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Original Research Article

Formation of γ-aminobutyric acid (GABA) during the natural lactic acid fermentation of cucumber

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ABSTRACT

This study investigated γ-aminobutyric acid (GABA) formation during natural lactic acid fermentation of cucumbers. Two lots of cucumbers were fermented or acidified in duplicate in 4 % sodium chloride (NaCl) brine and analyzed for GABA content over 6 months storage. GABA was present in fresh and acidified pickling cucumbers at 0.83 ± 0.16 mM and 0.56 ± 0.30 mM, respectively. Additional GABA was generated in fermented cucumbers (1.21 ± 0.35 mM) and remained stable over time and during further processing. Brine salt content (2 % - 6 %) affected GABA formation with greatest GABA formation in cucumber brined in 2 % NaCl (1.38 ± 0.31 mM). Commercially available, fermented cucumber pickles packed in their original fermentation brines contained more GABA (1.32 ± 0.25 mM) than acidified (0.46 ± 0.26 mM) or fermented, desalted and re-packed products (0.49 ± 0.32 mM). This work demonstrated that low-salt fermentation enhances GABA content in fermented cucumber products prepared for direct consumption.

1. Introduction

Lactic acid bacteria (LAB) fermented foods such as cheeses, yogurts, sourdough bread, cured meats, and pickled vegetables are recognized as dietary sources of health-promoting components with potential for improving physical and mental wellness (Marco et al., 2017; Selhub et al., 2014) Historically, benefits derived from LAB fermentation have been mostly attributed to the presence of live, probiotic microorganisms. Most fermented vegetables have a low pH (3.0−3.5) and may undergo thermal processing (i.e. pasteurization) to provide adequate shelf life, thereby compromising microbial viability. However, compounds produced during fermentation such as bioactive peptides, amino acids, organic acids, and vitamins may survive processing and provide a benefit to the consumer. Research into food-sourced bioactive compounds is an active field, and more recently, attention has been drawn to the generation of bioactive peptides and the liberation of amino acids in plant-based fermentations (Bartkiene et al., 2016; Curiel et al., 2015; Zhu et al., 2016).

LAB have robust proteolytic systems capable of producing peptides and free amino acids from food proteins (Savijoki et al., 2006). Recently, our group demonstrated that both total peptide content and the concentrations of four antihypertensive bioactive peptides were higher in fermented cucumbers than in raw or acidified cucumbers (Fideler et al., 2019). In addition to proteolytic systems, LAB contribute to changes in the free amino acid profiles of fermented foods through amino acid dependent acid resistance systems. In one such system, glutamic acid decarboxylase (GAD) is used to decarboxylate glutamic acid to GABA, consuming a proton and protecting against decreases in intracellular pH (Wu et al., 2017). In humans, GABA is a non-proteinogenic amino acid that serves as the main inhibitory neurotransmitter (McCormick, 1989). While GABA is primarily found in the brain, only miniscule amounts penetrate the blood-brain barrier (Boonstra et al., 2015; Hayakawa et al., 2004). Therefore, exogenous GABA sources positively impact human health via other organ systems such as the peripheral nervous system. Consumption of GABA from foods or supplements has been shown to reduce blood pressure (Hayakawa et al., 2004; Inoue et al., 2003; Pouliot-Mathieu et al., 2013), improve decision making (Steenbergen et al., 2015), reduce anxiety (Abdou et al., 2006; He et al., 2019), and boost immunity (Abdou et al., 2006). GABA content has been enhanced in various foods through fermentation including Pu-erh tea (Zhu et al., 2016), buckwheat sprouts (Koyama et al., 2013), dairy foods (Inoue et al., 2003; Wu et al., 2017),

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soy sauce (Yamakoshi et al., 2007), and sourdough bread (Rizzello et al., 2008; Venturi et al., 2019).

Cucumber pickles are widely consumed around the globe and represent the most commonly consumed fermented vegetable in the United States. During fermentation of cucumbers, LAB convert glucose and fructose to lactic acid (110−140 mM) and carbon dioxide to achieve a final equilibrated pH of 3.2–3.6 (Pérez-Díaz et al., 2013). While the conversion of sugars to acid is well-characterized in fermented cucumbers, little has been published regarding amino acid changes during fermentation and storage of these products. Costliow and Fabian (1953) studied changes in cysteine, glutamic acid, leucine, isoleucine, tryptophan and valine in the brines of fermented and non-fermented cucumbers but did not quantify GABA. The USDA National Nutrient Database reported that fresh cucumbers contain 0.17 g/100 g glutamate (USDA Food Composition Database, https://ndb.nal.usda.gov/ndb/search/list, Accessed 10.10.16), suggesting that glutamate is present in fresh cucumbers and could be available as a substrate for GAD. LAB most commonly associated with cucumber fermentation include the following in order of prevalence: *Lactobacillus pentosus, Lb. plantarum, Lb. brevis, Weissella* spp., *Pediococcus ethanolidurans, Leuconostoc* spp. and *Lactococcus* spp. (Pérez-Díaz et al., 2017). GAD activity has been demonstrated in *Lb. plantarum* isolated from a variety of Italian cheeses (Siragusa et al., 2007), *Lb. brevis* isolated from kimchi (Park and Oh, 2007), as well as a number of other LAB species (Cui et al., 2020). Therefore, we hypothesize that GABA is generated during natural lactic acid fermentation of cucumbers. Analysis of glutamate and GABA content will provide insight into the compositional changes during natural lactic acid fermentation as well as the potential for fermented cucumbers to influence consumers' health. In large commercial operations, cucumber pickles are commonly fermented and stored in large vats for up to nine months, re-packed into jars with fresh cover brine and pasteurized to produce finished pickle products. Accordingly, the stability of bioactive compounds over time and through processing is an important factor to investigate. The objectives of this study were to (1) determine whether GABA was generated during natural lactic acid fermentation of cucumber; (2) assess GABA stability during fermented cucumber storage and after subsequent processing into finished products; and (3) survey commercial pickle products for GABA content.

2. Materials and methods

2.1. Chemicals and materials

Pickling cucumbers, vinegar (acetic acid, 20 %), and pickling salt (sodium chloride (NaCl) ≥ 99 %) were obtained from Mount Olive Pickle Company (Mount Olive, NC, USA). LC–MS grade acetonitrile, LC–MS grade water, and calcium hydroxide ($Ca(OH)_2$, ≥ 95 %) were purchased from Fisher Scientific (Hampton, NH, USA). Sodium benzoate (≥ 99 %) was purchased from Acros Organics (Waltham, MA, USA). Ammonium formate (≥ 99 %), formic acid (≥ 98.5 %), lactic acid (≥ 85 %), hydrochloric acid (HCl, ≥ 37 %), sulfuric acid, calcium chloride ($CaCl_2$, ≥ 93 %), γ-aminobutyric acid (GABA, 99 %), glutamate (glu, 99.5 %), and glutamine (gln, 98 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following stable isotope labeled (SIL) standards were also purchased from Sigma-Aldrich: 4-aminobutyric acid-2,2,3,3,4,4-d_6 (97 atom %), and a universally labelled ^{13}C, ^{15}N mixture containing ala, arg, asn, asp, cys, glu, gln, gly, his, ile, leu, lys, met, phe, pro, ser, thr, trp, tyr, and val in water. Amicon Ultra-0.5 filters with a 10 kDa cutoff were purchased from Fisher Scientific.

2.2. Experimental design

Two sets of cucumber fermentations were prepared, the first to monitor GABA formation over time in fermented and acidified cucumbers and the second to monitor GABA formation in cucumbers fermented in brines of various salt levels.

The first experiment included three treatments of cucumbers: fresh, acidified, and fermented. Each treatment included two lots of cucumbers obtained from separate farms located in the United States and Mexico. Fresh cucumbers were analyzed at one time point, day 0. Acidified and fermented cucumbers were analyzed at 9 time points, days 1, 3, 7, 14, 21, 30, 48, 107, and 178. Acidified and fermented cucumbers were independently replicated in duplicate for each lot at each time point. Two additional replicates were prepared for finished product processing and pasteurization on the day 48 sampling point. Acidified cucumbers were prepared as a control to mimic the acid and salt content of fermented cucumbers, and sodium benzoate was added to acidified cucumbers to prevent fermentation.

The second experiment was performed on cucumbers fermented in four brines with various levels of salt: 2, 3, 4, or 6 % NaCl. Each treatment was repeated with three lots of cucumbers obtained from separate farms located in the United States and Mexico. Fresh and fermented cucumbers were analyzed at one time point, day 0 and day 27 (end of fermentation), respectively, while brines were aseptically sampled at eight time points throughout the fermentation. Fermentations were performed in duplicate for each treatment and cucumber lot.

2.3. Brining and fermentation of cucumbers: time course experiment

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 v/v ratio) and fitted with heat-sealed lids. Jars intended for finished product processing and pasteurization (at day 48) were sealed with a septum fitted lid to allow for brine sampling with a syringe. Brines for acidified and fermented treatments were prepared to attain equilibrated concentrations in brined cucumbers of 0.684 M (4 %) NaCl, 12 mM $CaCl_2$, 18 mM $Ca(OH)_2$, and 53 mM acetic acid. In addition to these components, acidified cucumber brine contained lactic acid to mimic fermented cucumber composition (110 mM, equilibrated), sodium benzoate to prevent fermentation (8 mM, equilibrated), and were adjusted with HCl to pH 2.75 to allow cucumbers to reach a final pH of 3.25 after equilibration. Brined cucumbers were incubated for 6 weeks at 28 °C.

2.3.1. Sample preparation

Each jar was processed on its appointed time point in the following manner: all cucumbers were removed from brine, cut into 1 inch sections, added to a blender (Waring, Model CB15 V, Waring Commercial, Torrington, CT, USA), and ground for 1 min at speed 3 and 2 min at speed 6 to form a slurry for analysis of sugars, organic acids, and amino acids. Brine was sampled for pH measurement. Raw cucumbers were similarly prepared on the day that brined cucumbers were packed. All samples were stored at −80 °C until analysis.

2.3.2. Finished product processing

Replicates designated for finished product processing were desalted, re-packed and thermally treated to mimic preparation of commercial, shelf-stable products. Cucumbers were removed from brine, placed into clean 1.36 L jars with water at a 55:45 cucumber:water v/v ratio, and allowed to equilibrate for 48 h at room temperature to achieve a target salt content of 376 mM NaCl. Following desalting, cucumbers were sliced into 3−6 mm thick chips, packed into clean 0.479 L (16 oz) jars and covered with fresh brine at a 58:42 cucumber:brine v/v ratio. Fresh cover brines were prepared for fermented and acidified cucumbers in order to attain the following equilibrated concentrations that mimic commercially available pickled cucumber products: 0.380 M NaCl, 25 mM $CaCl_2$, 100 mM acetic acid, 35 mM lactic acid and 4.0 mM sodium benzoate. Jars were closed with heat-sealed lids and pasteurized in batches in a boiling water canner to reach an internal temperature of 74 °C for 15 min. Jars were cooled in an ice water bath to room temperature. Products were sampled as described above 12 h after pasteurization and cooling.

Table 1
Glutamate, glutamine and GABA in whole cucumber slurries.

Amino Acid (precursor *m/z*)	Retention Time (min)	Product Ion	Reference Ion(s)	Standard Curve Range (μM)	Concentration (mM)*				
					Fresh	Acidified	Acidified Processed**	Fermented	Fermented Processed**
GABA $[M+H]^+$ 104.00	8.13	87.10 (−13.0 CE)	69.15 (-17.0 CE)	0.40 – 350	0.83 ± 0.16^{ab}	0.56 ± 0.30^{bc}	0.17 ± 0.06^{c}	1.21 ± 0.35^{a}	0.50 ± 0.17^{bc}
Glutamate $[M+H]^+$ 148.25	8.50	83.95 (−15.0 CE)	102.00 (-15.0 CE) 130.10 (-13.0 CE)	0.50 – 60	0.54 ± 0.09^{a}	0.23 ± 0.15^{b}	0.24 ± 0.08^{b}	0.23 ± 0.17^{b}	0.17 ± 0.14^{b}
Glutamine† $[M+H]^+$ 147.20	8.50	84.05 (−16.0 CE)	56.10 (-29.0 CE) 42.05 (-55.0 CE)	1.5 – 1450	7.40 ± 1.04^{a}	0.20 ± 0.03^{b}	0.11 ± 0.14^{b}	0.55 ± 0.30^{b}	0.56 ± 0.84^{b}

*Superscript letters within a row indicate significant differences between treatments ($p < 0.05$) using a one-way analysis of variance with post-hoc Tukey HSD. **Processed treatments underwent desalting in deionized water, re-packing in fresh cover brine, and pasteurization. †Glutamine values were averaged on day 0 for Fresh and day 48 for all other treatments.

2.4. Brining and fermentation of cucumbers: Brine salt experiment

Pickling cucumbers, size 2B (3.5–3.8 cm diameter), were packed into 760 mL jars (24 oz.) covered with brine (55:45 cucumber:brine ratio) and closed with septum fitted, heat-sealed lids to allow for brine sampling with a syringe. Four brines were prepared to reach equilibrated concentrations in brined cucumbers of 53 mM acetic acid, 12 mM $CaCl_2$, 18 mM $Ca(OH)_2$ and one of four salt concentration levels: 2, 3, 4, or 6 % NaCl. Jars were incubated at 28 °C for 27 days. Jars were inverted to mix brines prior to aseptic sampling on days 0, 1, 2, 3, 5, 7, 14, 20, and 27 for analysis of pH, organic acids, sugars, and GABA.

2.5. Fermentation biochemistry

Quantification of lactic acid, acetic acid, fructose, and glucose was performed using a Shimadzu UFLC (Shimadzu Corporation, Kyoto, Japan) per McFeeters and Barish (2003) with slight modifications. Slurry samples were thawed, vortexed, centrifuged at 12,000×*g* for 10 min and supernatants injected onto an Aminex HPX-87H resin column (300 × 7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA) held at 60 °C. Elution was performed with 0.01 N sulfuric acid mobile phase at 0.9 mL/min. Acids were detected with a SPD-20A UV–vis light detector (Shimadzu Corporation, Kyoto, Japan) at 210 nm and sugars were detected with an RID-10A refractive index detector (Shimadzu Corporation) connected in series. Eight-point calibration curves (0.5–100 mM) were prepared from external standards for all analytes.

2.6. Amino acid quantification by LC-QQQ-MS

Slurried samples were thawed and ground to release amino acids from intact cells using a Retsch mixer mill MM301 (Cole-Parmer, Vernon Hills, IL, USA). Fresh cucumber samples were prepared in duplicate for LC–MS analysis for each lot. Fresh cucumber slurries were diluted with a mock brine to achieve the same salt and acid composition as the acidified treatment to control for matrix effects. Slurry (2.5 g) was aliquoted into 25 mL stainless steel capsules containing three 9 mm stainless steel beads. Capsules were submerged in liquid nitrogen for 30 s then oscillated for 4 min at 30 Hz. Samples were poured into microcentrifuge tubes and spun at 12,000 *x g* for 5 min at 4 °C to remove cellular debris. Supernatant (90 μL) was mixed with 565 μL mobile phase A (85 % acetonitrile, 10 mM ammonium formate, 0.15 % formic acid) in microcentrifuge tubes and stored at 4 °C for 1 h to precipitate insoluble material. Tubes were centrifuged at 12,000 *x g* for 8 min at 4 °C and supernatant aliquoted into 10 kDa centrifugal cutoff filters. Filters were spun at 14,000 *x g* for 10 min at 4 °C. Filtrate (323 μL) and 10 μL internal standard mixture were added to LC–MS vials, placed in a chilled autosampler (4 °C) and analyzed within 36 h. One analytical replicate was performed for each sample.

Amino acids were quantified using a Shimadzu LCMS-8040 triple quadrupole LC–MS/MS (Shimadzu Corporation). Separation was achieved using an Atlantis HILIC column (100 × 4.6 mm, 3.0 μm) (Waters Corporation, Milford, MA, USA) held at 37 °C with a segmented gradient of 85 % acetonitrile with 10 mM ammonium formate and 0.15 % formic acid (A) and 10 mM ammonium formate with 0.15 % formic acid (B). Gradient elution at 0.6 mL/min was carried out as follows: 0–9.6 % B (0−3 min), 9.6–27 % B (3−7 min), 27 % B (7−8 min), 27–37 % B (8−9 min), 37−0 % B (9–10.5 min), 0 % B (10.5−19 min) (Qiu et al., 2020). The first 2 min of flow was diverted to waste to reduce salt accumulation on the ion source. The MS utilized multiple reaction monitoring in positive ion mode for all amino acids. Nebulizing and drying gas (nitrogen) flow rates were 3 L/min and 15 L/min, respectively. Heat block temperature was 400 °C. Loop time of 1 s was used and resolution for the first and third quadrupoles was set to 0.7 Da (Da) and 1.0 Da, respectively. Optimized collision energies, transitions, and reference ions used for quantification are reported in Table 1. Method validation for LC-QQQ-MS analysis of amino acids was performed by Qiu et al. (2020).

Eight-point standard curves were prepared using a mixture of amino acids. Concentration ranges for curves are reported in Table 1. Standard curves for all experiments were linear with $r^2 \geq 0.99$, 0.97 and 0.98 for GABA, glu and gln, respectively. Glu and GABA were prepared individually in 0.1 N HCl, mixed, and stored at −19 °C. Gln was prepared in LC–MS grade water to prevent degradation and stored at −19 °C. Final standards used for quantification were prepared in LC–MS vials by combining individual amino acids, internal standards and mobile phase A to a final volume of 1 mL. Fresh standard mixes were prepared every 48 h. LC–MS analyses and data processing utilized LabSolutions software ver. 5.8 (Shimadzu). Peak height was used for quantification.

2.7. Commercial product survey

Commercial cucumber pickle products were analyzed for amino acid content. Products were chosen from three categories: 1) fermented and packed in the original fermentation brines without desalting (n = 7); 2) fermented, desalted and re-packed in fresh cover brine (n = 12); and 3) acidified (n = 12). Except for one manufacturer who could provide only one lot, two independent lots of each product were purchased from local grocery stores, ordered online, or donated by manufacturers. Slurries of each lot of product were prepared and analyzed for amino acid content as described above.

2.8. Statistical analysis

Analysis of variance (ANOVA) and post-hoc Tukey test with $\alpha = 0.05$ was performed using JMP Pro v. 14 (SAS Institute Inc., Cary, NC, USA). A factorial model was fit for GABA production in fermented cucumbers including the variables treatment (acidified or fermented), cucumber lot, time point (days) and all interactions among variables. Time point alone was not a significant effect ($p = 0.81$); therefore, GABA content was averaged across fermentation time points for data reporting.

3. Results and discussion

3.1. Fermentation biochemistry

Fermentation of cucumbers in the time course experiment progressed normally in all replicates as indicated by decreases in glucose and fructose to less than 2 mM each, and an increase in lactic acid concentration (Fig. 1A). Raw pickling cucumber sugar content (glucose and fructose) was not significantly different between lots. Acidified cucumbers were formulated based on an industry average of 110 mM lactic acid in fermented cucumber. Sugar and acid concentrations did not change between equilibration (7–10 days) and final sampling (43 days) for the acidified cucumbers, indicating that no fermentation occurred (Fig. 1B). The fermented cucumbers contained less than 2 mM (< 0.05 %) residual sugars and accumulated 104.6 ± 12.9 mM lactic acid (Fig. 1A). The final pH of acidified and fermented cucumbers was 3.15 ± 0.05 and 3.5 ± 0.13, respectively.

3.2. Formation and stability of GABA in fermented and acidified cucumbers

GABA was present in fresh pickling cucumbers at 0.83 ± 0.16 mM (105 ± 21 mg/kg) and in acidified cucumbers at 0.56 ± 0.30 mM (73 ± 38 mg/kg) (Table 1, Fig. 2). GABA accumulates in living plants such as cucumbers in response to both biotic and abiotic stresses, including cold, heat, salinity, drought, oxygen deficit, ultraviolet light, mechanical damage, cytosolic acidification, and infections by *Agrobacterium* and *Pseudomonas* (Shelp et al., 2017; Yoon et al., 2017). Other fresh plant foods that contain GABA include carrot, 63 mg/kg (Ito et al., 2019); cabbage, 26 mg/kg (Ito et al., 2019); snap peas, 26 mg/kg (Ito et al., 2019); raw and germinating soy, ~26–205 mg/kg (Xu and Hu, 2014); spinach, ~17–328 mg/kg (Ito et al., 2019; Yoon et al., 2017); sweetpotato, 5–52 mg/kg (Ito et al., 2019; Qiu et al., 2020); tomato, 206–825 mg/kg (Akihiro et al., 2008); and raw and germinated wheat, ~142 mg/kg (Van Hung et al., 2015).

Acidified cucumber GABA content was consistent with the equilibration of water-soluble cucumber components with brine (55 % cucumbers, 45 % brine). GABA was formed in fermented cucumber with an average total content of 1.21 ± 0.35 mM (150 ± 44 mg/kg) (Table 1, Fig. 2), a 116 % increase over acidified cucumbers ($p < 0.001$). The majority of the GABA was formed within the first 24 h of fermentation and remained stable over time (Fig. 3A). Biological variability in the population of natural microbiota (Pérez-Díaz et al., 2017) as well as the initial cucumber composition may affect GABA formation during fermentation. As mentioned, natural GABA content in plants varies based on biotic and abiotic stressors. A comparison of the two lots used in the time course experiment revealed no significant difference in GABA or glu content between lots of fresh cucumbers. However, after fermentation, cucumber lot was a significant effect for total GABA ($p < 0.006$), suggesting that variability in native microbiota between the two lots may have contributed to differences in GABA formation. A comprehensive review by Cui et al. (2020) associated variability in GABA production among species and strains with differences in the enzymatic properties of GADs isolated from them, including amino acid sequence, optimal pH, temperature, K_m, V_{max}, and activators. The diversity in GAD systems and their organization within LAB genomes showed that some strains were found to contain multiple GAD genes leading to greater GAD expression and GABA production. The two lots represented in this study were grown in Florida and Mexico which allows for variation in native microbiota at both the species and strain level.

GABA was found in both the brine and cucumber portions of fermented and acidified cucumbers. A preliminary study showed that during the first 24 h of fermentation GABA was significantly higher ($p < 0.05$) in the cucumber slurry (1.10 ± 0.18 mM) than the brine (0.22 ± 0.04 mM) as it had not yet equilibrated into the brine and glutamate had not yet been fully converted to GABA. However, there were no significant differences ($p = 0.3892$) in GABA content between brine and cucumber from day 7 onward (1.50 ± 0.48 mM in slurry and 1.38 ± 0.29 mM in brine on average), which is consistent with the equilibration of other water soluble solutes in fermenting cucumber. While some consumers drink pickle brine, fermented cucumbers are the main portion consumed from jarred pickles; therefore, this study analyzed whole cucumber slurry for determining GABA content. Opportunity exists for

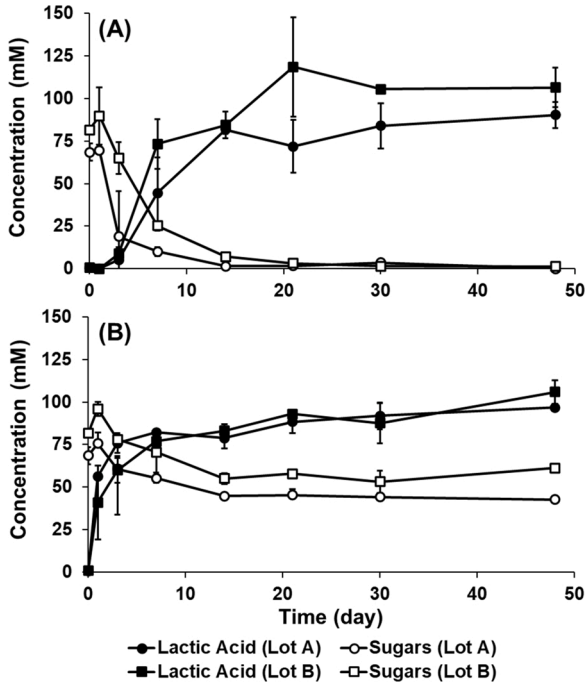

Fig. 1. Changes in lactic acid and sugar content in fermented and acidified cucumber. (A) Conversion of sugars to acid in fermenting cucumber; and (B) Equilibration of sugars and acid in acidified cucumber.

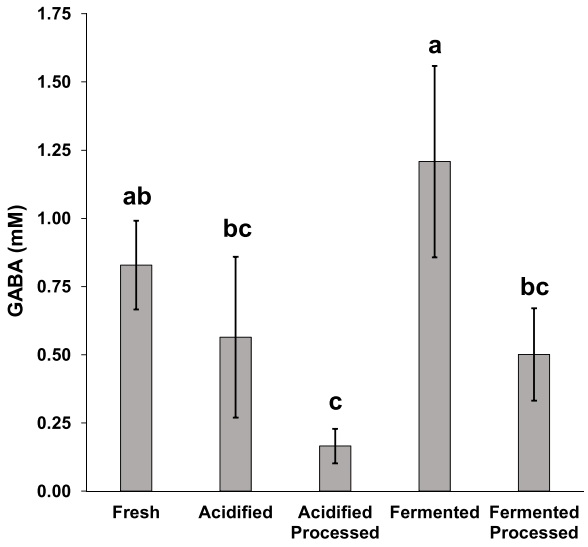

Fig. 2. Average GABA content in size 2B cucumbers by treatment. Acidified and fermented pickling cucumber were prepared with a 55:45 cucumber:brine ratio. Processed cucumber pickles were desalted in deionized water, sliced into chips, and re-packed in fresh cover brine at a 58:42 cucumber:brine ratio, then pasteurized at 74 °C for 15 min. Letters indicate significant differences between treatments ($p < 0.05$) using a one-way analysis of variance with post-hoc Tukey HSD test.

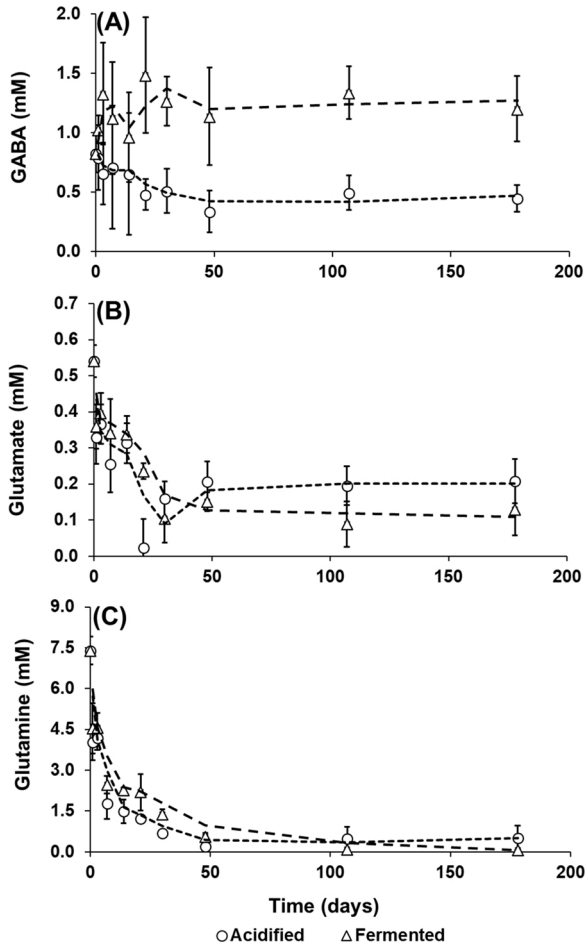

Fig. 3. Changes in amino acid contents in fermented and acidified cucumbers during fermentation and storage at 28 °C.

the development of GABA enhanced value-added products utilizing the typically discarded fermentation brine. Similarly, research has been conducted in the use of fermented olive wastewater for its natural health-promoting properties. Tafesh et al. (2011) found that hydroxytyrosol from olive mill wastewater was an effective antimicrobial agent against gram-positive bacteria, *Streptococcus pyogenes* and *Staphylococcus aureus,* as well as gram-negative bacteria, *Escherichia coli,* and *Klebsiella pneumoniae.* Belaqziz et al. (2017) determined that the phenolic compounds hydroxytyrosol and tyrosol were prevalent in olive processing wastewater, displayed high free radical-scavenging activity, and may be suitable additions to pharmaceutical, cosmetic, or consumable applications, as a source of antibacterial and polyphenolic compounds. The discovery of GABA in both whole cucumbers and fermentation brine opens the door for exploration into of the recovery of value-added ingredients and new uses for cucumber fermentation brine and processing waste streams.

Glu in fresh cucumbers averaged 0.54 ± 0.09 mM (98.0 ± 16.1 mg/kg) (Table 1) and decreased to 0.06 ± 0.08 mM in fermented cucumbers by the end of storage (Fig. 3B). Accounting for dilution of components by cover brine, if all glu in fresh cucumber were converted to GABA and all GABA remained stable, the maximum potential GABA present at the end of fermentation would be 0.75 ± 0.14 mM. GABA content of fermented cucumbers was 1.21 ± 0.35 mM, suggesting full conversion of free glu to GABA and perhaps conversion of other minor sources of glu to GABA as well. Glu levels in acidified cucumbers averaged 0.21 ± 0.14 mM at the end of storage (Fig. 3B) due to dilution into the cover brine as well as potential degradation to pyroglutamic acid (pGlu) (Exterkate and Stadhouders, 1971). Costliow and Fabian (1953) observed significant decreases in glutamate in the brines from only three of the six commercial cucumber fermentation tanks they surveyed, indicating that glutamate may have been preserved (i.e. not converted to GABA or degraded to pGlu) or generated in some commercial fermentations.

Interestingly, gln was present at much higher levels than glu in fresh cucumber (7.40 ± 1.04 mM, 1330 mg/kg) and precipitously decreased in both acidified and fermented treatments (Fig. 3C) to 0.20 ± 0.03 mM and 0.55 ± 0.30 mM, respectively (Table 1). Some bacteria, specifically enterics and select strains of LAB such as *Lb. brevis* possess the enzyme glutaminase which can convert gln to glu and ammonia (Kieronczyk et al., 2001). In this study, glu to GABA conversion averaged 160 % and net glu levels did not increase. Despite the abundance of gln available for conversion to glu, the GABA levels suggest that glutaminase activity was very low or absent in this natural fermentation system. Both gln and glu easily degrade to pGlu under acidic conditions (Exterkate and Stadhouders, 1971) which may explain the low levels of glutamine in both acidified and fermented cucumbers at the end of storage.

Treatments that mimicked common techniques for finished product processing resulted in a significant decrease in GABA content. Acidified processed cucumbers contained 70.6 % less GABA (0.17 ± 0.06 mM; 21.3 ± 8.2 mg/kg) and fermented processed cucumbers contained 58.5 % less GABA (0.50 ± 0.17 mM; 62.2 ± 21.0 mg/kg) than their respective non-processed treatments (Table 1, Fig. 2). These values are consistent with the expected dilution of water-soluble components due to desalting (55:45 cucumber:water ratio) and re-packing (58:42 cucumber:brine ratio) that would result in an expected overall dilution of 68.1 %. These results suggest that heat of pasteurization did not further degrade GABA and that this compound would be maintained in shelf-stable pickle products. Thermal stability of GABA is supported by its presence in sourdough bread both before and after baking (Venturi et al., 2019). Additionally, Ito et al. (2019) observed the effects of heat treatments on free amino acid content in vegetables and found that roasting carrots (15 min at 200 °C) and sweetpotatoes (90 min at 160 °C) resulted in significant increases in GABA. Conversely, boiling carrots, cabbage, snap peas, and spinach significantly reduced GABA content even after accounting for dilution into boiling water.

3.3. *Effect of brine salt concentration on GABA formation*

Brine salt concentration had a significant effect on GABA generation in fermented cucumbers. Fresh (1.04 ± 0.27 mM), 2 % NaCl (1.38 ± 0.31 mM), and 3 % NaCl (1.02 ± 0.14 mM) treatments were not significantly different from each other in GABA concentration but the 2 % NaCl treatment had higher GABA than the 4 % NaCl (0.73 ± 0.13 mM) and 6 % NaCl (0.87 ± 0.22 mM) treatments (Fig. 4A). GABA was formed early in each treatment's fermentation with no significant difference in brine GABA concentration between days 2 and 27 (Fig. 4B). These findings are similar to the previous experiment, supporting that GABA is formed within the first 24−48 h of natural cucumber fermentation and remains stable over time. Work by Wu et al. (2017) supports the concept of early GABA formation, observing that GABA was formed at the end of log phase (12 h) and into stationary phase (24 h) by *Lb. brevis* strain 145 grown in Lactobacilli MRS broth with added glu.

Microbial viability, gene expression and enzyme functionality are impacted by extrinsic factors such as pH and salt concentration. It is known that when salt content exceeds an organism's optimal range, the pH range in which it grows narrows (Jay et al., 2005). Additionally, salt plays an integral role in vegetable fermentations by selecting for particular LAB species, thus influencing the type and extent of microbial activity (Pérez-Díaz et al., 2013). The significantly lower concentrations of GABA in the 4 % and 6 % NaCl treatments may reflect the effect of salt or the combined effect of salt and acid on species selection, cell growth and production of GABA. These data show that GABA formation may be increased by lowering salt levels in natural fermentations.

Commercial fermentations typically contain ~6 % (1 M) salt after equilibration with brine which helps select for robust organisms that

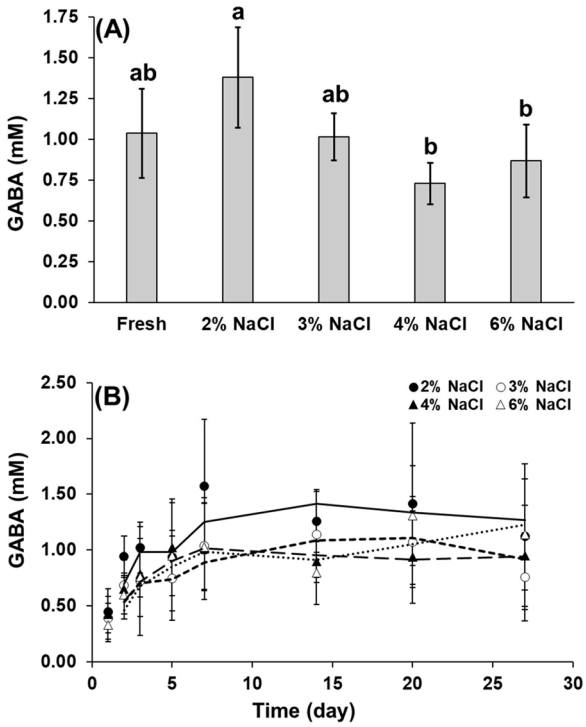

Fig. 4. (A) Average GABA content in fresh and fermented cucumber brined in varying sodium chloride salt concentrations. Letters indicate significant differences between treatments ($p < 0.05$) using a one-way analysis of variance with post-hoc Tukey HSD test. (B) GABA generation over time in the brines of cucumber fermented in varying salt concentrations.

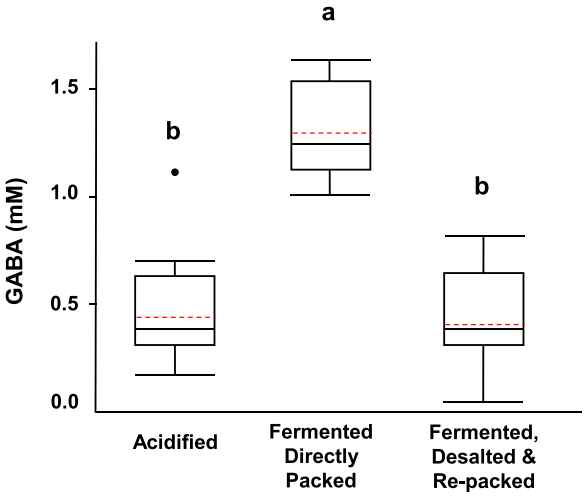

Fig. 5. GABA content in commercial cucumber pickle products by type of preservation process. Letters indicate significant differences between groups ($p < 0.05$) using a one-way analysis of variance with post-hoc Tukey HSD test. Red dotted line indicates average GABA content of each group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

will complete the fermentation, inhibits spoilage organisms, and maintains long-term stability of fermented cucumbers in tanks prior to finished product processing (Franco et al., 2016; Pérez-Díaz et al., 2013). Reducing salt in a fermentation allows for more organisms to grow, including both LAB and undesirable spoilage microorganisms which may produce off-flavors and odors and cause softening. Johanningsmeier and McFeeters (2013) found that NaCl content impacted the degree to which *Lb. buchneri* initiated secondary fermentation spoilage in fermented cucumber media. *Lb. buchneri* degraded lactic acid fermented cucumber media (pH 3.8) with adjusted NaCl concentrations ranging from 2 to 6 %, with the highest level of degradation at 2 % NaCl. Our study demonstrated that more GABA was produced in 2 % and 3 % NaCl fermentations, but these reduced salt fermentations may have an increased risk of long-term spoilage compared to most current commercial processes. Reducing in-tank storage time and/or pasteurizing products in their fermentation brine would both mitigate spoilage and maintain higher levels of GABA in low-salt fermentations.

3.4. GABA content of commercially available cucumber pickles

Fermented, directly packed cucumber pickles contained significantly more GABA (1.31 ± 0.23 mM) than fermented cucumbers that were desalted and re-packed with fresh cover brines (0.43 ± 0.23 mM) and acidified products (0.46 ± 0.26 mM) (Fig. 5). These data are consistent with laboratory scale experiments showing that GABA is present at lower levels in acidified products due to equilibration of GABA from raw cucumber with the cover brine and that the GABA formed in many commercially fermented products is significantly diluted during desalting and re-packing. Consumer trends encourage consumption of fermented products for probiotic benefits; however, most fermented cucumbers' pH levels are too low for organisms to remain viable, or products have been pasteurized to eliminate live organisms for increased shelf-stability. We have shown that refrigerated or pasteurized fermented cucumbers that are directly packed with their original fermentation brines retain GABA, a non-probiotic, health-promoting compound. Refrigerated products have a shorter shelf life than pasteurized ones and are most costly to stock and display at grocery stores. Our findings have potential to counteract the misconception that processed foods have less nutritional value than fresh ones.

Currently, there is no established clinical dosage for GABA and researchers have administered GABA to human and animal subjects at varying concentrations both in the form of a pure supplement as well as foods with naturally occurring GABA due to fermentation. Yamakoshi et al. (2007) administered GABA enriched soy sauce (97 mM GABA) fermented by *Lb. rennini* to spontaneously hypertensive rats at 0.33 mg GABA/kg body weight and observed a significant decrease in systolic blood pressure by 25.2 ± 8.0 mmHg within 8 h as compared with control soy sauce. He et al. (2019) repeatedly fed rats GABA at 2 mg/kg body weight and exposed them to stress-inducing conditions and found that GABA improved availability of nitric oxide to the brain's frontal cortex, inducing an anxiolytic-like effect. In another study, acrophobic human participants received 100 mg of GABA orally prior to crossing a suspension bridge (Abdou et al., 2006), which increased alpha brain waves associated with a calm mental state in the test group as compared with the control. Pouliot-Mathieu et al. (2013) found that consumption of 50 g of cheese containing 16 mg of GABA lowered blood pressure by 3.5 mmHg in human subjects. Commercially, cucumbers are most commonly fermented using high levels of salt (6 %, 1 M) that are not acceptable for direct consumption. Pickles are therefore desalted and re-packed in fresh cover brine with a final salt content of 2 %, in effect diluting the content of water-soluble compounds such as GABA. Using data obtained from our fermentation time course experiment, in order to consume 16 mg of GABA from fermented cucumbers, a person would need to eat ~3, 4" pickle spears (107 g) that were consumed directly (without further desalting or re-packing in fresh cover brines), or ~7, 4" pickle spears (257 g) that were desalted and re-packed as commonly practiced. We showed that lower salt fermentations produced higher levels of GABA. These products would not require desalting and could deliver greater levels of GABA to consumers if packed in their fermentation brine.

4. Conclusion

GABA, a stable health-promoting compound, was generated from its unstable precursor, glutamate, through natural lactic acid fermentation of brined cucumber. GABA did not degrade during pasteurization and remained stable in fermented and acidified cucumbers over a 6-month time period. Increasing the GABA content of ready to eat fermented cucumbers to clinically relevant levels may be achieved by enhancing GABA generation during fermentation, and/or fermenting cucumbers in lower salt brines and packing them in the original fermentation brines for direct consumption.

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CRediT authorship contribution statement

Jennifer Fideler Moore: Investigation, Writing - original draft, Conceptualization, Formal analysis, Methodology, Validation, Visualization. **Rachel DuVivier:** Investigation, Methodology, Writing - review & editing. **Suzanne D. Johanningsmeier:** Conceptualization, Formal analysis, Methodology, Supervision, Project administration, Resources.

Declaration of Competing Interest

Authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2020.103711.

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