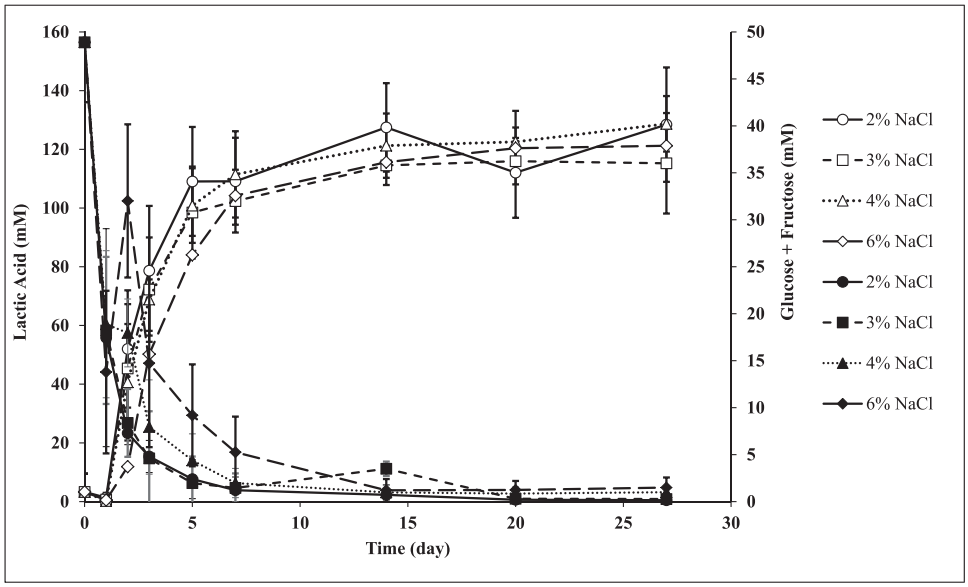

FIGURE 1 Changes in sugars and lactic acid over time of cucumbers fermented in 2%, 3%, 4%, and 6% NaCl brines. Open markers represent lactic acid, closed markers represent glucose + fructose

data demonstrate that, initially, fermentation progressed more rapidly in cucumbers fermented in 2% NaCl than 6% NaCl; however, by day 7 lactic acid production in the 6% NaCl cucumbers approached that of the 2% NaCl cucumbers. Salt considerably impacts which microorganisms are selected during natural LAB fermentations, thereby influencing metabolic products and their rate of formation during fermentation (McMurtrie et al., 2019; Pérez-Díaz et al., 2013). The higher amount of salt in the 6% NaCl brined cucumbers delayed the metabolic activity of the LAB, resulting in a slower conversion of sugars to lactic acid.

3.2 | Changes in free amino acids during cucumber fermentation

Significant changes were observed in the FAA profiles of cucumbers undergoing acidification and fermentation. These changes can be explained by three main factors: biological variation of cucumbers and native microbial population, diffusion of water-soluble components into the packing brine, and microbial metabolism.

The sum of FAA quantified in this study was 2,442 ± 39 mg/kg in fresh cucumbers (Table 1). FAA content of naturally fermented (1,302 ± 102 mg/kg) and starter culture fermented (1,250 ± 108 mg/kg) cucumbers were not significantly different, and both were two-fold greater than that of acidified cucumbers (635 ± 35 mg/kg; Table 1). The most abundant free amino acid in fresh cucumbers was gln (1,491.4 ± 69.3 mg/kg) followed by GABA (269.6 ± 21.4 mg/kg), asn (113.0 ± 6.4 mg/kg), and cit (110.3 ± 8.5 mg/kg; Table 1). Greater levels of AAA (33.7 ± 2.0 mg/kg), arg (114.6 ± 4.7 mg/kg), asn (113.0 ± 6.4 mg/kg), cit (110.3 ± 6.4 mg/kg), GABA (269.6 ± 21.4 mg/kg), and glu (67.4 ± 3.4 mg/kg) were observed in fresh cucumbers than in acidified or fermented cucumbers. Levels of arg, asn, and cit in acidified cucumber were consistent with dilution due to the 55:45 cucumber:brine pack ratio (Table 1; Figure 2a–c). Gln was significantly lower in all treatments for all experiments compared with fresh cucumbers. This change was consistent with that of acidified and fermented cucumbers reported by Moore et al. (2021) and was attributed to either degradation to pyroglutamic acid (pGlu) due to acidity and/or potential conversion to glu, and/or other metabolites by LAB. The most abundant free amino acids in fermented cucumber were GABA (181.3 ± 21.5 mg/kg), ile (165.2 ± 11.2 mg/kg), leu (129.8 ± 10.9 mg/kg), and lys (110.9 ± 5.0 mg/kg; Table 1). The total GABA content is a function of the GABA from the fresh cucumber plus that formed from the conversion of glutamate to GABA by lactic acid fermentation after accounting for the dilution of this water-soluble molecule that occurs during pickling processes (Moore et al., 2021). On the other hand, the LAB that are prevalent in cucumber fermentation are auxotrophic for several amino acids, including ile, leu, and lys (Pérez-Díaz et al., 2017). Therefore, the observed increases in certain free amino acids (Figure 2e, f, h, i) are likely derived from metabolism of cucumber protein during lactic acid fermentation. LAB possess both endo- and exopeptidases which act upon proteins and oligopeptides to generate peptides and release terminal amino acids (Christensen et al., 1999). Increases in FAA observed in this study indicate the release of amino acids from proteins and peptides

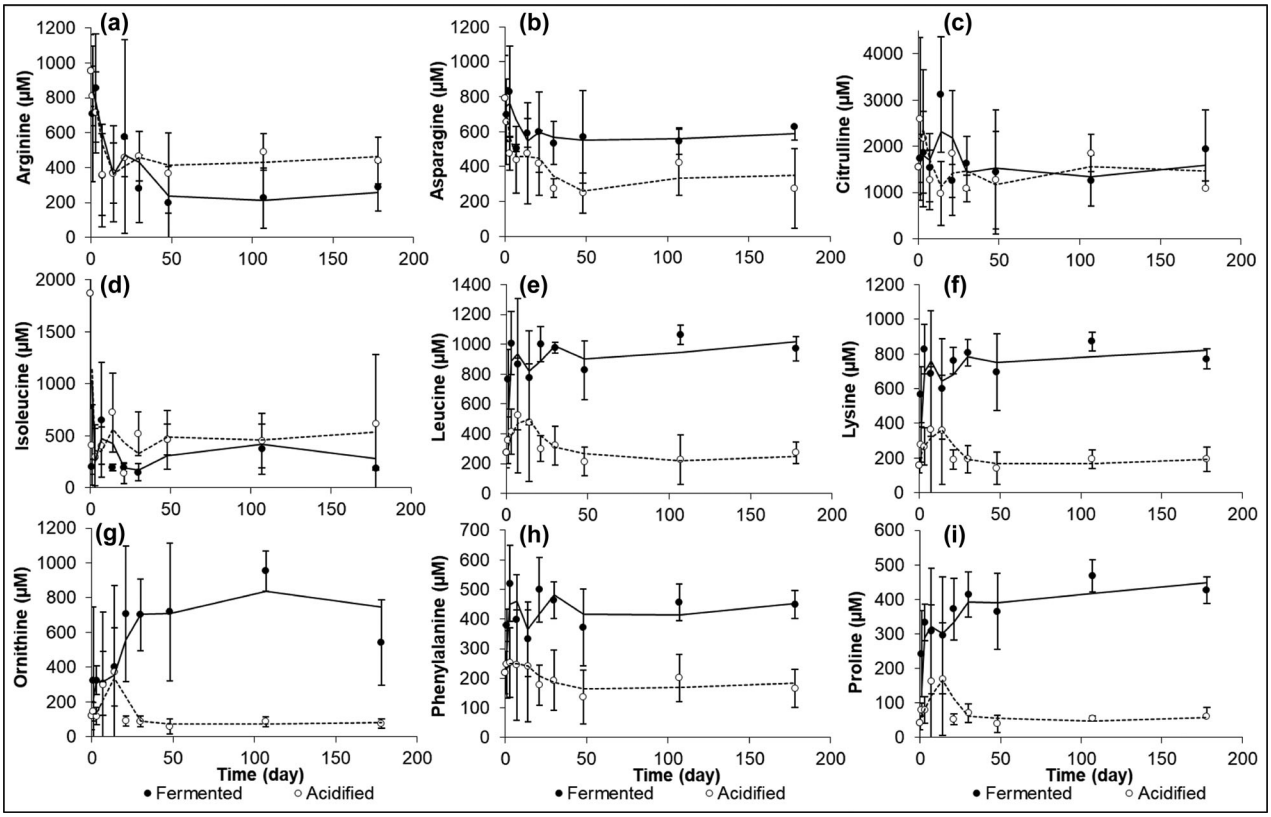

FIGURE 2 Amino acid content in fermented and acidified cucumbers over time

and is consistent with the findings of Fideler et al. (2019) that showed an increase in total peptides in fermented cucumber. Specifically, bioactive di- and tripeptides (arg-tyr, ile-pro-pro, leu-pro-pro, and lys-pro) containing some of the amino acids quantified in this study were also found in higher levels in fermented than acidified cucumbers. Further degradation of these peptides by aminopeptidases of lactic acid bacteria (Savijoki et al., 2006) would result in the increases in the constituent amino acids as observed in this study.

Six of the nine essential amino acids for human nutrition were quantified in this study: his, ile, leu, lys, met, and phe. Since humans cannot produce these amino acids, they must consume them from food sources. Upon consumption, food proteins undergo enzymatic hydrolysis by digestive enzymes to release their constituent amino acids. Alternatively, breakdown of food proteins prior to consumption increases digestibility of the food and results in quicker absorption of small peptides and amino acids in the gastrointestinal tract (Foltz et al., 2007; Terefe & Augustin, 2019). In the analysis of the *Lb. plantarum* WCFS1 genome, Kleerebezem et al. (2003) noted that while *Lb. plantarum* possesses many genes for amino acid synthesis it lacks the ability to produce the BCAA, leu, ile, and val, requiring it to obtain them through the uptake of FAA or the breakdown of environmental proteins such as those found in cucumber. This is also true of many of the prevailing LAB in cucumber fermentation (Pérez-Díaz et al., 2017). As a result, the availability of essential amino acids from cucumbers may increase due to LAB proteolytic activities. Higher levels of these six essential amino acids were found in fermented cucumbers compared to acidified cucumbers (Table 1). Similarly, slightly greater concentrations of leu and phe were produced in olive brines fermented with *L. mesenteroides* Lm51 and *L. pentosus* CECT5138, respectively (Montaño et al., 2000).

Biological variability impacts the FAA composition of cucumbers due to multiple factors including cultivation method (i.e., in soil vs. trellis grown), soil chemistry, and climate. Eppendorfer and Bille (1996) grew cauliflower, green beans, kale, potatoes, and spinach in soils with varying nutrient levels and found that the sums of FAA analyzed varied widely between treatments. Soils with increasing total nitrogen content correlated with higher FAA values. In the time course and brining salt studies, multiple cucumber lots were used and sourced from different farms in different states or countries to account for biological variation. We found that cucumber lot was a significant factor ($P < 0.05$) in fresh cucumber content of GABA, gln, glu, lys, orn, phe, and pro. Costilow and Fabian (1953) found that pickling cucumber size was inversely correlated to free amino acid concentration.

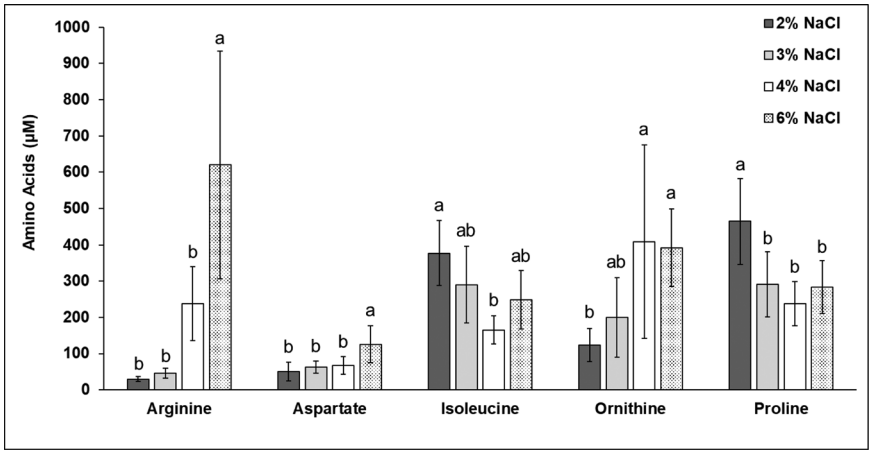

FIGURE 3 Differences in free amino acid content of fermented cucumber brined in varying salt concentrations. Letters indicate significant differences between treatments ($P < 0.05$) using a one-way analysis of variance with post-hoc Tukey HSD test

Although these authors focused on three grading classes of cucumbers (nos. 1, 2, 3), size variation within a grading class may also contribute to differences in amino acid concentration between individual cucumbers.

3.3 | Effect of starter culture on amino acid profile of fermented cucumbers

Higher levels of asp, GABA, ile, leu, lys, met, orn, phe, pro, and tyr were observed in natural and starter culture fermented cucumbers than acidified cucumbers (Table 1). Despite the 45% dilution factor due to packing in brine, significantly greater levels of ile, leu, lys, met, phe, pro, and tyr were observed in natural and starter culture fermented cucumbers than in fresh cucumbers. Most notably, orn levels in naturally fermented cucumbers (63.3 ± 31.5 mg/kg) were significantly higher than in starter culture fermented cucumbers (3.0 ± 0.7). It has been shown that starter cultures introduced to cucumber fermentations can outcompete the natural microbiota to dominate the fermentation (Breidt & Fleming, 1992). The large difference in orn observed indicates that the starter culture utilized in the study did not possess genes for the ADI pathway and outcompeted the natural microbiota that were responsible for orn production in the natural fermentation.

3.4 | Effect of brine salt concentration on amino acid composition

Average total FAA in cucumbers fermented with different salt concentrations were not significantly different and were similar to the total FAA found in naturally fermented and starter culture fermented cucumbers, ranging from 932 to 1221 mg/kg (Table 2). However, individual levels of asp, GABA, ile, orn, pro, and tyr were significantly different between the four brining salt treatments (Table 2; Figure 3), suggesting differences in proteolytic activity and utilization of amino acid dependent acid resistance systems. The effect of salt on FAA changes in fermented cucumbers differed from that reported in Spanish artisanal cheeses. Diana et al. (2014b) correlated higher salt levels (2% NaCl vs. 0.5% NaCl) in cheese with lower average FAA content due to the salt's inhibitory effect on microbial cultures. However, our study found no significant differences in FAA content across a range of salt levels. As noted in our previous work, higher levels of GABA were found in the 2% NaCl than the 6% NaCl fermentation and GABA was formed within the first 24–48 hours of fermentation (Moore et al., 2021). Fermentation biochemistry data show that 2% NaCl fermentations contained higher lactic acid than the 6% NaCl fermentations on day 2, demonstrating that fermentation progressed more quickly in 2% NaCl fermentation (Figure 1). The initial increased fermentation rate of the 2% NaCl treatment corresponds with the 48-hour window in which GABA is formed during cucumber fermentations, suggesting that higher brine salt levels inhibit not only conversion of sugars to lactic acid but also GAD expression in LAB.

Examination of the substrates and products of the ADI pathway shows that the 6% NaCl fermented cucumbers contained both greater arg and orn than the 2% NaCl fermented cucumbers, suggesting that although arg is being utilized to generate orn as an acid resistance mechanism, it is also being released from proteins. Orn levels for the 4% and 6% NaCl brine treatments were similar (Table 2), suggesting that above 3% NaCl, the ADI pathway or the organisms producing arginine deiminase are similarly active. The 2% and 3% NaCl treatments had lower levels of arg and orn (Figure 3), suggesting less proteolytic release of arg and less conversion of arg to orn. Examination of the microbial populations of fermented cucumbers in varying salt brine levels could help identify which organisms are responsible for these differences and could reveal why the GAD or ADI pathways are activated under such conditions.

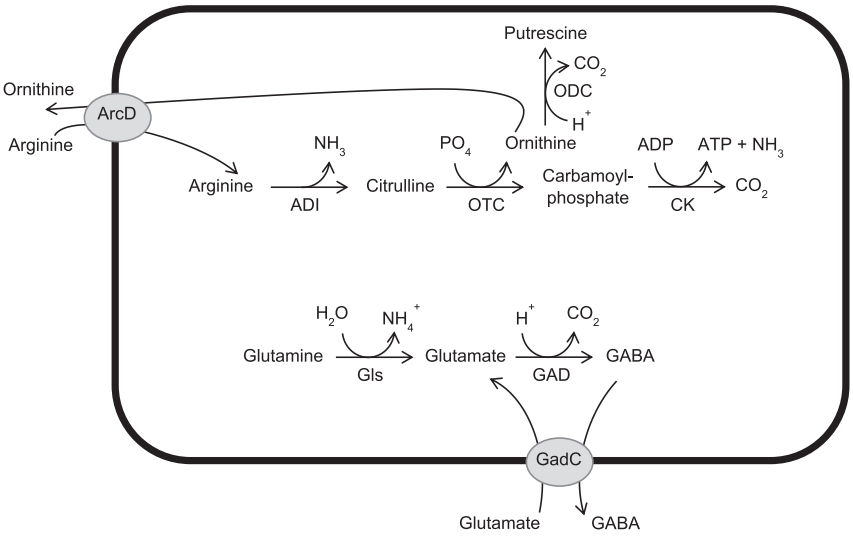

FIGURE 4 Schematic representation of glutamate decarboxylase and arginine deiminase pathways in lactic acid bacteria. ADI, arginine deiminase; ArcD, arginine/ornithine antiporter; CK, carbamate kinase; Gls, glutaminase; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; GadC, glutamate-GABA antiporter; ODC, ornithine decarboxylase; OTC, ornithine carbamoyl-transferase

3.5 | Formation of ornithine during cucumber fermentation

In addition to proteolytic activity, LAB metabolic pathways also contribute to compositional changes in amino acids. This study and previous work by our group demonstrated formation of GABA from glu by glutamate decarboxylase during cucumber fermentation (Table 1; Moore et al., 2021). GABA formation from glutamate is one of multiple amino acid-dependent acid-resistant mechanisms active in LAB as a means of protection against decreases in intracellular pH (Fernández & Zúñiga, 2006). Likewise, the formation of orn from arg also contributes to acid resistance. In this pathway, known as the arginine deiminase pathway (ADI), arg is deiminated to form cit and ammonia, and cit is decarboxylated to form orn and carbamoyl-phosphate (Figure 4). The ADI pathway is commonly distributed among LAB and produces 1 mole of ATP for every mole of arg catabolized, in addition to its function in pH regulation (Fernández & Zúñiga, 2006). In our experiments, we found that orn was present at low amounts in fresh (1.9 ± 0.1 mg/kg), acidified (0.6 ± 0.2 mg/kg), and starter culture fermented (3.0 ± 0.7 mg/kg) cucumbers and at a much greater level in naturally fermented cucumbers (63.3 ± 31.5 mg/kg; Table 1). Naturally fermented cucumbers also had lower levels of arg (54.8 ± 42.8 mg/kg) and cit (69.5 ± 6.0 mg/kg) compared to starter culture fermented cucumbers (arg, 131.3 ± 10.7 mg/kg; cit, 104.8 ± 27.8 mg/kg), which is consistent with the orn formation. When observed in cucumber fermentations over time, orn is nearly absent at day zero and increases through the end of fermentation with the greatest increase between days 0 and 21 (Figure 2g), which coincides with lactic acid formation and decrease in pH during typical cucumber fermentations (Figure 5).

According to the ADI pathway, as orn is formed, free arg is expected to decrease. Cit, however, is an intermediate in the conversion of arg to orn and its concentration may remain stable due to simultaneous deamidation of arg by ADI and decarboxylation of cit by OTC, or it may decrease if the reaction shifts to the right and access to free arg is limited (Figure 4). Cit was present at 110.3 ± 8.5 mg/kg in fresh cucumbers and decreased in naturally fermented cucumbers but levels remained stable in starter culture fermented cucumbers (Table 1). This decrease in cit and arg in the naturally fermented cucumbers coincides with the development of orn. Likewise, the unchanging cit and arg levels in starter culture fermented cucumbers corresponds with a lack of orn formation (Table 1).

Similar levels of orn were found in cucumber fermented in 4% NaCl (64.9 ± 42.3 mg/kg) and 6% NaCl (62.3 ± 17.0 mg/kg) brines, with lower levels in the 2% NaCl (19.6 ± 7.3 mg/kg) and 3% NaCl (31.8 ± 17.5 mg/kg) treatments (Table 2). There were also higher levels of arg in the 4% and 6% NaCl treatments compared with the 2% and 3% NaCl, yet no significant differences in cit levels, indicating that arg may have been liberated from proteins in order to fuel the ADI pathway and maintain a constant level of cit for decarboxylation to orn. In contrast, we found that significantly greater GABA was produced in the 2% NaCl fermentations (170.68 ± 38.3 mg/kg) than any other treatment (Table 2). Therefore, pH cannot be the only determining factor in the production of GABA or orn. The addition of salt to fermentation brines aids in the selection of particular LAB species (Jay et al., 2005; Pérez-Díaz et al., 2013). Although both the ADI and GAD systems are widely distributed among LAB (Fernández & Zúñiga, 2006; Wu et al., 2017), individual species and strains possess differing levels of activity for these two acid resistance systems. Therefore, selection of bacteria by different salt

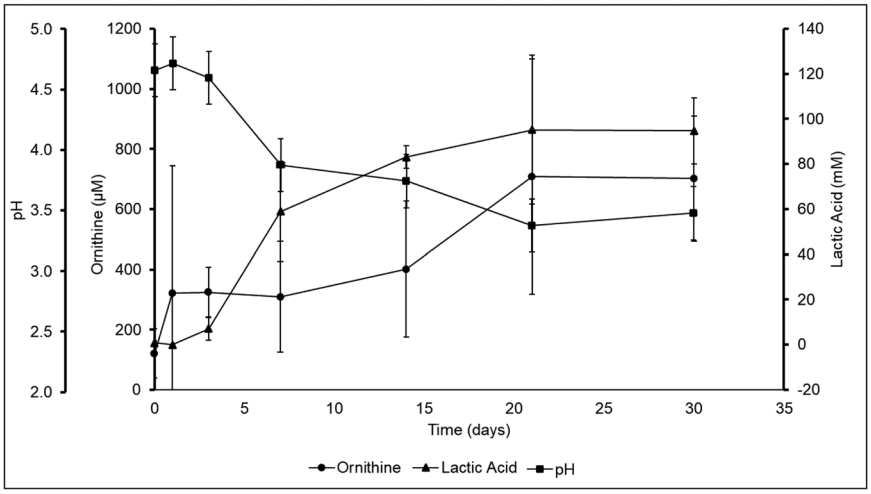

FIGURE 5 pH and formation of ornithine and lactic acid over time in naturally fermented cucumbers

concentrations may contribute to varying production of GABA and orn. These data suggest that the organisms responsible for orn production either proliferate more efficiently or have greater expression of ADI pathway enzymes in higher salt environments.

Orn values remained stable in fermented cucumbers up to 3 months of storage. There was a significant decrease between 3 and 6 months, suggesting that longer storage times may result in further FAA changes (Figure 2g). Diana et al. (2014b) found that semi-ripened (≤1 month) cheeses had greater orn levels than fresh and ripened (≥3 months) cheeses suggesting that orn peaked and then underwent degradation. Arginase has the capability of interconverting between orn and arg, and ornithine decarboxylase converts ornithine to putrescine. No increase in arg was observed in the stored fermented cucumbers (Figure 2a); therefore, orn may have been converted to other compounds such as carbamoyl-phosphate (Figure 4) and/or further metabolized to the polyamine putrescine.

3.6 | Health beneficial amino acids

Certain amino acids provide health benefits beyond the fulfillment of nutritional requirements and the availability as FAA in foods has prompted much investigation. Arginine, citrulline, and ornithine, present in pickling cucumber or its fermented product, are among those of interest. The conditionally essential amino acid arg is required by mammalian infants and by adult humans with specific health complications, including renal failure, trauma, small-bowel resection, and severe burns (Flynn et al., 2002). For healthy children and adults, arg plays a major role as a component of the nitric oxide (NO) production pathway (Romero et al., 2006), contributing to vasodilation of arterial and venous blood vessels (Schwedhelm et al., 2008). Kaore and Kaore (2016) proposed that increasing arg availability in foods may serve as a therapeutic benefit to hypertensive patients. However, oral delivery of pure arg was only marginally effective in increasing NO production due to its degradation by cytosolic and mitochondrial arginases (EC 3.5.3.1) in the gut wall and liver (Flynn et al., 2002; Schwedhelm et al., 2008; Shearer et al., 1997). In contrast, cit is not degraded by these enzymes and serves as a precursor for arg formation in human endothelial cells (Waugh et al., 2001). In fact, oral supplementation of pure cit in humans increased plasma arg concentration and NO-dependent signaling in proportion to its dosage (Schwedhelm et al., 2008).

Plants from the Cucurbitaceae family including melons, squash, pumpkins and cucumbers are natural sources of cit. Watermelon is the most abundant source of citrulline among cucurbits and therefore has been studied most extensively (Fish, 2012). Collins et al. (2007) found that long term (3 week) feeding of watermelon juice (1,280 mg/kg cit) delivering 1 or 2 g cit per serving resulted in increased fasting arg concentration of 12% and 18%, respectively. In contrast, fasting cit did not increase compared to the control, suggesting that plant derived cit was converted to arg and consumption from watermelon may serve as a means to boost plasma arg. In our study, we found that whole fresh pickling cucumber contained 110.3 ± 8.5 mg/kg cit (Table 1), which is similar to the 112.4 mg/kg cit reported in fresh market cucumbers (Fish, 2012). Cucumbers contain roughly 10-fold less cit than watermelon and are comprised of approximately 97% water. Lyophilization or other drying technologies would serve as appropriate methods for concentrating amino acids resulting in about a 33-fold increase. Indeed, this technology has already been employed for watermelon to administer 3 g cit/day to postmenopausal women, resulting in a significant reduction in aortic systolic blood pressure and arterial stiffness (Figueroa et al., 2013).

Like cit, orn is a nonessential amino acid and is not synthesized by humans; however, it has been linked to sports-related health benefits such as stimulating human growth hormone (Demura et al., 2010) and promoting tissue protein synthesis (Tujioka et al., 2012). Sugino et al. (2008) found that oral administration of 2 g orn/day in female human subjects significantly improved fatigue after physical performance tests through the promotion of protein and lipid catabolism. However, a recent critical review of supplements concluded that existing studies on orn do not provide sufficient evidence for its safety at the levels in which it was administered (4–12 g/day) nor did they assess short- or long-term effects on muscle mass (Valenzuela et al., 2019). These studies provided orn to subjects in pure form rather than in a food matrix in which it was naturally produced and the effect of consuming foods with enhanced orn has yet to be investigated. We found that while fresh pickling cucumbers contained only 1.9 ± 0.1 mg/kg orn, natural fermentation enhanced this concentration 10- to 34-fold (19.6–64.9 mg/kg; Table 2). As with cit, concentration of naturally occurring orn through moisture reduction technologies might be necessary to achieve a therapeutic dosage of orn from fermented cucumbers.

3.7 | Taste-active amino acids

FAA, peptides and amino acid derivatives significantly impact taste and consumer liking in fermented foods (Zhao et al., 2016). Innovation using LAB starter cultures that produce savory, umami- or salt-potentiating amino acids may serve as a means for reducing sodium while maintaining consumer acceptability. In the case of fermented cucumber pickles, chloride reduction in wastewater has been achieved by replacing high levels of NaCl (1.03 M) with low levels of $CaCl_2$ (0.1 M) and inoculating with *Lb. plantarum* (Pérez-Díaz et al., 2015). Despite a successful fermentation without NaCl, pickle processing involves re-packing fermented cucumbers in fresh cover brine to prepare finished products with approximately 2% NaCl to meet consumer expectations (Wilson et al., 2015). While development of reduced sodium cover brines is feasible, consumer liking is essential for commercialization of these new products. Zhao et al. (2015) demonstrated that sourdough breads produced with 1% NaCl and either GABA- or glu-producing *Lb. reuteri* starter cultures were equivalent in saltiness to bread produced with 1.5% NaCl, showing that accumulation of glu or GABA allowed for NaCl reduction without compromising taste. Tang et al. (2018) demonstrated significant differences in the amino acid profiles of aseptic model sausage fermentations inoculated with four different LAB strains in both pure and co-culture fermentations. Specifically, a 5- to 7-fold increase in glu was observed in fermented sausage (1,400—1,800 mg/kg) compared with the control (240 mg/kg). Although these glu values are far above that of fresh (67.4 ± 3.4 mg/kg) and fermented cucumber (33.2 ± 24.4 mg/kg; Table 1), other studies have shown that low levels of certain amino acids enhance saltiness perception when combined with NaCl. Lioe et al. (2005) found that adding either 0.5 mM phe or 1.5 mM tyr to a mixture of 80 mM NaCl and 4.0 mM MSG significantly enhanced umami perception. Both phe and tyr were added at concentrations below their sensory thresholds, indicating that a synergistic effect is observed when combined with other umami and salty tastants. Fresh cucumbers contained 0.06 mM phe and 0.21 mM tyr whereas these amino acids were increased in fermented cucumbers to 0.37 mM phe and 0.43 mM tyr. Although these levels remain below threshold values, these amino acids may contribute to overall flavor perception. Understanding the composition of individual FAA and how to manipulate their concentration can aid in the development of novel fermented foods with optimal sensory characteristics and warrants further research.

4 | CONCLUSION

Free amino acid profiles of fresh, acidified, naturally fermented and starter culture fermented cucumbers were compared, revealing that fermentation significantly increased the concentration of several amino acids in cucumbers compared to direct acidification. Additionally, brine salt level and the use of a starter culture both affected free amino acid composition. Specifically, ornithine content was limited when a starter culture was used or cucumbers were naturally fermented in low salt brines, while more GABA was produced in low salt fermentations. Understanding the amino acid profile of fermented cucumbers and how to manipulate it has value for processing innovation and creation of new products that benefit consumer health and meet consumer preferences.

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AUTHOR CONTRIBUTIONS

Jennifer Fideler Moore: Data curation; formal analysis; investigation; methodology; writing – original draft; writing – review & editing. Rachel DuVivier: Investigation; writing – review & editing. Suzanne D. Johanningsmeier: Conceptualization; formal analysis; investigation;

methodology; project administration; resources; supervision; writing – review & editing

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

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