ABSTRACT

MOORE, JENNIFER FIDELER. Generation of Bioactive Peptides and γ-aminobutyric acid (GABA) During Natural Lactic Acid Fermentation of Cucumbers. (Under the direction of Drs. Suzanne D. Johanningsmeier & Rodolphe Barrangou).

Lactic acid (LA) fermentation has been used for centuries to preserve perishable foods and transform their sensory properties. During fermentation, lactic acid bacteria (LAB) promote compositional changes in foods due to their metabolic activity, resulting in production or release of bioactive compounds such as peptides, amino acids, vitamins, antioxidants and neurotransmitters. Fermented cucumbers are the most commonly consumed fermented vegetable in the United States with two-thirds of Americans consuming various types of pickles, yet little research exists regarding health promoting compounds developed during cucumber fermentation. We hypothesized that bioactive peptides and γ-aminobutyric acid (GABA) would be formed during LA fermentation of cucumbers. Research objectives were to identify and quantify bioactive peptides in fresh and fermented cucumbers and monitor changes in free amino acids during cucumber fermentation.

In the first study, bioactive peptides were identified in fresh, acidified, and fermented cucumbers using direct analysis infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) mass spectrometry (MS). Natural and starter culture fermented cucumbers were prepared in triplicate in sodium chloride brines and acidified cucumbers served as the control. Five bioactive peptides were putatively identified by IR-MALDESI-MS, confirmed by IR-MALDESI MS/MS and quantified by LC-MS/MS. Three angiotensin converting enzyme (ACE) inhibitory peptides were formed in natural and stater culture fermented cucumbers: isoleucine-proline-proline (0.42-0.49 mg/kg), leucine-proline-proline, (0.30-0.33 mg/kg), and valine-proline-proline (0.32-0.35 mg/kg). Additionally, ACE inhibitory lysine-proline increased 3 to 5-

fold in fermented cucumbers (0.93-1.5 mg/kg) compared to acidified cucumbers. This work demonstrated that fermentation by LAB can enhance bioactive peptide content in vegetables.

In the second study, GABA formation in fermented cucumbers was studied using three experiments. In the first, GABA formation and stability was determined over time in naturally fermented and acidified cucumbers. GABA was found in fresh and acidified cucumbers at 0.83 ± 0.16 mM and 0.56 ± 0.30 mM, respectively. Additional GABA was formed in fermented cucumbers (1.21 ± 0.35 mM) and remained stable during pasteurization and through 6 months storage. In the second experiment, the effect of brine salt content (2%, 3%, 4%, 6%) on GABA formation in naturally fermented cucumbers was studied. The highest GABA formation was observed in cucumbers brined in 2% NaCl (1.38 ± 0.31 mM). In the third experiment, commercially available pickle products were analyzed for GABA content. Acidified products contained 0.46 ± 0.26 mM GABA and fermented, desalted, and re-packed products contained 0.49 ± 0.32 mM GABA. Directly packed, fermented products contained the most GABA (1.32 ± 0.25 mM). This work demonstrated that GABA formation is enhanced in low-salt fermentations and in products prepared for direct consumption.

In the third study, the impact of initial brine acidification, starter culture addition and glutamate addition upon GABA formation during LA cucumber fermentation in 2% NaCl brine was investigated. Fermentations were conducted in acidified or non-acidified brines with and without added glutamate (10 mM) and with and without starter cultures. LAB with genes encoding for glutamate decarboxylase (*Lactiplantibacillus plantarum*) and glutaminase (*Lactobacillus gasseri*) were selected as starter cultures. Significantly higher GABA was produced in treatments with added glutamate (10.39 – 14.86 mM) compared to naturally fermented cucumber (1.12 ± 0.16 mM). Glutaminase production by starter cultures and

autochthonous microbiota was minimal or absent under these conditions as evidenced by no significant increases in glutamate or downstream GABA formation. Nonetheless, addition of glutamate to natural cucumber fermentations in 2% NaCl brines resulted in directly consumable pickles containing clinically relevant levels of GABA.

In summary, these studies provide evidence that natural LA fermentation enhances the GABA and bioactive peptide content of pickled cucumbers. Optimization of fermentation conditions and the addition of glutamate have the potential to further enhance GABA formation during cucumber fermentation.

Generation of Bioactive Peptides and γ-aminobutyric acid (GABA) During Natural Lactic Acid Fermentation of Cucumber.

by
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I would like to dedicate this dissertation to my family. To my grandpa and grandma who taught me that I must always be curious and who inspired me to finally go back to school and pursue an advanced degree. To my dad and mom, who have always supported me and provided me with incredible opportunities. You taught me to work hard, to keep going when things get tough – really tough – and you passed down your tenacity, compassion, intelligence and your incredible senses of humor and wit. To Andrea Jo, my original roommate and big sister; thank you for being a role model in many ways and for blazing trails for me, inspiring me to learn Spanish and live abroad, and for showing me how to be independent and take care of myself. To Eric Peter, my beloved brother; you were always my protector, my friend, and the one I admired for your intuitive mechanical skills and your innate ability to put others above yourself. I will always miss you. To my husband, David, who has supported me immensely and will always be the best part of life.

BIOGRAPHY

Jennifer Fideler Moore was born in Mason City, IA and raised in Beaverton, OR by her parents Randy and Michele Fideler and accompanied by her older siblings, Andrea and Eric. She developed a love of cooking at a young age, even playing the role of "Julia Child's Child" in a family home-movie, complete with her trusty Easy Bake Oven. She became interested in the science of cooking and found her way to Oregon State University where she completed her Bachelors of Science in Food Science and Technology (2011) with concentrations in food science and fermentation science as well as minors in Spanish and chemistry.

Jennifer's first permanent position was as a food scientist at Betty Lou's Inc, a small natural foods co-manufacturer in McMinnville, OR. During her time at Betty Lou's, she established a shelf-life testing program, helped direct the organic, kosher, non-GMO and vegan certification programs, ensured label compliance with the FDA Code of Federal Regulations, and was responsible for developing and maintaining over 80 products from nutrition bars to cookies, crackers, and ready-to-mix gluten-free breads.

In January 2015 Jennifer moved to Raleigh, NC to serve in a church planted from Portland, OR and to explore options for graduate school at NC State. She connected with Dr. Suzanne D. Johanningsmeier and was immediately interested in both the project available and the mentorship offered by Dr. Johanningsmeier. In May 2015 Jennifer began her graduate studies in the Department of Food, Bioprocessing and Nutrition Science. Since then, Jennifer has been heavily involved with her church as a youth leader and with the Food Science Club at NC State serving as Vice President (2016-2017) and proudly earning the Dairy Bar "Manager of the Year" award for three consecutive years. Jennifer plans to return to industry upon completion of her degree with a focus on manufacturing and ingredient applications.

ACKNOWLEDGMENTS

conceal things, but the glory of kings is to search things out" (Proverbs 25:2) and am thankful

that the wonders of this world have been concealed that we might search them out as scientists

and further understand God's creation.

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CHAPTER 1: Bioactive Peptide and γ-aminobutyric acid (GABA) Generation in Lactic Acid Fermented Foods

1.1 Abstract

Lactic acid fermentation has been used for thousands of years as a food preservation method and, more recently, has gained interest for its potential to increase the healthfulness of foods. Lactic acid bacteria (LAB) are fastidious microorganisms with robust enzymatic systems capable of releasing essential nutrients from food, including the hydrolysis of proteins to peptides. Some of these peptides have demonstrated antihypertensive, anticarcinogenic, anticholesterolemic, antidiabetic, and antioxidant activities and are known as bioactive peptides. Typically, these peptides are short, 2 – 6 amino acid residues in length, with the most studied peptides being di- and tripeptides. Extensive work has been performed to understand bioactive peptide development in high protein foods including lactic acid fermented dairy, cured meats, and legumes, with limited research on lactic acid fermented vegetables and fruits. In addition to bioactive peptides, lactic acid fermented foods have received notable attention for their γ-aminobutyric acid (GABA) content. Certain LAB are capable of decarboxylating glutamate to form GABA as part of an amino acid dependent acid resistance mechanism. GABA has demonstrated antihypertensive, antianxiety, anticancer, antioxidant, antistress, and immune-boosting properties. Numerous studies have analyzed GABA development in and bioactive peptide composition of lactic acid fermented foods. Efforts have also been made to increase these health promoting compounds through starter culture selection and manipulation of fermentation substrates and conditions. This review summarizes bioactive peptide and GABA generation in lactic acid fermented foods, their health promoting properties in human and animal studies, and assesses their potential formation in LA fermented cucumbers.

1.2 Introduction

Lactic acid fermented foods are consumed throughout the world, comprising between 5-40% of human diets in certain populations (Borresen et al., 2012). Vegetables, fruits, meats, grains, and milk are transformed from their fresh, perishable state into preserved forms such as pickles, sauces, breads, sausages, cheeses and yogurt through the use of lactic acid bacteria

(LAB). During lactic acid fermentation, glucose and fructose are converted to primarily lactic acid with small amounts of acetic acid, ethanol and carbon dioxide (CO_2). Common microbiota involved in these types of food fermentations include *Lactiplantibacillus plantarum* (*Lactobacillus plantarum*), *Lactobacillus acidophilus*, *Levilactobacillus brevis (Lactobacillus brevis), Fructilactobacillus sanfranciscensis (Lactobacillus sanfranciscensis), Lactococcus lactis, Leuconostoc mesenteroides, Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (Marco et al., 2017).

While the primary objectives of lactic acid fermentation include preservation and the enhancement of organoleptic qualities, other major drivers include economic value and healthfulness. Economically, fermentation reduces raw product spoilage by processing seasonal, perishable crops into products with extended shelf lives that may be consumed long after harvest. Healthfulness of raw foods can be preserved or enhanced via lactic acid fermentation due to microorganisms that pre-digest macronutrients into their subcomponents, release bound nutrients, and contribute vitamins and metabolites not natively present in the food (Acosta-Estrada et al., 2014; Marco et al., 2017; Singh et al., 2015). Additionally, some fermented foods contain probiotic microorganisms capable of surviving the digestive process, colonizing in the gastrointestinal tract, and providing benefits to the host (Stanton et al., 2005; Turkmen et al., 2019; Panda et al., 2017; Park et al., 2014)

Worldwide, a common conception has spread among consumers that fermented foods are healthful. Many consumers attribute this healthfulness to probiotic content; an association which may be credited to the abundance and popularity of fermented dairy products, especially yogurt, which serve as suitable delivery matrix for probiotic bacteria. Some controversy has arisen regarding the term "probiotic" and the accuracy of consumers' perceptions of the products that bear this claim. In fact, in 2012 the European Commission banned the word "probiotic" from food packaging, considering it to be an unauthorized health claim as it confers health benefits without substantiated evidence (European Parliament, 2017). This legislation suggests that consumers consciously look to fermented foods for health benefits. That said, various marketing terms such as "probiotic", "live and active cultures", "cultured", and "fermented" are used to appeal to consumers by associating fermented food with beneficial bacteria. However, viability of these organisms is often compromised due to the accumulation of acid during lactic acid

fermentation or post-fermentation heat treatments used to increase shelf stability (i.e., pasteurization).

Health benefits of fermented foods are not limited to live organisms but include those that may be influenced by compositional changes incurred during fermentation. Stanton et al. (2005) differentiated direct and indirect functional properties of fermented foods as the "probiotic effect" and "biogenic effect", respectively. Direct health benefits are those derived from ingesting live microorganisms whereas and indirect health benefits are those experienced by ingesting microbial metabolites. In agreement, Takano (2002) described these biogenic metabolites as "food components derived through microbial activity which provide health benefits without involving microbial microflora". Furthermore, Mitsuoka (2014) attributed immunomodulatory, anticancer, tumor suppressive, anticholesterolemic, and antiputrefactive qualities to biogenic compounds. Great leaps and bounds have yet to be made in confirming the generally purported healthfulness of fermented foods and elucidating the specific mechanisms at work. Nonetheless, a number of reviews summarize studies investigating the connection between fermented foods and human health in areas such as gastrointestinal wellbeing, thyroid regulation, cancer prevention, immunomodulation, weight maintenance, and cardiovascular health (Dimidi et al., 2019; Melini et al., 2019; Şanlier et al., 2019; Xiang et al., 2019). Furthermore, there is an emerging field of nutritional psychiatry that postulates psychological effects of consuming fermented foods such as reduced anxiety and depression-like behaviors, and influence on neurotransmitter production (Selhub et al., 2014; Aslam et al., 2020).

Recently, fermented foods have gained considerable interest from consumers for these purported health benefits. While only 11% of consumers claimed to eat fermented foods to improve their gut health and microbiome in 2017, 57% claimed to do so in 2019 (Mintel, 2020). In tandem with this interest, restaurants increased menu offerings featuring fermented ingredients by 39% between 2016 and 2019, with dill pickle offerings spiking 186% (Mintel, 2019b). Yogurt, kefir and kombucha are well-known products in the United States (US) and often consumed for their probiotic content (Mintel, 2020), while fermented fruits and vegetables are lesser known but gaining in popularity. A 2019 survey revealed that 44% of consumers were interested in fermented condiments such as pickles, kimchi, olives, and sauces for their health benefits (Mintel, 2019a). This rise in consumer interest has prompted investigation into the compounds responsible for the purported health promoting properties of fermented foods.

Furthermore, understanding the metabolic role of LAB in producing these bioactive compounds is essential for manipulating fermentations to optimize their production. Great opportunity exists for developing foods with enhanced bioactive compounds through lactic acid fermentation. This review seeks to summarize currently available literature regarding bioactive compounds in lactic acid fermented foods with a specific emphasis on bioactive peptides and γ-aminobutyric acid (GABA).

1.3. Bioactive Peptides

Bioactive peptides are sequences of amino acids that produce a positive physiological effect or conditions for such an effect on the human body. Bioactive peptides may be naturally present in a food in its raw form or produced through a biological or chemical processing step. Bioactive amino acid sequences are most often encrypted in a latent form within a parent protein and do not exhibit functional properties until they are liberated via enzymatic hydrolysis (Meisel and Bockelmann 1999). This hydrolysis occurs by one of three means: in the gastrointestinal tract through the action of endogenous digestive enzymes, through the use of exogenous proteases prior to food ingestion, or by microbial fermentation inside or outside the body (Korhonen and Pihlanto 2006).

In the field of peptidomics, protein fragments comprised of 2 to 500 amino acid residues are studied. While biologically active peptides are present throughout this range, the shortest peptides are of highest interest due to an observed inverse relationship between peptide length and potency (Panchaud, Affolter & Kussmann, 2012). Roberts, Burney, Black & Zaloga (1999) explored this structure-function relationship *in vivo* in rats using hormones of 3, 10, and 51 amino acids in length. Responses to enteral and intravenous delivery methods were compared and, when introduced directly to the intestine, researchers found that as peptide length increased its effectiveness decreased. Specifically, thyroid-stimulating hormone, a tripeptide, demonstrated equal effectiveness in both delivery methods while luteinizing hormone-releasing hormone, a decapeptide, performed with only 10% effectiveness when administered enterally as compared to intravenously. Insulin, a hormone with 51-amino acid residues was absorbed in the intestine and increased circulating levels of insulin yet failed to produce a significant hypoglycemic response. This study suggested that while medium and large sized peptides may be absorbed through the gastrointestinal tract, the most bioavailable and potent peptides are significantly shorter.

1.3.1 Health Benefits Associated with Bioactive Peptides

Bioactive peptides have been shown to perform anticancer (Rizzello, Nionelli, Coda & Gobetti, 2012), antimicrobial (Kwak et al., 2014; McCann et al., 2006; Robert et al., 2015), antioxidative (Chen & Decker, 1994; Karnjanapratum, O'Callaghan, Benjakul, O'Keeffe, Fitzgerald & O'Brien, 2017; Kudoh, Matsuda, Igoshi & Oki, 2001; Moayedi, Hashemi & Safari, 2016; Pihlanto, Akkanen & Korhonen, 2008), antithrombotic (Chabance et al., 1995; Khiari, Rico, Martin-Diana & Barry-Ryan, 2014; Yu, Wang, Zhang & Fan, 2016), antihypertensive (Boschin, Scigliulo, Resta & Arnoldi, 2014; Escudero, Aristoy, Nishimura, Arihara & Toldrá, 2013; FitzGerald, Murray & Walsh, 2014; Ishiguro, Sameshima, Kume, Ikeda, Matsumoto & Yoshimoto, 2012; Nakamura, Yamamoto, Sakai, & Takano, 1995), hypocholesterolemic (Hori et al., 2001; Jiang et al., 2020; Siow, Choi & Gan, 2016), opioid (Fukudome & Yoshikawa, 1993; Takahashi, Fukunaga, Kaneto, Fukudome & Yoshikawa, 2000), and immunomodulatory (Horiguchi, Horiguchi & Suzuki, 2005; Kayser & Meisel, 1996; Morris et al., 2007; Takahashi, Moriguchi, Yoshikawa & Sasaki, 1994) functions, among others.

A large proportion of the studies published on bioactive peptides focus on promotion of cardiovascular health. Cardiovascular diseases (CVDs) encompassing coronary heart disease, cerebrovascular disease and strokes, among others, are the number one cause of death globally with 31% of all deaths in 2016 attributed to CVDs (World Health Organization, 2017). High blood pressure is a significant contributor to the development of CVDs, and even small reductions in blood pressure (2 – 5 mm Hg) can significantly impact public health (Appel et al., 2006). The primary clinical mechanism for lowering blood pressure is angiotensin converting enzyme (ACE) inhibition. ACE converts angiotensin I to angiotensin II which causes vasoconstriction, while simultaneously inactivating the vasodilator, bradykinin, resulting in heightened blood pressure via two mechanisms (Erdmann et al., 2008). Unfortunately, ACE inhibitory drugs produce a range of side effects in patients including cough, fatigue, edema, skin rashes and loss of taste (Antonios & MacGregor, 1995) which may reduce patient adherence to prescribed medication and exacerbate disease severity (Aluko, 2015). This obstacle has prompted researcher to identify and develop foods with naturally containing ACE-inhibitory bioactive peptides to replace or reduce dosages of ACE inhibitors and reduce their unwanted side effects.

The blood pressure lowering effect of ACE inhibitory bioactive peptides has been observed in a variety of studies in which animal or human subjects ingest test foods naturally containing bioactive peptides with *in vitro* ACE inhibitory activity. The most-studied ACE inhibitory bioactive peptides are isoleucine-proline-proline (IPP) and leucine-proline-proline (LPP), first identified in milk fermented by *Lactobacillus. helveticus* (Nakamura, Yamamoto, Sakai, Okubo, Yamazaki & Takano 1995). A meta-analysis of studies administering IPP and LPP to adults showed that these peptides were effective in reducing blood pressure in Japanese individuals (Fekete, Givens & Lovegrove, 2015). Seppo, Jauhiainen, Poussa & Korpela (2003) conducted a placebo-controlled human study with *Lb. helveticus* fermented milk and found that long term consumption (21 weeks) significantly lowered blood pressure. Due to the complex nature of foods, non-peptide components may work in synergy with bioactive peptides to lower blood pressure. These factors include other antihypertensive biogenic compounds (i.e., GABA), the mineral profile of the food (i.e., calcium or sodium), interaction of live microorganisms with the host, and the release of other vasodilatory substances such as prostaglandin I2, nitrous oxide, and carbon monoxide (Seppo et al., 2003). Sipola, Finckenberg, Santisteban, Korpela, Vapaatalo & Nurminen (2001) observed in a rat model that the hypertension development was attenuated to a greater degree by a fermented milk product containing IPP and LPP than by equal ingested concentrations of these peptides. This finding suggests that these bioactive peptides alone are not responsible for the antihypertensive effects of the fermented milk and that the delivery matrix may affect the peptides' potency. Therefore, consumption of naturally fermented foods in whole form may provide greater health benefits than consuming isolated bioactive compounds.

1.3.2 Formation of Bioactive Peptides During Food Fermentation

LAB are not able to synthesize all necessary amino acids for their growth and survival and have therefore developed a sophisticated proteolytic system allowing them to obtain amino acids from environmental proteins (Pessione, 2012). The proteolytic systems of LAB include cell-envelope proteinases to hydrolyze oligopeptides, transport systems to uptake these oligopeptides, and intracellular peptidases to break down oligopeptides into shorter peptides and amino acids (Pessione & Cirrincione, 2016). From a food quality standpoint, proteolysis is an important component of LA fermentation, impacting the texture, flavor profile and nutritional quality of foods. For example, during sourdough fermentation LAB acidify and reduce disulfide

bonds of gluten and increase cereal protease activity (Gänzle, Loponen & Gobetti, 2008). In turn, LAB have greater access to peptides for breakdown to amino acids to meet their nutritional needs (Kunji, Mierae, Hagting, Poolman & Konings, 1996). Simultaneously, liberated amino acids are made available for participation in chemical reactions such as Maillard browning that contribute to the flavor and color of baked bread. Similarly, generation of dipeptides in soy fermentation has also been demonstrated to impact the flavor profile and quality of soy sauces (Yamamoto et al., 2014).

Proteolysis is associated with bioactive peptide generation; therefore, it is common for researchers to measure proteolysis, often associating it with LAB with a high potential for generating bioactive peptides. Proteolytic activity may be measured semi-quantitatively and expressed as degree of hydrolysis (DH), which is defined as the percentage of peptide bonds cleaved. One method for determining DH is the trinitrobenzenesulfonic acid (TNBS) method in which TNBS reacts with primary amines to form a chromophore which can be detected spectrophotometrically (Adler-Nissen, 1979). Solieri, Rutella & Tagliazucchi (2015) utilized the TNBS method to screen 39 non-starter LAB isolated from cheese for their ability to hydrolyze milk proteins. The authors selected the two strains with the highest proteolytic activity, *Lactobacillus casei PRA205* and *Lactobacillus rhamnosus PRA331*, for inoculation into bovine milk, producing fermented milks containing the ACE-inhibitory peptides IPP and VPP. A more recently developed protocol, the ophthaldialdehyde (OPA) method, involves the primary reaction between amino groups and OPA in the presence of dithiothreitol to produce a chromophore detectable at 340 nm by a spectrophotometer (Nielsen, Petersen & Dambmann, 2001). Utilizing the OPA method, Pihlanto, Virtanen & Korhonen (2010) found that DH and ACE inhibitory activities were correlated in 25 milks fermented with different strains of LAB. Similarly, Gómez-Ruiz, Taborda, Amigo, Recio & Ramos (2006) found that Spanish cheeses with higher degrees of proteolysis also had greater ACE inhibitory activities. Limitations of these methods include background color of samples interfering with absorbance, interference of N-terminal amines of proteins and the R-group of lysine with the reagents, and the presence of non-peptide material that absorbs at the same wavelength.

Proteolytic activity may also be measured quantitatively and expressed as a concentration in the food (i.e., mg/kg). Quantitative peptide analysis typically involves spectrophotometric or fluorescence measurements of peptide bonds or aromatic residues. Absorbance methods have

been used to quantify both protein and peptides utilizing absorbance of peptide bonds at 205 nm or 214 nm, or of aromatic amino acid residues at 280 nm (Simonian & Smith, 2001; Kuipers & Gruppen, 2007). Due to their limitations, these methods work optimally with defined proteins (i.e., bovine serum albumin) or pure protein digests (i.e., tryptic digest of casein). Absorbance at 205 nm requires that the tryptophan and tyrosine content of the protein be known, while absorbance at 280 nm is only possible if the peptides contain tryptophan or tyrosine. In contrast to digests of isolated, defined proteins such as casein or whey, whole food fermentations contain a variety of proteins and the concentration of tryptophan and tyrosine may not be consistent between lots or across growing or fermentation conditions. Additionally, the presence of LAB and their contribution to the proteome of the food in question would need to be taken into consideration. Interfering substances such as proteins which have peptide bonds and aromatic amino acids could be quantified as peptides in these methods. While peptide assay kits are also available, the standards provided with these kits are not similar enough to many plant-based fermented foods making the results more relatively quantitative than absolute. An alternative approach for describing proteolytic activity is to perform comprehensive analyses of the peptides in raw and fermented products. Ebner, Arslan, Fedorova, Hoffmann, Küçükçetin & Pischetsrieder (2015) demonstrated proteolytic activity by LAB and yeast in kefir, a fermented milk product, by performing a comprehensive analysis of the peptide profile in the raw and fermented milks. The authors found 257 peptides in the milks with 236 uniquely detected in kefir, 16 of which were previously associated with a variety of bioactivities in the literature. This approach demonstrated that fermentation increased proteolytic activity 1.7- to 2.4-fold compared to raw milk and the authors suggested using individual peptides to track authenticity of kefir.

Tracking proteolysis during fermentation is useful for determining optimal peptide formation during fermentation and which LAB strains are most efficient at hydrolyzing proteins. Peptide formation has been shown to increase over time in fermented foods with ripening processes such as cured meats and cheeses (Sentandreu & Toldrá, 2007). For example, Bütikofer, Meyer, Sieber and Wechsler (2007) found that hard cheeses with characteristic long ripening periods contained considerably higher IPP and VPP (100 mg/kg) compared with semi-hard (51.6 mg/kg) and soft (3.4 mg/kg) cheeses aged for less time. In contrast, Solieri et al. (2015) observed that IPP and VPP content in cow's milk fermented with a non-starter LAB peaked at 96 hrs and decreased by 30-40% after 111 hrs. A variety of factors contribute to the

formation, stability, and breakdown of the peptides in these two examples, including the source of the milk (bovine, caprine, ovine, etc.), the microbiota involved in the fermentation and their associated enzymatic capabilities, the activity of endogenous milk enzymes, and the incubation or storage conditions of the products. Bioactive peptides formed during fermentation are susceptible to further hydrolysis by bacterial peptidases (Christensen, Dudley, Pederson & Steel, 1999) therefore it is useful to track their concentration throughout the shelf life of a product to determine what concentration of peptides will be delivered to consumers.

1.3.3 Discovery of Bioactive Peptides in Fresh and Fermented Foods

Bioactive peptides have been found in fresh vegetables (Lee, Bae, Lee & Yang, 2006; Suetsuna, 1998) and a variety of fermented products (Sánchez & Vázquez; 2017); however, they are more prevalent in the latter. Therefore, the majority of literature published on food-derived bioactive peptides focuses on fermented products. Furthermore, a large proportion of these studies involve foods of animal, grain, or legume origin such as dairy (FitzGerald & Murray, 2006), cured meats (Escudero et al., 2013), wheat (Rizzello, Cassone, Di Cagno & Gobbetti, 2008) and soy (Gibbs and others 2004; Vallabha & Tiku, 2014) due to their rich protein content. A summary of bioactive peptides and food sources can be found in Table 1.

A pioneering study performed by Nakamura et al. (1995a) purified and identified two ACE-inhibitory tripeptides, IPP and VPP responsible for blood pressure lowering effects of milk fermented by *Lactobacillus helveticus* (Nakamura et al., 1995a). Since this discovery, a multitude of research has been conducted on bioactive peptide generation in dairy products with several reviews covering their presence in milk, cheese, yogurt, kefir and other dairy products (Choi, Sabikhi, Hassan & Anand, 2012; Mohanty, Mohapatra, Misra & Sahu, 2016). Several commercial fermented milk products have capitalized on this research to make heart health claims, including CALPIS from the Calpis company (Japan) and Evolus® from Valio Ltd. (Finland).

Numerous studies on bioactive peptide generation involve hydrolysis with exogenous enzymes or *in vitro* digestion to simulate how food proteins may be digested to liberate bioactive peptides in the gastrointestinal tract. Common approaches involve digesting proteins with trypsin, chymotrypsin, pepsin, Alcalase ® (subtilisin) or a combination of these enzymes and analyzing the digests for specific bioactivities (Chen, Liu, Ye, Cai, Ji & Wu, 2013; He, Malomo,

Alashi, Girgih, Ju & Alulzo, 2013; Panchaud et al., 2012). While this approach holds value for the discovery of peptides and their functionalities, food hydrolysates are not commonly consumed products and would require further processing or refinement to be delivered in an acceptable format to consumers. Conversely, fermented foods are widely available and commonly consumed, serving as ideal candidates for delivery of bioactive peptides.

Compared to dairy, meats and grains, fruits and vegetables have a relatively low protein content and have not been primary areas of bioactive peptide research. However, due to their suitability for lactic acid fermentation and the potent nature of bioactive peptides, fruits and vegetables are worthy substrates for development of fermented foods with enhanced bioactive peptides.

1.3.4 Bioactive peptide discovery and identification in foods

A multitude of studies have been published on bioactive peptide discovery and identification in foods utilizing either empirical or bioinformatic strategies as well as targeted studies aiming to find specific peptides and nontargeted approaches that survey all peptides present (Sánchez-Rivera, Martínez-Maqueda, Cruz-Huerta, Miralles & Recio, 2014). Empirical methods rely heavily on chromatography and mass spectrometry (MS) while bioinformatic methods, also known as *in silico* sequencing, apply enzymatic functions to known protein sequences to predict bioactive sequences that may be liberated. In both of these approaches, databases containing bioactive peptide sequences and activities as well as MS fragmentation information for confirmation of peptide identities are highly utilized (Minkiewicz, Dziuba, Iwaniak, Dziuba & Darewicz, 2008).

The most commonly used approach to bioactive peptide discovery is a "fragment and test" workflow. First, peptides are released through fermentation or enzymatic hydrolysis, Second, the peptides are separated and purified into fractions using high performance liquid chromatography (HPLC) or filtration techniques. Third, peptide fractions are concentrated, fourth, peptides are tested for *in vitro* or *in vivo* bioactivity. Finally, peptides are identified by MS or peptide sequencing (Figure 1) (Arihara, 2013; Pihlanto-Leppälä, Koskinen, Piilola, Tupasela & Korhonen, 2000)). Peptides may also be screened for targeted bioactivity immediately after formation (Sánchez-Rivera et al., 2014). This workflow has been used to identify peptides in milk (Pihlanto et al., 2010; Contreras, Carrón, Montero, Ramos & Recio,

2009), Spanish dry-cured ham (Escudero et al., 2013), soy protein (Vallabha et al., 2014), broccoli (Lee et al., 2006), garlic (Suetsuna, 1998), canary seeds (Valverde, Orona-Tamayo, Nieto-Rendón & Paredes-López, 2017) and oats (Yu et al., 2016) among other foods.

Beginning with the first step of the workflow, hydrolysis, whole foods or protein isolates are fermented naturally, fermented with starter cultures, or subjected to enzymes native to microorganisms or the human digestive tract. During this process an abundance of peptides are released, requiring separation and/or fractionation prior to performing MS analysis, typically carried out by HPLC. Modes utilized for this separation include reversed-phase HPLC, ion exchange chromatography, size exclusion chromatography, hydrophilic interaction chromatography (HILIC), and affinity chromatography (Arihara, 2013). Depending on the food matrix, additional steps may be required to remove interfering substances (i.e., proteins, fats, salt) to prepare them for MS analysis. As instruments have advanced, mass spectrometers have been increasingly used for peptide identification while the popularity of protein and peptide sequencing has decreased (Picariello, Mamone, Addeo & Ferranti, 2012). Due to the complexity of peptide mixtures generated in shotgun approaches, MS with high resolving power are required, such as ion trap (Contreras et al., 2009; Rizzello et al., 2008), orbitrap (Yu et al., 2016), triple quadrupole (QQQ) (Lahrichi, Affolter, Zolezzi & Panchuad, 2013), quadrupole time-of-flight (QTOF) (Rutella et al., 2016; Babini, Tagliazucchi, Martini, Più & Gianotti, 2017), and matrix-assisted laser desorption ionization (MALDI) TOF (Pihlanto et al., 2010). Peptide identity is confirmed using tandem MS (MS/MS) by fragmenting putative precursors and comparing their mass spectra to those of known standards.

More recently, bioinformatic-driven strategies have been developed for bioactive peptide discovery, offering cost, resource and labor-saving alternatives to empirical approaches (Girogorov & van Bladeren, 2007). This *in silico* prediction approach combines knowledge of LAB genomes with food protein amino acid sequences to predict proteolysis of dietary proteins and release of functional peptides (Panchaud et al., 2012). Enzymes produced by LAB as well as human digestive enzymes may be considered in the approach to better predict peptide stability and release during the digestive process. The most promising food and LAB or enzyme combinations can be predicted using this approach, thus reducing the number of fermentation trials and bioactivity tests used in traditional empirical studies. Proteomes of many crops are available, including that of the cucumber *(Cucumis sativus)* allowing for evaluating proteins'

potential for releasing bioactive peptides (Uniprot, Accessed 8/8/2017). Various authors have performed these analyses to discover natural food sources with encrypted bioactive peptide sequences. Khaket, Redhu, Dhanda & Singh (2015) performed an *in silico* analysis of 69 proteins from 17 food commodities looking for peptides with dipeptidyl peptidase-III (DPP-III) inhibitory properties which may be promising treatments for pain management. The analysis found 2659 peptides encrypted within the proteins that shared sequences of previously published DPP-III inhibitory activity. Foods with the highest occurrence frequency of these peptides were corn, pumpkin, soy and rapeseed. Similarly, Lacroix & Li-Chan (2012) evaluated 34 food commodity proteins for DPP-IV inhibitory peptides for treatment of type 2 diabetes. Using the *in silico* approach, they found 2256 fragments matching sequences of previously identified DPP-IV inhibitory peptides with highest occurrence in cow's milk, beef collagen and salmon. Identification of bioactive peptide sequences encrypted within proteins is valuable for assessing the suitability of a food for bioactive peptide release. The next step is to perform the hydrolysis or fermentation to achieve the peptide release and verify the *in silico* predictions.

In addition to the food proteome, fully annotated genomes of LAB commonly responsible for lactic acid food fermentations are available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) database, GenBank, allowing for prediction of proteolytic products based on putative LAB enzyme activity. In line with these resources, Ravenschot et al., (2018) proposed that researchers could utilize *in silico* tools to evaluate proteolytic enzymes of lactic acid bacteria, group strains with similar genetic profiles, then select appropriate substrates to produce bioactive peptides during fermentation. This proposed method would also incorporate components of the empirical approach such as bioactivity testing and sequencing of hydrolysates to verify that the predicted peptides were produced. Both empirical and *in silico* approaches to bioactive peptide discovery hold much promise and the combination of the two will further advance research in the field of bioactive peptides.

1.4 GABA

GABA, a non-protein amino acid, acts as the primary inhibitory neurotransmitter in the mammalian central nervous system, responsible for counteracting excitatory neurotransmitters and inducing a state of calm (McCormick, 1989). The brain synthesizes and metabolizes GABA in a closed-loop system known as the GABA shunt (Figure 2), primarily fed by glucose

metabolites from the Krebs cycle (Olsen and DeLorey 1999). During this process, α-ketoglutarate is transaminated by GABA α-oxoglutarate transaminase (GABA-T) to glutamate. Glutamate is decarboxylated to GABA by glutamate decarboxylase (GAD, EC 4.1.1.15). GABA is metabolized by the same enzyme with which it was formed, GABA-T, into succinic semialdehyde. In general, α-ketoglutarate must be present to receive the amino group removed from GABA which ensures that GABA is conserved. Succinic semialdehyde is oxidized to succinic acid by succinic semialdehyde dehydrogenase and reenters the Krebs cycle. The primary precursor to GABA is L-glutamate, yet others including pyruvate and amino acids have been documented. It is significant to note that in the human body only cells which use GABA as a neurotransmitter express GAD, in effect confining its synthesis to the brain (Olsen and DeLorey, 1999). Despite this fact, positive health effects have been reported due to the consumption of GABA and efforts have been made to develop foods with enhanced GABA content.

1.4.1 Health Benefits Associated with GABA Consumption

It is commonly accepted in the medical community that GABA does not pass the blood-brain barrier thereby rendering GABA supplementation questionable with regards to directly effecting brain chemistry. In contrast, a handful of studies suggest mechanisms by which the blood brain barrier may become more permeable to GABA. Kakee et al., (2001) found that excessive levels of GABA in the brain were excreted into the bloodstream in an animal model, while Shyamaladevi, Jayakumar, Sujatha, Paul & Subramanian (2002) suggested that nitric oxide production can increase the permeability of the blood brain barrier to GABA. Despite GABA's extremely limited passage from the blood stream to the brain, oral supplementation of GABA and consumption of foods containing GABA have been shown to decrease blood pressure (Hayakawa, Kimura, Kasaha, Matsumoto, Sansawa & Yamori, 2004; Nishimura et al., 2016; Zareian, Oskoueian, Forghani & Ebrahimi, 2015), reduce anxiety (Abdou, Higashiguchi, Horie, Kim, Hatta & Yokogoshi, 2006; Hinton, Jelinek, Viengkhou, Johnston & Slade, 2019; Yoto et al., 2011), improve decision making (Steenbergen, Sellaro, Stock, Beste & Colzato, 2015), improve short-term working memory (Reid, Ryu, Kim & Jeon, 2018), improve temporal attention (Leonte, Colzato, Steenbergen, Hommel & Ayürek, 2018) and aid sleep (Yamatsu, Yamashita, Pandharipande, Maru & Kim, 2016) (Table 2). Effective doses of GABA

administered to participants in the aforementioned studies ranged from 2.01 mg to 800 mg and were either administered once or on a daily basis for one week. Over-the-counter GABA supplements containing between 100 and 750 mg GABA are available in the United States, and bear claims to reduce stress and promote relaxation.

The positive health effects associated with oral GABA intake are hypothesized to be related to its effects on the peripheral nervous system. Hayakawa, Kimura & Kamata (2002) explored this mechanism in spontaneously hypertensive rats, determining that the antihypertensive effect of orally administered GABA was due to GABA's interaction with presynaptic GABA$_B$ receptors, thus reducing noradrenaline release and reducing blood pressure. Both GABA and GABA$_B$ receptors have been found throughout the human gastrointestinal (GI) tract, including the enteric nerves, endocrine-like cells, epithelial layers and muscle cells, suggesting that GABA plays a role in GI function and endocrine mediation (Hyland & Cryan, 2010). This knowledge has even led to the development of probiotics genetically engineered to increase GABA production *in vitro* (Park, Ji, Park & Oh, 2005).

1.4.2 Formation of GABA by LAB

In addition to GABA formation within the mammalian nervous system, prokaryotic bacteria are also capable of producing GABA. In an effort to protect themselves from intracellular pH decline, bacteria utilize several amino acid-dependent acid resistance systems, one of which results in the production of GABA. During this reaction, glutamate is decarboxylated by GAD to form GABA, consuming a proton and aiding in cytosolic pH homeostasis (Figure 3) (Feehily & Karatzas, 2013). The GAD system is widely distributed in LAB (Cui, Miao, Niyaphorn & Qu, 2020) as well as human pathogens such as *Listeria monocytogenes* (Feehily, O'Byrne & Karatzas, 2013) and *Escherichia coli* (Plokhov, Gusyatiner, Yampolskaya, Kaluzhsky, Sukhareva & Schulga, 2000). Among LAB specifically, the GAD system has been characterized in *Lactobacillus lactis* (Sanders, Leenhouts, Burghoorn, Brands, Venema & Kok, 1998), *Lactobacillus brevis* (Ueno Hayakawa, Takahashi & Oda, 1997), *L. delbrueckii* subsp. bulgaricus (Siragusa, De Angelis, Di Cagno, Rizzello, Coda & Gobbetti, 2007), *Lactobacillus paracasei* (Komatsuzaki, Nakamura, Kimura & Shima, 2008), and *Lactobacillus plantarum* (Shin et al.¸ 2014).The main genes involved in the GAD system present in LAB include *gadB* which encodes GAD and *gadC* which encodes a glutamate-GABA

antiporter known as GadC, both of which are organized in an operon, *gadCB* (Feehily et al., 2013). Sanders et al. (1998) determined that a transcriptional activator, *gadR*, regulates a promotor, *Pgad*, of the *gadCB* operon and that while *gadR* is constitutively expressed, *gadCB* is maximally expressed during the onset of stationary phase when glutamate and sodium chloride (NaCl) are present and pH drops below 6. The common distribution of the GAD system in LAB make lactic acid fermented foods likely sources of GABA.

1.4.3 Presence of GABA in Fresh and Fermented Fruits, Vegetables, and Other Foods

GABA is naturally found in fresh and fermented products; however, it is more prevalent in the latter. GABA content of fermented foods typically falls within the mg/kg range (Table 3). Interest in enhancing lactic acid fermented foods' natural concentration of GABA has resulted in the identification of suitable organisms with high-GABA producing capabilities to serve as starter cultures. These starter cultures may be used as the main LAB responsible for fermentation or may be used to compliment natural microbiota or starter cultures already employed in the production of a food. For example, Pouliot-Mathieu et al., (2013) found that cheddar cheese fermented with a standard starter culture of *Lactococcus lactis* spp. *cremoris* W62 did not contain GABA; however, when a GABA-producing of *L. lactis* (spp. ULAAC-H13) was utilized in addition to the starter culture, 320 mg/kg GABA was produced. Lee, Shim, Yao, Kim & Kim (2018) found that naturally fermented kimchi contained a maximum of 620 mg/kg GABA and that inoculation with GABA-producing *Lactobacillus zymae* isolated from kimchi resulted in a product with slightly greater GABA, at 690 mg/kg. Altering the ingredients in the kimchi by adding sea kelp containing glutamate increased the GABA concentration to 822 mg/kg, demonstrating that starter cultures as well as substrates may be manipulated during development of GABA-enhanced foods.

GABA formation in fermented foods can vary significantly depending on growth conditions, geographical regions, the population of natural microbiota, fermentation technique and environmental conditions. Marseglia, Palla & Caligiani (2014) measured GABA content in fermented cocoa beans from Africa, Asia, Central/South America and Oceania finding that a minimum of 317 mg/kg GABA was formed in cocoa beans from Grenada and a maximum of 1,020 mg/kg GABA was formed in Ecuadorian cocoa beans. In addition to the geographical differences in cocoa bean samples, fermentation technique also varies between regions with

some manufacturers using a system of boxes and others using piles for fermentation. Additionally, microbiota introduced from the environment, especially from banana leaves used to cover cocoa beans during fermentation, may affect GABA formation. Similarly, the impact of microbiota and fermentation technique upon GABA formation in cheese was studied by Siragusa et al. (2007). The authors surveyed 22 commercially produced Italian cheese varieties, reporting a range of GABA concentrations from 0.260 to 391 mg/kg, noting that GABA content was positively correlated with ripening time. Furthermore, 440 LAB were isolated from the cheese samples and screened for GABA producing capabilities. Only 61 isolates, mostly *Lb. plantarum* and *Lb. paracasei*, were able to synthesize GABA, confirming that GABA-forming microbiota are the major prerequisite for manufacturing GABA-enriched cheeses. Fermentation techniques also have the capacity to effect GABA formation in tea. Wu et al., 2014 found that repeated anaerobic incubation periods increased GABA formation in Maoyecha tea (*Camellia ptilophylla* H.T. Chang).

Despite high GAD expressing activity of LAB, GABA production may be limited due to the amount of glutamate present in the food matrix. For this reason, glutamate, often in the form of monosodium glutamate (MSG) may be added to fermentations to provide more substrate for GABA production, potentially reaching concentrations in the g/kg range. Kim, Lee, Ji, Lee & Hwang (2009) developed a GABA-enhanced, lactic acid fermented black raspberry juice by adding 2% w/v (~118 mM) MSG prior to fermentation. The resulting product contained 26.9 mg/ml (260 mM, ~25.2 g/kg) GABA, suggesting that both naturally present and exogenously added glutamate were converted to GABA during the fermentation. Similarly, water dropwort *(Oenanthe javanica* DC) with 3% added MSG was fermented using a co-culture of *Leuconostoc mesenteroides* SM and *Lb. plantarum* K154, resulting in a final GABA content of 10 mg/ml (96.9 mM, ~9.35 g/kg) (Kwon, Garcia, Song & Lee, 2016). Fermentation using LAB and added glutamate to enhance GABA in value-added products has also been investigated. Di Cagno et al. (2010) converted an agricultural surplus product, grape must, into a GABA-enhanced functional beverage by adding 18.4 mM L-glutamate and fermenting with a GABA producing strain isolated from cheese, *L. plantarum* DSM19463. The resulting beverage contained 4.83 mM (~465 mg/kg) GABA. Not all foods supplemented with glutamate result in large concentrations of GABA, however. Honey, supplemented with 507 mM glutamic acid and fermented for 53 hrs with LAB isolated from bee stomach produced only 4.3 mM (~306 mg/kg) GABA, suggesting

that the organism chosen for the fermentation did not efficiently convert glutamate to GABA (Gharehyakheh, Elhami Rad, Nateghi & Varmir, 2019). While addition of glutamate to food substrates prior to fermentation may result in increased GABA content in the final product, consumers may be weary of added ingredients on the label, especially MSG. Product developers creating these types of products must take into consideration consumer attitudes and may want to focus on consumer education and marketing to explain the technology used in the fermentation/manufacturing process.

1.5 Lactic Acid Fermentation of Cucumbers

1.5.1 Cucumber Composition and Fermentation Biochemistry

Compositionally, cucumbers (*Cucumis sativus*) contain ~97.5% water, 2% sugar (Breidt, McFeeters, Pérez-Díaz & Lee 2013) and 0.65% protein (USDA Food Composition Database, https://ndb.nal.usda.gov/ndb/search/list, Accessed 10.10.16). Sugars – glucose and fructose – are the primary energy sources utilized by LAB, and over the course of fermentation 110-140 mM lactic acid will be produced, resulting in an equilibrated pH of 3.2-3.6 (Pérez-Díaz et al., 2013). Cucumber proteins may serve as substrates for bioactive peptide formation by either microbial or cucumber-associated proteolytic enzymes. A full free amino acid profile of cucumbers has not been published, however an early manuscript by Costilow & Fabian (1953) demonstrated that six amino acids essential for growth of *Lb. plantarum* were present in cucumber (leucine, isoleucine, valine, tryptophan, glutamate, and cysteine). More recently, the USDA Food Composition Database provided a total amino acid profile for cucumbers, identifying glutamate as the most abundant amino acid at 0.169 g/100g (USDA Food Composition Database, https://ndb.nal.usda.gov/ndb/search/list, Accessed 10.10.16). It is likely that this value is the sum of both glutamine and glutamate given that no glutamine value was listed. Often amino acid analyzers either cannot chromatographically separate glutamate and glutamine, or glutamine is converted to glutamate for quantification, leading to the declaration of both amino acids as glutamate. Despite this ambiguity, these two data sources support that cucumbers may serve as glutamate sources for GABA production by LAB during fermentation.

1.5.2 Commercial Cucumber Fermentation and Processing Practices

Fermentation of pickling cucumbers is practiced on both commercial and artisan scales. For commercially produced pickles, fresh cucumbers are added to large capacity, outdoor tanks, some reaching up to 40,000 L in volume. A high-salt brine (0.6-1.7 M NaCl) is added to the tanks and the cucumbers undergo fermentation by autochthonous LAB until less than 0.05% sugar (glucose and fructose) remains. After fermentation, cucumbers are stable in fermentation brine for up to 9 months prior to further processing. The next step in processing typically involves desalting which is necessary to remove excess NaCl and make pickles acceptable to consumers (typically ~2% NaCl). Next, fermented cucumbers are packed into containers either as whole fruit, chips, or spears. Containers are filled with fresh cover brine containing NaCl and vinegar and may also include preservatives (i.e., potassium sorbate), coloring agents (i.e., turmeric, yellow #5), and flavorings (i.e., dill oil). Containers are sealed and some are pasteurized for additional shelf stability. The majority of commercially produced products are manufactured in this manner, however alternative processes exist. Some fermented cucumbers are packed directly in their fermentation brine and either pasteurized or stored refrigerated for immediate consumption, or they may be desalted, packed in fresh cover brine and stored refrigerated.

LAB primarily involved in cucumber fermentation include *Lactobacillus pentosus, Lb. plantarum, Lb. brevis, Leuconostoc spp.*, *Lactococcus spp., Pediococcus ethanolidurans* and *Weissella spp*. (Pérez-Díaz et al., 2016). Starter cultures are not currently used on a widespread level commercially; however, several researchers have investigated development of these cultures for quality improvement. Etchells, Bell, Fleming, Kelling & Thompson (1973) proposed the use of starter cultures to reduce defects found in naturally fermented cucumbers including bloaters, shriveled, discolored, or malodorous pickles. Furthermore, Zhai, Pérez-Díaz, Diaz, Lombardi & Connelly (2018) evaluated malic acid decarboxylase-deficient starter cultures for their ability to reduce the occurrence of bloaters in NaCl-free cucumber fermentations. Development of a starter culture appropriate for manufacturing kosher certified products has also been completed (Pérez-Díaz and McFeeters, 2011)

1.5.3 Opportunities for Bioactive Compound Production in Fermented Cucumbers

Limited research has been performed to elucidate potential health promoting compounds in fermented cucumbers. Lactic acid fermented foods are often touted for containing live organisms, however due to long-term storage and/or pasteurization, fermented cucumbers are not suitable candidates for delivering live organisms to consumers. While the inclusion of probiotic bacteria in refrigerated cucumbers was explored by Fan, Breidt, Price & Pérez-Díaz (2017), only one probiotic strain of *Lb. casei* was found to survive for 63 days at 10^8 CFU/ml, which is a relatively brief period when considering food distribution systems. Significant advances would need to be made in the development of acid-resistant probiotic LAB in order to produce pickle products with probiotic benefits. That said, research into the non-probiotic benefits of lactic acid fermented cucumbers should be performed to assess their potential to impact human health.

Fresh cucumbers contain protein, however, little knowledge exists regarding compositional changes in protein, the formation of peptides, release of free amino acids or the conversion of glutamate to GABA during cucumber fermentation. Quantification of these compounds before and after fermentation will provide important information about LAB's proteolytic activity within this specific matrix, whether or not peptides are formed during the process, and the capability of cucumber associated LAB to convert glutamate to GABA.

1.6 Hypotheses & Objectives

Lactic acid fermentation of cucumbers is an established, commercially relevant process that transforms a perishable, agricultural commodity into a shelf-stable product. Apart from economic benefits of cucumber fermentation, potential exists for the discovery and enhancement of health-promoting compounds through manipulation of fermentation conditions. We hypothesize that bioactive peptides will be liberated from cucumber protein during fermentation by LAB-associated proteolytic enzymes and that the naturally occurring LAB will convert glutamate to GABA during fermentation. The research objectives are to identify and quantify bioactive peptides in fresh, acidified and fermented cucumbers and to quantify changes in free amino acids during cucumber fermentation.

1.7 Significance

  The results of this investigation will contribute to the both the academic and public sector's limited knowledge of health promoting compounds produced during vegetable fermentations. These findings may provide credence and practical explanations for the popularly held belief that fermented foods are healthy, leading to their increased consumption. Analysis of peptide and amino acid profiles of fresh and fermented cucumbers will provide new insight into the microbial impact on and biochemistry of fermented vegetables. The approach and methods developed in this study may also be applied to similar fermented vegetable products such as kimchi and sauerkraut to investigate their potential bioactive compounds. Finally, stakeholders and members of the pickling industry will benefit from the discovery of heretofore unknown compositional changes in proteins, peptides, and amino acids during fermentation. Future studies may explain how changes in protein and protein-derived components relate to manufacturing, fermentation attenuation, and product quality. Furthermore, evidence of beneficial compounds in the brine fraction may prompt development of value-added products and novel uses for spent brine.

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Table 1. Bioactive peptides identified from fermented and hydrolyzed foods

Food	Hydrolysis Source	Sequence	Bioactivity	IC$_{50}$	Reference
Anchovy	Natural LA fermentation	KP	ACE inhibitory	22 µM	Ichimura et al., 2003
Anchovy, bonito	Natural LA fermentation	RP	ACE inhibitory	21 µM	
Anchovy, sardine, bonito	Natural LA fermentation	AP	ACE inhibitory	29 µM	
Barley	Hydrolysis - pancreatin & trypsin		Antithrombotic	N/A	Yu et al., 2016
Bonito	Hydrolysis - chymotrypsin, pepsin & trypsin	IKP	ACE inhibitory	1.7 µM	Yokoyama et al., 1992
Bovine skin hydrolysate	Hydrolysis - Alcalase ®, collagenase & Pronase E	GPV	ACE inhibitory	4.67 µM	Kim et al., 2001
Broccoli	Raw	YPK	ACE inhibitory	23.7 µM	Lee et al., 2006
Buckwheat	Hydrolysis - chymotrypsin, pepsin & trypsin	AY	ACE inhibitory	100 µM	Li et al., 2002
		FY		25 µM	
		ITF		49 µM	
		LGI		29 µM	
		PSY		16 µM	
		VK		13 µM	
		YQ		628 µM	
Buckwheat	Raw	GPP	ACE inhibitory	23 µM	Ma et al., 2006
Buckwheat	Hydrolysis - pancreatin & trypsin		Antithrombotic	N/A	Yu et al., 2016
Buckwheat sprouts	*Lactobacillus plantarum* KT	FQ	ACE inhibitory	Not reported	Koyama et al., 2013
		VAE		Not reported	
		VVG		Not reported	
		DVWY		Not reported	
		FDART		Not reported	
		WTFR		Not reported	

Table 1. (cont.)

Canary Seeds	Hydrolysis - pancreatin & pepsin	LSLGT	Antioxidant	N/A	Valverde et al., 2017
		TDQPAG		N/A	
		QQLQT		N/A	
		FEPLQLA		N/A	
		KPQLYQPF		N/A	
Canola	Hydrolysis - Alcalase ®	VSV	ACE inhibitory	0.15 μM	Wu et al., 2008
Cauliflower	Hydrolysis - Alcalase ®	Unknown	Antioxidant	8.3 μM	Caliceti et al., 2019
Cumin seeds	Hydrolysis - amylase, lipase & pepsin	Unknown	Hypocholesterolemic	N/A	Siow et al., 2016
Fermented soybean paste	Natural LA fermentation	HHL	ACE inhibitory	5 μM	Shin et al., 2001
Fig tree latex	Boiled	LVR	ACE inhibitory	14 μM	Maruyama et al., 1989a
Fish (*Aluterus monocero*)	Hydrolysis - glycyl endopeptidase	EGPLG	Antioxidant	N/A	Karnjanapratum et al., 2017
Garlic	Raw	GF	ACE inhibitory	277.9 μM	Suetsuna 1998
		FY		3.74 μM	
		GY		72.1 μM	
		NF		46.3 μM	
		SY		66.3 μM	
		NY		32.6 μM	
		SF		130.2 μM	
Jellyfish	Hydrolysis - Alcalase ®	VKP	ACE inhibitory	1.3 μM	Li et al., 2014
		VKP	Antioxidant	N/A	
Kamut khorasan flour	*Lb. plantarum* 98a or *Lb. rhamnosus* C249	VLPPQQQY	Antioxidant	N/A	Babini et al., 2017
Lupin	Hydrolysis - pepsin	Mixture	ACE inhibitory		Boschin et al., 2014
Lupine flour	Hydrolysis - neutrase	FVPY	Antioxidant	N/A	Babini et al., 2017
Mackerel	Hydrolysis - aspartic protease & pepsin	Mixture	ACE inhibitory		Khiari et al., 2014
		Mixture	Antithrombotic	N/A	

Table 1. (cont.)

Maiz	Synthesized	LPP	ACE inhibitory	9.6 µM	Maruyama et al., 1989b
Maiz	Hydrolysis - thermolysin	FY	ACE inhibitory	25 µM	Miyoshi et al., 1991
		LRP		0.27 µM	
		LSP		1.70 µM	
		IRA		6.4 µM	
		LAA		13 µM	
		LAY		3.9 µM	
		LLP		57 µM	
		LNP		43 µM	
		LRP		0.27 µM	
		LQP		1.9 µM	
		LQQ		100 µM	
		VAY		16 µM	
Micro algae	Hydrolysis - pepsin	FAL	ACE inhibitory	26.3 µM	Suetsuna et al., 2001
		IAE		34.7 µM	
		IAPG		11.4 µM	
Milk	Hydrolysis - thermolysin	LLF	ACE inhibitory	79.8 µM	Hernández-Ledesma et al., 2002
Milk	*Lb. helveticus*	IPP	ACE inhibitory	5 µM	Nakamura et al., 1995a
		VPP		9 µM	
Milk	Hydrolysis - chymotrypsin, pepsin & trypsin	YGL	ACE inhibitory	409 µM	Pihlanto-Leppälä et al., 2000
Milk	*in vitro* digestion	AW	ACE inhibitory	5 µM	Rutella et al., 2016
		VY		5 µM	
		FFVAP		6 µM	
Milk	*Lb. casei, Lb. rhamnosus*	Not reported	ACE inhibitory	54.57 µM	Solieri et al., 2015
		Not reported	Antioxidant	N/A	
Milk	Hydrolysis	LN	ACE inhibitory	Not reported	van Platerink et al., 2008

Table 1. (cont.)

Milk	*Lactobacillus helveticus* CPN4	YP	ACE inhibitory	720 μM	Yamamoto et al., 1999
Milk (casein)	Hydrolysis - neutrase	TDVEN	Hypocholesterolemic	N/A	Jiang et al., 2020
		LQPE		N/A	
		VAPFPE		N/A	
		VLPVPQ		N/A	
Milk (casein)	Hydrolysis - carboxypeptidase A & chymotrypsin	FGK	ACE inhibitory	160 μM	Maruyama et al., 1987a
		PQR		400 μM	
		PYP		220 μM	
		PLW	Antihypertensive	18 μM	
		TTMPLW		16 μM	
Milk (casein)	Hydrolysis	AVP	ACE inhibitory	340 μM	Meisel 1993
Milk (casein)	Hydrolysis - trypsin	TVY	ACE inhibitory	15 μM	Tauzin et al., 2002
Milk (whey protein)	Hydrolysis	II	Cellular glucose uptake stimulator	N/A	Morifuji et al., 2009
		IV		N/A	
		IL		N/A	
		LI		N/A	
		LL		N/A	
		LV		N/A	
		VL		N/A	
Muscle tissue	Naturally occurring	AH	Antioxidant	N/A	Boldyrev et al., 1988
Muscle tissue	Naturally occurring	AH	Antioxidant	N/A	Chan et al. 1994
Mushroom	Naturally occurring	GEP	ACE inhibitory	3200 μM	Lee et al., 2004
Oat	Hydrolysis - pancreatin & trypsin		Antithrombotic	N/A	Yu et al., 2016
Pea	Hydrolysis - Alcalase ®	EF	ACE inhibitory	2980 μM	Li et al., 2010
		IR		2250 μM	
Pea flour	Hydrolysis - flavourz	TVTSLDLPVLRW	Antioxidant	N/A	Babini et al., 2017
		VTSLDLPVLRW	Antioxidant	N/A	

Table 1. (cont.)

Porcine muscle	Hydrolysis - porcine dipeptidyl peptidases	AA	ACE inhibitory	51.4 µM	Sentandreu et al., 2007
		AR		95.5 µM	
		GP		66 µM	
		GR		162.2 µM	
		KA		31.5 µM	
		RP		15.2 µM	
		RR		267.1 µM	
Porcine skeletal muscle	Hydrolysis - thermolysin	MNP	ACE inhibitory	66.6 µM	Arihara et al., 2001
		NPP		290.5 µM	
		TNP		207.4 µM	
Rapeseed	Hydrolysis - pepsin, pancreatin	GHS	ACE inhibitory	1740 µM	He et al., 2013b
Rapeseed	Hydrolysis - subtilisin	VW	ACE inhibitory	1.6 µM	Marczak et al., 2003
		IY		3.7 µM	
		RIY		28 µM	
		VWIS		30 µM	
Red-mold rice extracts	*Monascus purpureus* IFO 44	VF	ACE inhibitory	47.9 µM	Kuba et al., 2009
		IY		4.0 µM	
		VW		3.1 µM	
		VVY		22.0 µM	
Rice	Hydrolysis - Alcalase ®	VNP	ACE inhibitory	6.4 µM	Chen et al., 2013
		VWP		4.5 µM	
Rice protein	Hydrolysis - trypsin	GYPMYPLPR	Immunomodulatory	N/A	Takahashi et al., 1994
Sake and sake lees	Fermentation - yeast	RY	ACE inhibitory	10.5µM	Saito et al., 1994
		GGY		1.3 µM	
		IY		2.4 µM	
		PR		4.1 µM	
Sardine hydrolysate	Hydrolysis - *Bacillus licheniformis* protease	AKK	ACE inhibitory	3.13 µM	Matsufuji et al., 1994
		KW		1.63 µM	
		RY		51 µM	

Table 1. (cont.)

Sesame	Hydrolysis - thermolysin	IVY	ACE inhibitory	14.74 μM	Nakano et al., 2006
		LSA		7.81 μM	
		LVY		1.8 μM	
Sesame	Hydrolysis - Alcalase ®, pepsin & trypsin	LAN	Zinc chelating	N/A	Wang et al., 2012
		NCS		N/A	
		SM		N/A	
Sourdough	LAB - various	Mixture	Antioxidant	N/A	Coda et al., 2012
Soy	Hydrolysis - protease S	PHH	Antioxidant	N/A	Chen et al., 1996
Soy	Hydrolysis - endoprotease (B*acillus* sp.)	KA	Hypotriglyceridemic	N/A	Inoue et al., 2011
Soy (tempeh)	Hydrolysis - multiple enzymes	Mixture	Antithrombotic	N/A	Gibbs et al., 2004
Soy protein hydrolysate	Hydrolysis - pepsin	IA	ACE inhibitory	153 μM	Chen et al., 2002
Soy protein hydrolysate	Hydrolysis - Alcalase ®	DG	ACE inhibitory	12.3 μM	Wu et al., 2002
		DLP	ACE inhibitory	4.8 μM	
Sweet potato	Hydrolysis - trypsin	AH	ACE inhibitory	523.5 μM	Huang et al., 2008
		RF		392.2 μM	
		FK		265 μM	
		GFR		94 μM	
		IMVAEAR		84 μM	
		GPCSR		62 μM	
		CFCTKPC		1.3 μM	
		MCESASSK		76 μM	
Sweet potato	Hydrolysis - Thermoase PC10F, Protease S, Proleather FG-F	GQY	ACE inhibitory	52.3 μM	Ishiguro et al., 2012
		IIP		80.8 μM	
		ITP		9.5 μM	
		STYQT		300.4 μM	
Tilapia	Hydrolysis - Protamex	Mixture	Antibacterial	N/A	Robert et al., 2015

Table 1. (cont.)

Wheat	Hydrolysis - *Bacillus licheniformis* alkaline protease & *Aspergillus niger* protease	AF	ACE inhibitory	15.2 μM	Matsui et al., 1999
		VF		9.2 μM	
		IVY		0.48 μM	
		TF		17.8 μM	
Wheat	Hydrolysis - pepsin & Protease M	IAP	ACE inhibitory	2.7 μM	Motoi et al., 2003
Wheat	Autolysis	LRP	ACE inhibitory	0.21 μM	Nogata et al., 2009
		VY		21 μM	
		TF		18 μM	
Whey protein	Hydrolysis - proteinase K	FP	ACE inhibitory	315 μM	Abubakar et al., 1998
		GKP		352 μM	

Table 2. Health benefits of GABA

Health benefit	Subject	Dose	Food/Format	Organism	Reference
Anti-anxiety	Rats	2 mg/kg for 21 days	Drinking water	N/A	Yongjian et al., 2019
Antihypertensive	Rats	0.5 mg GABA/kg body weight, single dose	Fermented milk beverage	*Lb. casei* strain Shirota, *Lactococcus. lactis* YIT 2027	Hayawaka et al., 2004
Antihypertensive	Human	10 - 12 mg daily for 12 weeks	Fermented milk beverage	*Lb. casei* strain Shirota, *Lactococcus. lactis* YIT 2027	Inoue et al., 2003
Antihypertensive	Human	168 mg daily for 8 weeks	GABA-enriched white rice	N/A	Nishimura et al., 2016
Antihypertensive	Human	16 mg daily for 12 weeks	Cheese	*Lc. lactis* ssp. *lactis* ULAAC-H13	Pouliot-Mathieu et al., 2013
Antihypertensive	Rats	3.9% w/w soy sauce diet for 6 weeks	Reduced sodium soy sauce (1 g/100 ml (~9.26 g/kg GABA)	*Lactobacillus rennini*	Yamakoshi et al., 2007
Antiobesity	Mice	0.12 - 0.2% GABA for 20 weeks	Drinking water		Xie et al., 2014
Immunity enhancement	Human	100 mg, single dose	GABA in water	N/A	Abdou et al., 2006
Improve temporal attention	Human	800 mg, single dose	GABA in orange juice	N/A	Leonte et al., 2018
Improved decision making	Human	800 mg, single dose	Powder	N/A	Steenbergen et al., 2015
Improved sleep, antidepressant	Human	26.4 mg, single dose	Defatted rice germ with added GABA	N/A	Okada et al., 2000
Occupational fatigue	Human	50 mg, single dose	Hypotonic beverage	N/A	Kanehira et al., 2011

Table 2 (cont.)

Short-term working memory	Human	Not reported	Fermented sea tangle (*Laminaria japonica* A)	*Lactobacillus brevis* BJ20	Reid et al., 2018
Sleep	Human	100 mg per day for 1 week	Capsule	N/A	Yamatsu et al., 2016
Stress reduction	Human	100 mg, single dose	GABA in water	N/A	Abdou et al., 2006
Stress reduction	Human	2.01 mg, single dose	Oolong tea	N/A	Hinton et al., 2019
Stress reduction	Human	28 mg, single dose	Chocolate with added GABA	N/A	Nakamura et al., 2009
Stress reduction	Human	100 mg per day for 2 days	Capsule	N/A	Yoto et al., 2011
Weight management - increased lean body mass	Human	100 mg per day for 12 weeks	Whey protein shake	N/A	Sakashita et al., 2019

Table 3. GABA content in foods

Food/Format	Glutamate Added?	Concentration	Organism	Author
Black raspberry juice	X	26.9 mg/ml (~25.2 g/kg)	*Lb. brevis* GABA 100	Kim et al., 2009
Cheese, cheddar		320 mg/kg	*Lc. lactis* ssp. *lactis* ULAAC-H13	Pouliot-Mathieu et al., 2013
Cheese, Italian		0.260 to 391 mg/kg	Various LAB	Siragusa et al., 2007
Cheese, Spanish		100 - 980 mg/kg	Various LAB	Diana et al., 2014a
Cocoa beans		317 - 1012 mg/kg	Various LAB	Marseglia et al., 2014
Grape must	X	4.83 mM (~465.9 mg/kg)	*Lb. plantarum* DSM19463	Di Cagno et al., 2010
Honey	X	4.3 mM (~305.8 mg/kg)	*Lactobacillus sp.* Makhdzir Naser-1 (GQ451633)	Gharehyakheh, et al., 2019
Kimchi		616.5 mg/kg	*Lb. buchneri*	Cho et al., 2011
Kimchi		620 mg/kg	Natural fermentation	Lee et al., 2018
Kimchi	X	1.18 g/kg	*Lb. zymae* GU240	Lee et al., 2018
Kimchi with sea kelp extract	X	822 mg/kg	*Lb. zymae* GU240	Lee et al., 2018
Maoyecha tea		0.12 - 2.70 mg/g dry weight basis (~0.11 - 1.8 mg/kg)	Natural fermentation	Wu et al., 2014
Milk		27.1 ug/ml (~26.2 mg/kg)	*Lc. lactis* ssp. *lactis*	Nomura et al., 1998
Milk		144.5 mg/kg	*Lc. lactis* DIBCA2, *Lb. plantarum* PU11	Nejati et al., 2013
Milk		109.7 mg/kg	*Lb. helveticus*	Sun et al., 2009
Red seaweed (*Gracilaria fisheri*)	X	4156 mg/L (~3.9 g/kg)	*Lb. plantarum* DW12	Ratanaburee et al., 2011
Sourdough		504 mg/kg	*Lb. plantarum* C48	Coda et al., 2010
Sourdough		39 mg/kg	*Lb. brevis* A7, *Lb. farciminis* A11	Venturi et al., 2019
Sourdough		258.71 mg/kg	Natural fermentation	Rizzello et al., 2008
Sourdough		242 mg/kg	*Lb. brevis* CECT 8183	Diana et al., 2014b

Table 3 (cont.)

Sourdough with added protease		350.5 mg/kg dry weight basis (~218.7 mg/kg)	*Lb. brevis* CECT 8183	Penas et al., 2015
Water dropwort (*Oenanthe javanica* DC)	X	10 mg/ml (~9.35 g/kg)	*Lc. mesenteroides* SM, *Lb. plantarum* K154	Kwon et al., 2016
Wheat and rice fermentation (*dosa*)		143 mg/kg	*Lb. plantarum*	Zareian et al., 2015
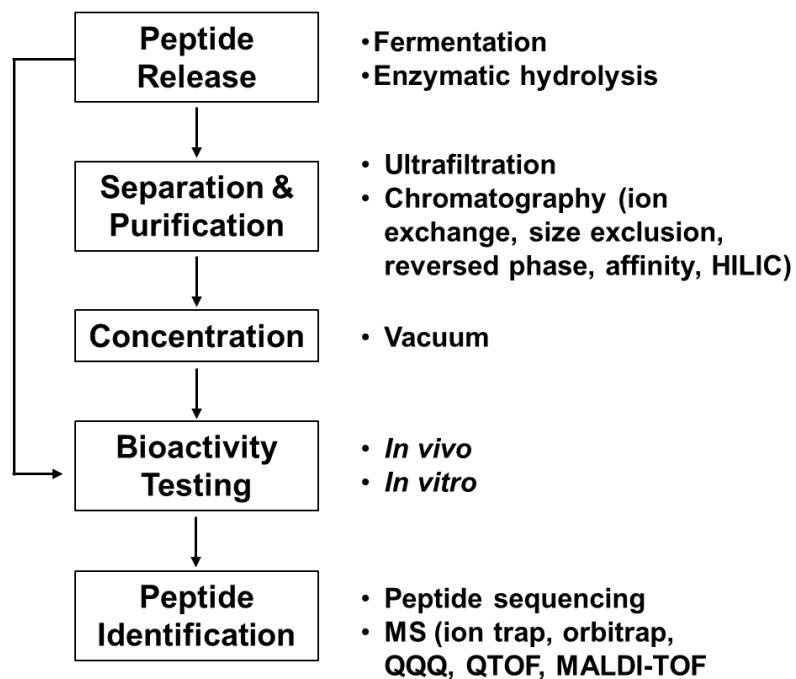
Figure 1. Empirical bioactive peptide discovery, testing and identification workflow.
HILIC, hydrophilic interaction liquid chromatography; MS, mass spectrometry; QQQ, triple
quadrupole; QTOF, quantitative time-of-flight; MALDI-TOF, matrix-assisted laser desorption
ionization time-of-flight.
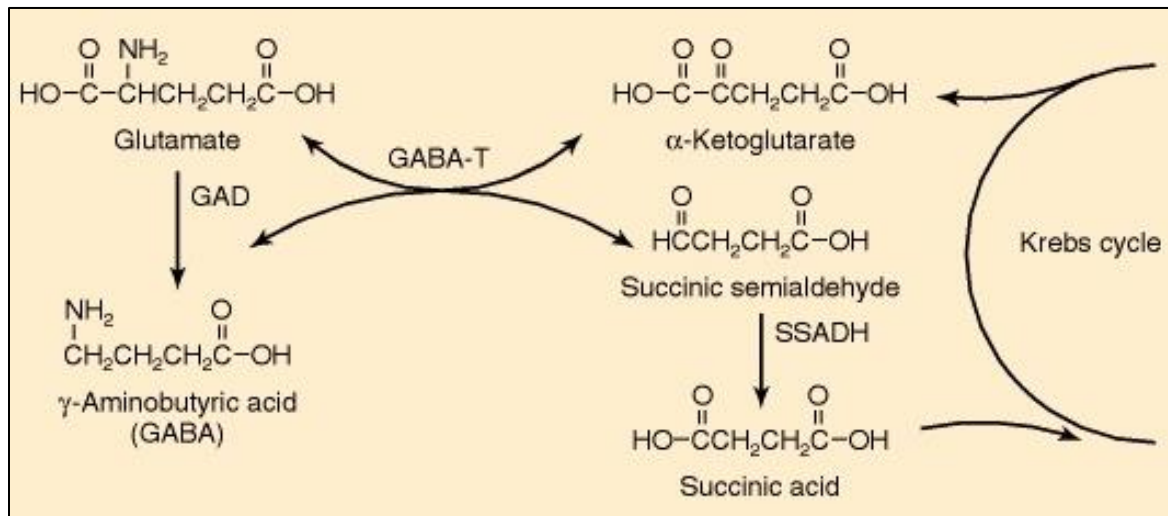
Figure 2. Mammalian GABA shunt (Olsen et al., 1999)
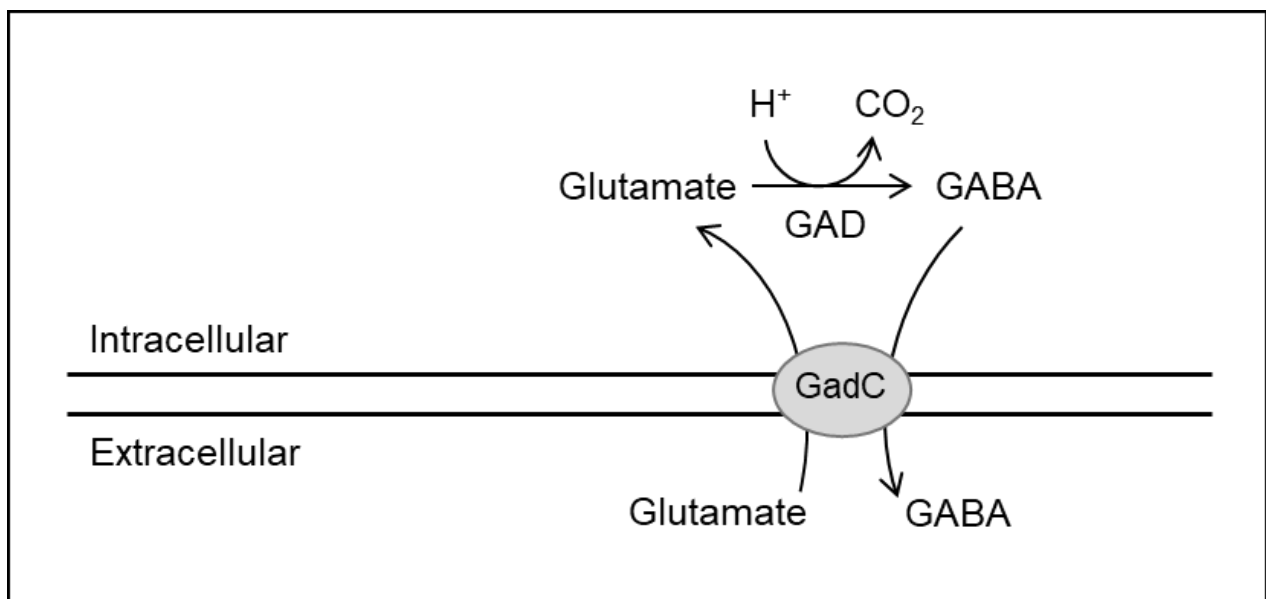
Figure 3. Schematic representation of glutamate decarboxylase pathway in lactic acid bacteria. GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; GadC, glutamate-GABA antiporter.

Chapter 2: Discovery and quantification of bioactive peptides in fermented cucumber by direct analysis IR-MALDESI mass spectrometry and LC-QQQ-MS

2.1 Abstract

Bioactive peptides have been identified in lactic acid bacteria fermented foods including cultured milk, sourdough, and cured meats; however, their presence has not been investigated in fermented vegetables. Here, direct analysis infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) mass spectrometry (MS) was employed to identify bioactive peptides in fermented cucumbers. Natural and starter culture fermented cucumbers were prepared in triplicate in sodium chloride brines and compared to acidified cucumbers. Putative matches of known food-derived bioactive peptides were identified by direct analysis using IR-MALDESI-MS. Peptides were confirmed by IR-MALDESI MS/MS and quantified by LC-MS/MS. Three angiotensin converting enzyme (ACE) inhibitory peptides, IPP (0.42-0.49 mg/kg), LPP (0.30-0.33 mg/kg), and VPP (0.32-0.35 mg/kg) were formed in fermented cucumbers. A fourth ACE inhibitory peptide, KP (0.93-1.5 mg/kg), was enhanced 3-5 fold in fermented cucumbers compared with acidified cucumbers. This work demonstrates that lactic acid bacteria fermentation can enhance bioactive peptide content in vegetables.

2.2 Introduction

Worldwide, consumption of fermented foods is commonly perceived as healthful. Research on the potential health benefits of fermented foods has primarily focused on the ingestion of live microorganisms and subsequent probiotic effect. More recently, emphasis has been placed on the discovery of health-promoting compounds derived from microbial activity, known as "bioactives". Lactic acid bacteria (LAB) are the most prominent microbial group responsible for fermentation of meat, dairy, grains, and vegetables. Apart from their primary metabolic role of converting sugars to acid, LAB are fastidious microorganisms that possess complex proteolytic systems. These systems include cell envelope proteases to hydrolyze food proteins, transport systems to uptake peptides, and intracellular peptidases to metabolize peptides

into amino acids and nitrogen essential for survival (Savijoki, Ingmer & Varmanen, 2006). During LAB fermentation, hydrolysis of food proteins leads to the formation of both free amino acids and peptides.

Bioactive peptides are sequences of amino acids encrypted in a latent form within food proteins that are liberated via enzymatic hydrolysis by one of three means: application of exogenous proteases during food processing; digestive enzymes post-consumption; or microbial fermentation (Meisel & Bockelmann, 1999). The resulting short peptides (2-20 amino acids) that contain specific sequences of amino acids exert biological activity locally within the gastrointestinal tract or systemically in the blood and organs (Kussmann & Van Bladeren, 2011). Reported benefits include antioxidative, antithrombotic, antihypertensive, hypocholesterolemic, or immunomodulatory effects (Gibbs, Zougman, Masse, & Mulligan, 2004; Karnjanapratum, O'Callaghan, Benjakul, O'Keeffe, Fitzgerald, & O'Brien, 2017; Kayser & Meisel, 1996; Koyama, Naramoto, Nakajima, Aoyama, Watanabe, & Nakamura, 2013; Nagaoka et al., 2001; Seppo, Jauhiainen, Poussa, & Korpela, 2003),and the growing interest in natural alternatives to chemical pharmaceuticals has led researchers to investigate bioactive peptide formation for either therapeutic consumption of foods or commericialization as active pharmaceutical ingredients. While much research has been done on bioactive peptides in meats, fermented dairy and select grains, there is limited information regarding their presence in raw or fermented vegetables.

Cucumber pickles are the most commonly consumed fermented vegetable in the United States. Commercially, fresh cucumbers are submerged in high-salt brine (0.6-1.7 M NaCl) in large capacity vats and undergo fermentation by the LAB naturally present on the cucumbers until less than 0.05% sugar remains. Microorganisms responsible for cucumber fermentation typically include *Lactobacillus plantarum or Lb. pentosus, Lb. brevis, Enterococcus faecalis, Leuconostoc mesenteroides,* and *Pediococcus cerevisiae* (likely *Pediococcus pentosaceus* and/or *Pediococcus acidilactici* after recent reclassification) (Pérez-Díaz et al., 2013). Inoculation of cucumber fermentations with a known starter culture is not common practice but has been performed in both laboratory and industrial settings. During fermentation, between 110-140 mM lactic acid is produced and the pH equilibrates near 3.2-3.6 (Pérez-Díaz et al., 2013). Many cucumber pickle products are stored for extended periods of time and/or pasteurized prior to consumption; therefore, health-promoting properties of these foods depend on the chemical composition rather than the presence of live LAB. In addition to fermented cucumber pickles,

non-fermented, acidified cucumber pickles are commonly consumed. The latter are produced by packing fresh whole or sliced cucumbers into jars and covering them with an acidified brine, typically containing acetic acid, salt, and sodium benzoate and/or potassium sorbate to prevent fermentation and spoilage. Cucumbers contain 0.65% protein (USDA Food Composition Database, https://ndb.nal.usda.gov/ndb/search/list, Accessed 10.10.16) that may serve as a substrate for microbial or endogenous enzymes in the production of bioactive peptides, and we hypothesize that fermented cucumbers possess greater concentrations of bioactive peptides than raw or acidified cucumbers due to the fermentation process.

Discovery of bioactive peptides in plant-based foods typically employs either bioactivity-guided or targeted approaches. In a bioactivity-guided approach, prepared samples undergo several stages of separation and fractionation using combinations of size-exclusion (SEC), ion-exchange (IEX), or high-performance liquid chromatography (HPLC) for fractionation of samples prior to bioactivity testing (Panchaud, Affolter, & Kussmann, 2012; White, Sanders, & Davis, 2014). Collected fractions are tested *in vitro* for specific bioactivities and those with the highest activity are further fractionated and analyzed. Peptide sequences in the final fractions are identified by comparison to synthetic standards using LC tandem mass spectrometry (LC-MS/MS) and in some cases MS[3]. Conversely, targeted workflows exclude bioactivity testing and consist of analyzing peptide standards by LC-MS[n] to obtain separation and spectral data for comparison to food samples. Bütikofer, Meyer, Sieber, & Wechsler (2007) and Solieri, Rutella, & Tagliazucchi (2015) utilized this targeted route to confirm and quantify IPP and VPP in fermented dairy products, and Yamamoto et al. (2014) screened soy sauce for 337 hypothesized dipeptides, confirming the presence of 237. While these two approaches are commonly used for bioactive peptide discovery, they often require lengthy method development and extensive sample preparation, including lyophilization, cryopulverization, desalting, precipitation, filtration and solvent extraction (Lee, Bae, Lee, & Yang, 2006; Rizzello, Cassone, Di Cagno & Gobetti 2008).

Infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) mass spectrometry (MS) is a salt-tolerant, atmospheric pressure, soft-ionization technology capable of ionizing analytes directly desorbed from intact tissue samples using endogenous water as the energy-absorbing matrix (Bokhart & Muddiman, 2016; Sampson, Murray, & Muddiman, 2009). Direct analysis IR-MALDESI circumvents sample preparation and separation steps for biological

samples and can be used for mass spectrometry imaging (MSI) in which a molecule's spatial location within the tissue is displayed as a heat map. This novel method has been demonstrated for identification of small molecules in fermented cucumbers, which are not directly amenable to traditional ESI due to their high salt (1 M NaCl) content (Ekelöf, McMurtrie, Nazari, Johanningsmeier, & Muddiman, 2017). The objectives of this study were to: 1) apply direct analysis IR-MALDESI MS for identifying small bioactive peptides in raw, acidified, and fermented cucumbers; and 2) determine whether bioactive di- and tri-peptides are formed as a consequence of lactic acid fermentation.

2.3 Materials and Methods

2.3.1 Chemicals and Materials

Pickling cucumbers, pickling salt (sodium chloride, NaCl, $\geq 99\%$), and vinegar (acetic acid, 20%) were obtained from Mount Olive Pickle Company (Mount Olive, NC, USA). Calcium chloride ($CaCl_2$, $\geq 93\%$), hydrochloric acid (HCl, $\geq 37\%$), sulfuric acid (H_2SO_4 3N) and lactic acid ($\geq 85\%$) were purchase from Sigma-Aldrich (St. Louis, MO, USA); calcium hydroxide ($Ca(OH)_2$, $\geq 95\%$) was purchased from Fisher Scientific (Hampton, NH, USA); sodium benzoate ($\geq 99\%$) was purchased from Acros Organics (Waltham, MA, USA).

For IR-MALDESI analyses, LC-MS-grade methanol and water were purchased from Burdick and Jackson (Muskegon, MI, USA); MS-grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitrogen gas ($\geq 99.999\%$) for the higher energy collision (HCD) cell was purchased from Arc3 Gases (Raleigh, NC, USA). Glass slides were purchased from VWR (Radnor, PA, USA).

For LC-MS/MS analyses, LC-MS grade methanol and water were purchased from Fisher Scientific (Hampton, NH, USA). Peptide standards were purchased from Bachem (Bubendorf, Switzerland): isoleucine-proline-proline (IPP, $\geq 98\%$), valine-proline-proline (VPP, $\geq 99.5\%$), leucine-proline-proline (LPP, $\geq 99\%$), arginine-tyrosine (RY, $\geq 99\%$), and lysine-proline (KP, $\geq 99\%$). Amicon Ultra-0.5 filters with a 3 kDa cutoff were purchased from Fisher Scientific (Hampton, NH, USA).

2.3.2 Experimental Design

Four treatments were prepared: raw cucumber, acidified cucumber, naturally fermented cucumber, and starter culture fermented cucumber. Treatments were independently replicated in triplicate from one lot of pickling cucumbers. The acidified cucumbers served as a control by mimicking the salt and acid content of a fermented cucumber and preventing fermentation by the addition of sodium benzoate.

2.3.3 Brining and Fermentation of Cucumbers

Size 2B pickling cucumbers (3.5-3.8 cm diameter) were rinsed, packed into 1.36 L glass jars, and covered with brine (55:45 cucumber:brine ratio). Jars were sealed with a septum fitted lid to allow for brine sampling with a syringe. Cucumbers fermented with a starter culture were brined and inoculated with *Lactobacillus pentosus* strain LA0445 (Food Science Research Unit Culture Collection, USDA-ARS, Raleigh, NC, USA) to a final concentration of 6.4 x 10^5 CFU/mL prior to sealing the jars. Acidification and fermentation brines were prepared so that the equilibrated concentrations in the brined cucumbers were 0.684 M NaCl, 12 mM $CaCl_2$, 18 mM $Ca(OH)_2$, and 53 mM acetic acid. Acidified cucumber brines also contained lactic acid to mimic fermented cucumber acid content (110 mM, equilibrated), sodium benzoate to prevent fermentation (8 mM, equilibrated), and were adjusted with HCl to pH 2.75 so the cucumbers would reach a final pH of 3.25 after equilibration. Brined cucumbers were incubated at 28 °C for 6 weeks. On day 43, three cucumbers were sampled from each replicate treatment, cut into 2 cm cross sections, then into three lobes, and stored at -80 °C. Approximately 200 g of cucumber from each treatment and replicate was blended into a slurry. Raw cucumbers were prepared similarly the same day that brined treatments were packed. All samples were stored immediately at -80 °C until the time of analysis.

2.3.4 Fermentation Biochemistry

Quantification of acetic acid, lactic acid, glucose, and fructose was performed on an Agilent 1260 Infinity system (Agilent Technologies Inc., Santa Clara, CA, USA) according to McFeeters and Barish (2003) with some modifications. Brine samples were aseptically sampled on days 0, 1, 3, 7, 14, 21, 28, and 43, and stored at -80 °C. Prior to analysis, brine samples were thawed and centrifuged at 9,000 x g for 10 min. Brine supernatants were injected onto an

Aminex HPX-87H resin column (300 × 7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA) maintained at 37 °C with a flow of 0.03 N H_2SO_4 mobile phase at 0.6 mL/min. Acids were detected by an Agilent 1260 Infinity diode array detector (Agilent Technologies Inc., Santa Clara, CA, USA) set at 210 nm, and sugars were detected by an Agilent Infinity refractive index detector (Agilent Technologies, Santa Clara, CA, USA) connected in series. External standard calibration was performed using eight-point calibration curves (0.5 to 100 mM) for all analytes.

2.3.5 Total Peptide Quantification

Total peptides were quantified using a Pierce Quantitative Fluorometric Peptide Assay (Thermo Scientific, Bremen, Germany). An eight point linear standard curve ranging from 0 - 329.5 μg/mL (0 - 1013.3 μM) was created using IPP in 0.1 N HCl. Samples were prepared from cucumber slurry stored at -80°C. Raw cucumber slurries were equilibrated with mock brine to mimic acidified cucumber composition (350 mM NaCl, 62 mM lactic acid, 25 mM acetic acid, 8 mM sodium benzoate). Prior to analysis, each cucumber slurry was thawed and 2.5 g was placed in a 25 mL stainless steel capsule with four 12 mm ball bearings. Capsules were submerged in liquid nitrogen for 30 s then oscillated for 4 min at 30 Hz using a MM301 Mixer Mill (Retsch Gmbh, Haan, Germany). Samples were centrifuged at 11,000 x g for 5 minutes. Supernatants were filtered through 3 kDa centrifugal filters for 15 min at $11,000 \times g$ and the filtrate used for the assay. Filtrates were diluted 4 fold in 0.1 N HCl and analyzed according to the manufacturer's instructions. Fluorescence was detected at Ex 390nm/Em 475nm using a Tecan Safire[2] microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Differences in total peptide concentrations were determined by ANOVA and post-hoc Tukey test with α = 0.05 using JMP®, Version *13* (SAS Institute Inc., Cary, NC, 1989-2007). Results are reported as mM and mg/kg IPP equivalents. .

2.3.6 Bioactive peptide discovery by IR-MALDESI

2.3.6.1 IR-MALDESI MS and MS/MS

Cucumber lobes were removed from -80 °C storage and further sectioned into 100 μm thick slices using a Leica CM1950 cryostat (Buffalo Grove, IL, USA). Slices were thaw-mounted onto pre-cleaned glass slides, stored at -20 °C, and analyzed within 48 hours. Full MS

analysis to determine putative bioactive peptide matches was performed directly from whole slices following Ekelöf et al. (2017) with minor modifications. Briefly, the IR-MALDESI source consisted of an electrospray ionization (ESI) emitter and mid-IR laser (IR-Opolette 2371; Opotek, Carlsbad, CA, USA). The ESI operated at 4 kV with a 50:50 mixture of methanol and water with 0.2% formic acid flowing at 2.0 µL/min. Sample tissue was desorped by the laser (λ = 2.94 µm) with two 7 ns pulses (20 Hz). The source was coupled to a Q Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany) in positive ion mode with 140,000 resolving power (FWHM, *m/z* 200). Scans (n=1000) were acquired from 100-400 *m/z* for each tissue sample including portions of the exocarp and mesocarp as well as the glass slide which served as a blank for imaging. Putative peptide matches were confirmed by MS/MS parallel reaction monitoring (PRM) using the same instrument parameters. Precursor ions were isolated with a 1.0 Da (± 0.5 Da) window and fragmented using HCD. Normalized collision energies (NCE) were optimized for each peptide to produce the strongest signal for characteristic transitions referenced in the METLIN database (Smith et al., 2005, accessed 10.27.16). Tissue was sampled with 200 scans per peptide, and product ion data was acquired from 50-200 *m/z* and 100-400 *m/z*.

2.3.6.2 IR-MALDESI MS Data Analysis

An internal database of 86 bioactive di- and tripeptides previously discovered in foods was compiled from the scientific literature **(Table E.1, Appendix E)**. Following MS analysis, composite images of all treatments and replicates were generated using MSiReader ver. 0.06 (Robichaud, Garrard, Barry, & Muddiman, 2013). Images of $[M+H^+]^+$, $[M+H^+-H_2O]^+$, and $[M+Na^+]^+$ adducts of these di- and tripeptides (n = 240) were generated with a *m/z* bin width of 5 ppm (± 2.5 ppm). Images with clear distinction between tissue and blank areas, and within the dynamic range of the instrument were selected for MS/MS (Fig. 1c, e). Mass spectra were analyzed using Xcalibur ver. 2.3.26 (Thermo Scientific, Bremen, Germany) and cross referenced with the METLIN database (Smith et al. accessed 01.28.17) and Fragment Ion Calculator (Institute for Systems Biology, http://db.systemsbiology.net/proteomicsToolkit/, accessed 01.28.17). Absolute abundance was calculated by multiplying the reported abundance, in counts per second, by the C-trap injection time of 110 ms.

2.3.7 Peptide Quantification by LC-QQQ-MS

Fermented and acidified cucumber slurries were thawed, diluted two-fold with 0.2% formic acid and homogenized on ice with a Tekmar Ultra-Turrax TP 18/10S1 Homogenizer (IKA, Staufen, Germany) for 60 s at 80% power to rupture plant tissue and extract intracellular peptides. Raw cucumber slurries were similarly prepared but diluted with a mock brine to mimic the salt and acid content of the other treatments after the 1.8-fold dilution (350 mM NaCl, 62 mM lactic acid, 25 mM acetic acid, 0.2% formic acid). Samples were then filtered through 3 kDa centrifugal filters for 15 min at $11,000 \times g$ and the filtrate transferred to glass autosampler vials for immediate analysis. Extraction efficiency of cucumber samples was evaluated by adding 450 µL 0.2% formic acid to the 3 kDa filter retentate, incubating with agitation for 60 min at 4 °C, centrifuging with a 3 kDa filter, and analyzing the filtrate. Technical variability from sample preparation through LC-MS analysis was less than 5% RSD.

Peptides were quantified on a Shimadzu LCMS-8040 triple quadrupole LC-MS/MS (Shimadzu, Kyoto, Japan). Sample components were separated using a Restek Pinnacle DB biphenyl column (100 × 2.1 mm, 1.9 µm) (Restek, Bellefonte, PA, USA) held at 45 °C with a segmented gradient of 0.2% formic acid in water (A) and 0.2% formic acid in methanol (B). Gradient elution at 0.3 mL/min proceeded as follows: 0-25% B (0-3 min), 25-30% B (3-5 min), 30-35% B (5-8 min). Initial column conditions were restored after 0.1 min and held for 2 min to re-equilibrate prior to the next injection. The first 1.25 min of flow was diverted to waste to reduce salt deposit in the ion source. The MS was operated in positive ion mode using multiple reaction monitoring (MRM). Capillary voltage was set to 4.5 kV. Nitrogen drying gas and nebulizing gas flow rates were 15 L/min and 3 L/min, respectively. Heat block temperature was set to 400 °C. Resolution for the first and third quadrupoles was set to 0.7 Da with loop time of 1 s. Collision energies, transitions, and reference ions used for quantification were optimized for each peptide as reported in Table 1.

A five-point external standard calibration curve was generated with mixtures of IPP, LPP, VPP, and RY from 0.05 to 0.5 ng/µL and KP from 0.25 to 2.5 ng/µL. Standards were solubilized in mock brine to mimic fermented and acidified cucumber sample conditions (350 mM NaCl, 62.5 mM lactic acid, 25 mM acetic acid, 0.2% formic acid), and peak height was used for quantification. LabSolutions software ver. 5.8 (Shimadzu, Kyoto, Japan) was used for all LC-MS analyses and data processing. Statistical analysis of differences between treatments was

determined by ANOVA and post-hoc Tukey test with α = 0.05 using SAS software v. 9.4 (SAS Institute Inc., Cary, NC, USA). Limit of detection (LOD) was defined as three times the signal to noise ratio. Lower and upper limits of quantification (LOQ) were defined as the minimum and maximum concentrations of the calibration standards for each peptide.

2.4 Results and Discussion

2.4.1 Fermentation biochemistry

Fermentation of cucumbers progressed normally in all replicates as indicated by decreases in glucose and fructose, and an increase in lactic acid concentration (Fig. 2a). The acidified treatment was formulated based on an industry average value of 110 mM lactic acid. Sugar and acid concentrations did not change between equilibration (7 – 10 d) and final sampling (43 d) for the acidified cucumbers indicating that no fermentation occurred (Fig. 2b). The natural and starter culture fermented cucumbers contained less than 2 mM (< 0.05%) residual sugars and accumulated 136 (± 11.7) and 141 (± 3.6) mM lactic acid, respectively. The final pH of acidified, naturally fermented and starter culture fermented cucumbers was 3.25 (± 0.03), 3.40 (± 0.04), and 3.36 (± 0.07), respectively.

2.4.2 Total peptide quantification

Total peptides were quantified using a fluorometric peptide assay and values expressed as mg IPP equivalents per kg of sample. Raw cucumber contained 344.3 ± 29.3 mg/kg (1.1 ± 0.09 mM) total peptides. Both acidification and fermentation resulted in statistically significant increases in total peptide content to 518.6 ± 29.7 mg/kg (1.7 ± 0.10 mM), 459.7 ± 32.1 mg/kg (1.5 ± 0.11 mM), and 564.8 ± 41.2 mg/kg (1.8 ± 0.13 mM) for acidified, fermented, and starter culture fermented cucumber, respectively.

2.4.3 Bioactive peptide discovery by IR-MALDESI Mass Spectrometry

MS data was examined for the $[M+H^+]^+$, $[M+H^+-H_2O]^+$, and $[M+Na^+]^+$ adducts of the peptides curated in the internal database within the 100-400 *m/z* range, totaling 240 *m/z* values. Images of cucumbers from the three technical replicates of naturally fermented and acidified cucumbers were collated into a single image, converted to heat maps for each *m/z*, and evaluated

(Fig. 1). Images with non-zero abundance but ubiquitous distribution were indistinguishable from chemical noise and thus not considered for further analysis (Fig. 1c, d). Evaluation of spectral images of naturally fermented and acidified cucumbers resulted in the putative identification of 12 peptides. The distinction between the sample tissue and background was especially evident in the images generated for *m/z* 244.1656 (putative lysine-proline) (Fig. 1a, b).

IR-MALDESI MS/MS analysis of the twelve putative assignments resulted in identification of five peptides in fermented cucumber: IPP/LPP, VPP, KP, and RY (Fig. 3). Acidified cucumbers contained four of these peptides: IPP/LPP, KP and RY. For IPP/LPP, VPP, and KP, target product ion abundances were much higher in the naturally fermented cucumbers than the acidified cucumbers, suggesting that these peptides were produced during fermentation. Most of these potentially anti-hypertensive peptides were first identified in other lactic acid fermented foods. Ichimura et al. (2003) isolated KP (IC_{50} = 22 µM) from fermented fish sauce and RY (IC_{50} = 10.5 µM) was isolated from yeast fermented sake lees (Saito, Wanezaki, Kawato, & Imayasu, 1994). LPP (IC_{50} = 9.6 µM) was first identified in the maize endosperm protein γ-zein (Maruyama, Miyoshi, Kaneko, & Tanaka, 1989). The two most prominently studied bioactive peptides, IPP (IC_{50} = 5 µM) and VPP (IC_{50} = 9 µM), were discovered in *Lb. helveticus* fermented milk (Nakamura, Yamamoto, Sakai, Okubo, Yamazaki, & Takano, 1995). IPP and VPP have been extensively investigated in human trials and a recent meta-analysis concluded that inclusion of these peptides in the diet produces a small yet clinically significant hypotensive effect (Fekete, Givens, & Lovegrove, 2015). Although IPP and LPP are structural isomers that are indistinguishable from each other by direct analysis MS/MS (Fig. 3b), they possess similar ACE inhibitory activities with IC_{50} values of 5 µM (Nakamura et al., 1995) and 9.6 µM (Maruyama et al., 1989), respectively. Therefore, the presence of either peptide is of interest. These and many other bioactive peptides have been identified in a variety of foods, including fermented milk (Nakamura et al., 1995; Solieri et al., 2015), cheese (Bütikofer et al., 2007), fish sauce (Ichimura, Hu, Aita, & Maruyama, 2003), fish skin (Karnjanapratum et al., 2017), sourdough (Rizzello et al., 2008), wheat, kamut, and emmer (Babini, Tagliazucchi, Martini, Piu, & Gianotti, 2017), fermented buckwheat sprouts (Koyama et al., 2013), and raw garlic (Suetsuna, 1998). Peptides isolated from these sources and the corresponding IC_{50} values can be found in Table E.1, appendix E.

Previously, bioactive peptides were discovered in foods through bioactivity-guided or targeted approaches requiring potentially lengthy and costly processes. In this study an alternative approach was utilized. IR-MALDESI MS, which had not been previously used for bioactive peptide analyses, performed a rapid, targeted discovery of bioactive peptides in raw, acidified and fermented cucumbers. This instrument's direct analysis nature required minimal method optimization, simple sample preparation, and no prior chromatographic separation. Additionally, the instrument's high resolving power and mass measurement accuracy allowed for putative identification of hundreds of compounds based on accurate mass, followed by confident identification by MS/MS in PRM mode. A traditional LC-MS based approach for non-targeted bioactive peptide discovery would have required the use of 86 standards for the food associated peptides listed in the internal database. The majority of these peptides are only available through custom synthesis, making discovery and confirmation with a traditional approach cost prohibitive. Conversely, the use of direct analysis IR-MALSDESI MS/MS narrowed the pool of potential bioactive peptides down to five confirmed identifications without necessitating standards. This initial screening process enabled the economically feasible targeting and quantification of these five peptides by LC-QQQ-MS. In future studies, direct analysis IR-MALDESI with high resolution MS could also be coupled with non-targeted data analysis to more comprehensively identify the compounds (including longer peptides) that are generated by LAB fermentation. Once identified, unique compounds of interest could be synthesized and screened for in vitro bioactivity to identify novel bioactive compounds generated during fermentation.

2.4.4 Quantification of peptides by LC-QQQ-MS

The five peptides identified by IR-MALDESI MS/MS were quantified in all treatments using LC-MS with multiple reaction monitoring (MRM). Chromatographic separation of target peptides was achieved on a biphenyl stationary phase without ion pairing reagents. Peptide retention times were 1.84 min (KP), 3.05 min (RY), 6.24 min (VPP), 7.29 min (IPP), and 7.79 min (LPP) (Fig. 4). Baseline resolution was achieved for all peptides except IPP and LPP, therefore quantification was performed using peak height for all peptides. IPP and LPP were sufficiently resolved to be quantified, and the chromatographic separation of these two structural isomers showed that both were present in fermented cucumber (Fig. 4). The effects of salt and

organic acid on signal intensity and resultant peak height were tested during method development by dissolving peptide standards in solutions of acetic and lactic acid (15 and 70 mM, respectively) or sodium chloride (350 mM) to mimic concentrations normally found in fermented cucumbers. In the presence of organic acid, peak height was significantly reduced for IPP ($p < 0.01$) and RY ($p < 0.05$) and increased for KP ($p < 0.05$); no significant difference was observed for LPP or VPP. In the presence of salt, peak heights were significantly reduced for KP ($p < 0.01$) and RY ($p < 0.01$) with no significant difference observed for IPP, LPP or VPP. Given these results, all peptide standards were dissolved in mock brine to mimic a fermented cucumber matrix and account for the effect of salt and organic acids on ionization. Similarly, raw cucumber was diluted with mock brine to the same final concentration of salt and organic acids. Fermented cucumber extraction efficiencies were 85.1% (IPP), 89.0% (VPP), 75.1% (KP) and 89.2% (LPP), and quantities reported in Table 1 were adjusted accordingly. Extraction of RY from fermented cucumber resulted in values below the standard curve; therefore, a conservative estimate that assumed 100% extraction was calculated for samples containing RY.

Both natural and starter culture fermented cucumber contained IPP, LPP, and VPP at quantifiable levels while the acidified and raw cucumbers did not (Table 1), demonstrating that these peptides were formed due to fermentation. KP was found in all four treatments with more than twice as much in naturally fermented cucumbers (0.93 ± 0.04 mg/kg) and three times as much in starter culture fermented cucumber (1.5 ± 0.10 mg/kg) than in raw cucumber (0.44 ± 0.04 mg/kg). Acidified cucumber contained less KP (0.29 ± 0.03 mg/kg) than raw cucumber, consistent with the dilution of raw cucumber during brine addition. These data suggest that KP was naturally present in raw cucumber and potentially resistant to degradation during a 6 week incubation in acidified conditions. RY was detected in starter culture fermented (0.24 ± 0.04 mg/kg) and acidified cucumbers (0.22 ± 0.03 mg/kg) but absent in raw and naturally fermented cucumbers. Although RY was present at similar levels in the starter culture fermented and acidified cucumbers, fermentation biochemistry results indicate that fermentation did not occur in the latter treatment which suggests RY may be formed due to non-fermentation related processes.

Sequences of all five bioactive peptides are present in the proteomes of cucumber (*Cucumis sativus*) as well as two prominent LAB species responsible for cucumber fermentation: *L. pentosus* and *L. plantarum* (Uniprot, http://www.uniprot.org/, accessed August 2017).

Environmental conditions including amino acid availability due to endogenous hydrolytic enzymes along with microbial metabolic capabilities dictates which microorganisms commonly ferment a specific food. While dairy-associated *Lactococcus lactis* and *L. helveticus* have cell envelope proteases for casein hydrolysis and subsequent peptide formation (Kunji, Mierau, Hagting, Poolman, & Konings, 1996), it has been suggested that LAB species of plant origin naturally encode fewer proteolytic enzymes due to the fiber-rich ecological niches they occupy (Liu, Bayjanov, Renckens, Nauta, & Siezen, 2010). Interestingly, Vermeulen, Pavlovic, Ehrmann, Gänzle, & Vogel (2005) demonstrated that initial proteolytic activity in sourdough fermentation is due to endogenous wheat enzymes with subsequent peptide generation by lactobacilli peptidases. Genome sequencing of *L. plantarum* WCFS1 (Kleerebezem et al., 2003) and *L. pentosus* KCA1 (Anukam et al., 2013) revealed the absence of genes encoding for Prt, the primary enzyme system known to be involved in protein hydrolysis and polypeptide utilization. Kleerebezem et al. (2003) also determined that *L. plantarum* WCFS1 cannot synthesize branched chain amino acids (BCAAs) and has genes encoding for 57 ATP-binding cassette transporters, including numerous amino acid and peptide importers which may make up for its inability to synthesize BCAAs. While it is known that endogenous proteases are active during meat and cereal grain fermentation, the comprehensive proteolytic activity of cucumbers has not been determined. Independently of or in conjunction with microbial proteases, endogenous cucumber proteases may act upon cucumber proteins to provide the poly- and oligopeptide substrates necessary for LAB metabolic activities and bioactive peptide formation during fermentation.

It is well documented that LAB possess species and strain specific proteolytic enzyme systems (Savijoki et al., 2006; Solieri et al., 2015), and numerous researchers have screened microbial strains for those which produce the greatest concentration of a specific bioactive peptide or highest bioactivity. For example, Sun, Zhao, Wang, Cai, Chen, and Zhang (2009) screened 81 strains of *Lb. helveticus* and identified 16 that produced ACE inhibitory peptides in milk. The statistically significant increase in IPP ($p < 0.05$), KP ($p < 0.01$), and RY ($p < 0.01$) observed in the starter culture fermented cucumbers compared to the naturally fermented cucumbers suggests that the addition of a pure culture of *L. pentosus* enhanced production of these peptides. Starter culture use is not common in commercial pickle manufacturing as the natural microbiota are robust enough to complete fermentations; however, starter cultures may gain traction if a strain that enhances bioactive peptide content is identified. The higher

concentrations of bioactive peptides in cucumbers fermented with starter culture in this study indicates that this property has potential to be optimized for vegetable fermentations, suggesting that culture selection may provide benefits beyond optimal fermentation attenuation and organoleptic properties.

The abilities of IPP and VPP to lower blood pressure have been well studied in clinical trials of hypertensive patients yet there exists debate over their effectiveness. A recent meta-analysis of randomized controlled trials with strict inclusion criteria identified publication bias and a "small study effect" yet still concluded that IPP and VPP produce a small (-2.95 ± 1.2 mmHg) yet significant decrease in systolic blood pressure (SBP) (Fekete et al., 2015). According to the American Heart Association, even a 2-5 mmHg reduction in SBP applied to an entire population could have a significant impact, reducing mortality rates 3-7% (Appel et al., 2006). IPP and VPP levels in the fermented cucumbers in this study did not reach the known clinically relevant dosages; however, other ACE-inhibitory peptides were present and these peptides' cumulative potential for antihypertensive effects has not been assessed. Furthermore, since the bioactive peptides quantified in this study comprised only a small fraction of the increase in total peptides during fermentation, it may be useful to apply established protocols to determine whether novel bioactive peptides are generated during cucumber fermentation. These findings offer promising directions for future research including screening of microbial strains with increased production of bioactive peptides and the development of value-added products from fermented cucumber pickles or the spent brine waste stream.

2.5 Conclusion

The bioactive peptides IPP, LPP, VPP, KP and RY were identified for the first time in fermented cucumber by a novel mass spectrometry technology, direct analysis IR-MALDESI-MS/MS. LC-MS quantification of these peptides demonstrated that IPP, LPP, VPP, and KP were formed by lactic acid fermentation. This study supports the idea that the composition of vegetables may be enhanced through lactic acid fermentation. Further research is needed to understand the source and stability of these peptides for optimizing their content in fermented cucumber products.

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Table 1. Quantification of bioactive peptides in raw, acidified and fermented cucumbers

Peptide (precursor *m/z*)	Retention Time (min)	Product Ion	Reference Ions	Peptide Concentration (mg/kg)[*]			
				Raw	Acidified	Natural Fermentation	Starter Culture Fermentation
IPP [M+H]$^+$ 326.15 *m/z*	7.29	213.10 (-17.0 CE)	116.1 (-26.0 CE) 183.15 (-17.0 CE)	< LOD[a]	<LOD[a]	0.42 ± 0.03[b]	0.49 ± 0.02[c]
LPP [M+H]$^+$ 326.15 *m/z*	7.79	213.10 (-17.0 CE)	116.1 (-26.0 CE) 183.15 (-17.0 CE)	< LOD[a]	< LOD[a]	0.30 ± 0.02[b]	0.33 ± 0.03[b]
KP [M+H]$^+$ 244.20 *m/z*	1.84	84.05 (-22.0 CE)	116.05 (-26.0 CE) 129.20 (-13.0 CE)	0.44 ± 0.04[a]	0.29 ± 0.03[†a]	0.93 ± 0.04[b]	1.5 ± 0.10[c]
RY [M+H]$^+$ 338.15 *m/z*	3.05	175.10 (-21.0 CE)	112.05 (-27.0 CE)	< LOD[a]	0.22 ± 0.03[b]	< LOQ[a]	0.30 ± 0.04[b]
VPP [M+H]$^+$ 312.15 *m/z*	6.24	197.15 (-13.0 CE)	169.10 (-17.0 CE)	< LOD[a]	< LOQ[a]	0.32 ± 0.04[b]	0.35 ± 0.01[b]

[*]Superscript letters within a peptide row indicate significant differences between treatments ($p < 0.05$) using a one-way analysis of variance with post-hocTukey HSD.
[†]KP values obtained for the acidified treatment were below quantification limits (< 0.25 ng/µL) prior to adjustments for dilution factor and extraction efficiency (0.217 ± 0.2 mg/ml); extrapolation below the standard curve was used to provide this value.
LOD: limit of detection. LOQ: limit of quantification.
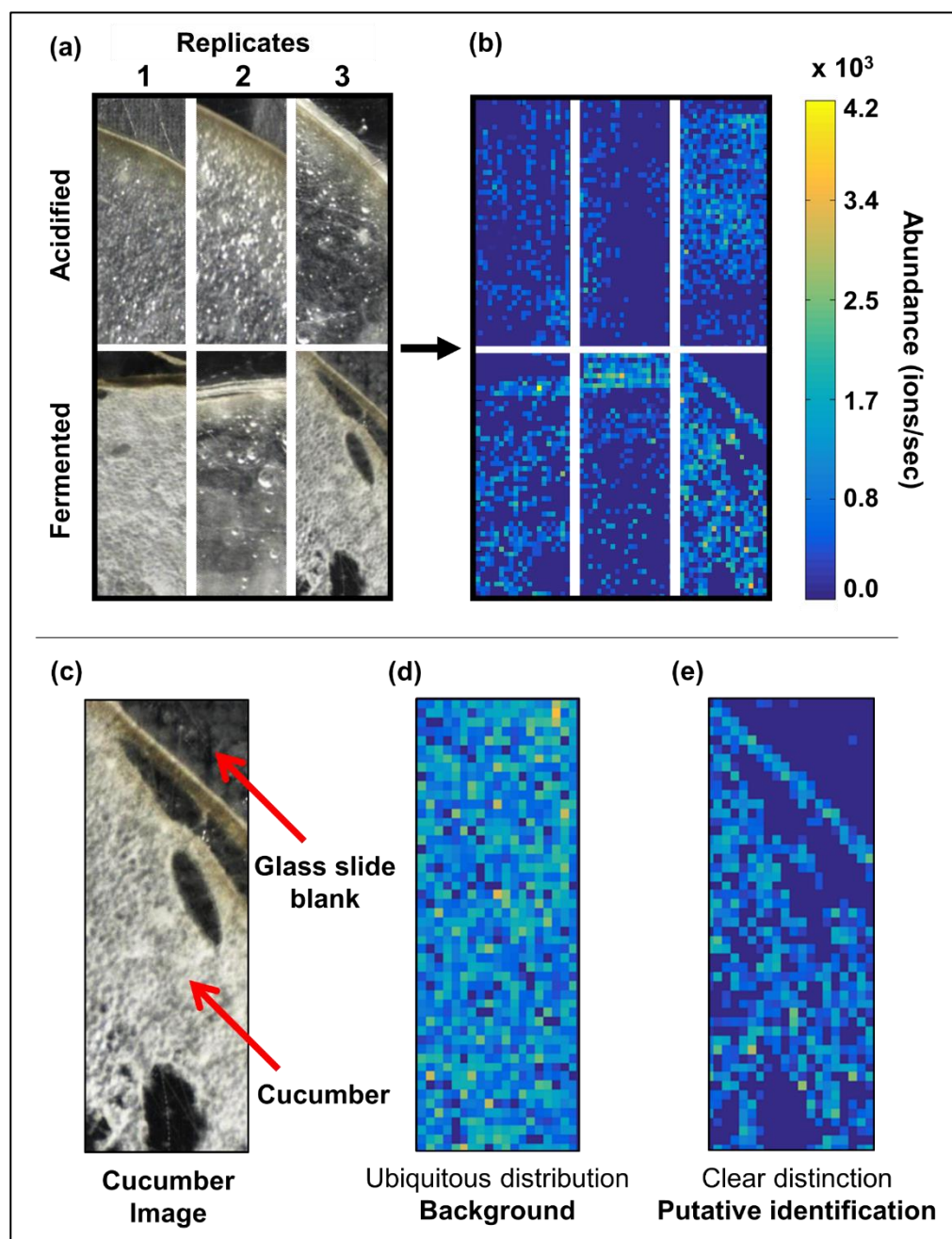
Figure 1. MS imaging interpretation (a) Image orientation of acidified and fermented cucumber areas sampled for MS analysis (b) Abundance (AU) heat map of lys-pro $[M+H^+]^+$ (m/z 244.1656) in acidified and fermented cucumbers (c) Magnified image of fermented cucumber replicate 3 (d) MS heat map of gly-glu-phe $[M+H^+]^+$ (m/z 302.1347) in fermented cucumber replicate 3 demonstrating background noise (e) MS heat map of fermented cucumber replicate 3 demonstrating putative identification of gly-his-ser [M+H+]+ (m/z 300.1302).
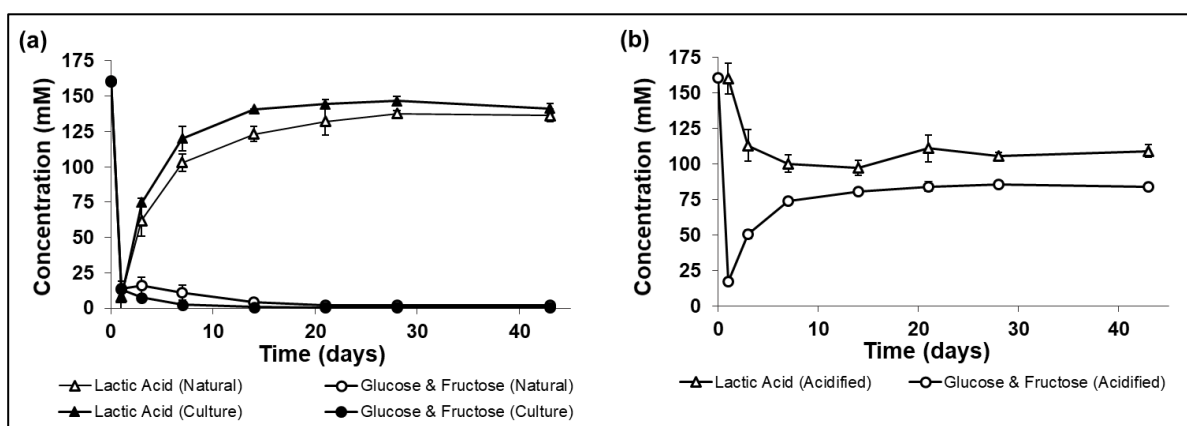
Figure 2. Changes in sugars and lactic acid content in fermented and acidified cucumber brines (a) Fermentation biochemistry of naturally fermented (Natural) and starter culture fermented (Culture) cucumbers and (b) equilibration of components in acidified cucumber over time.
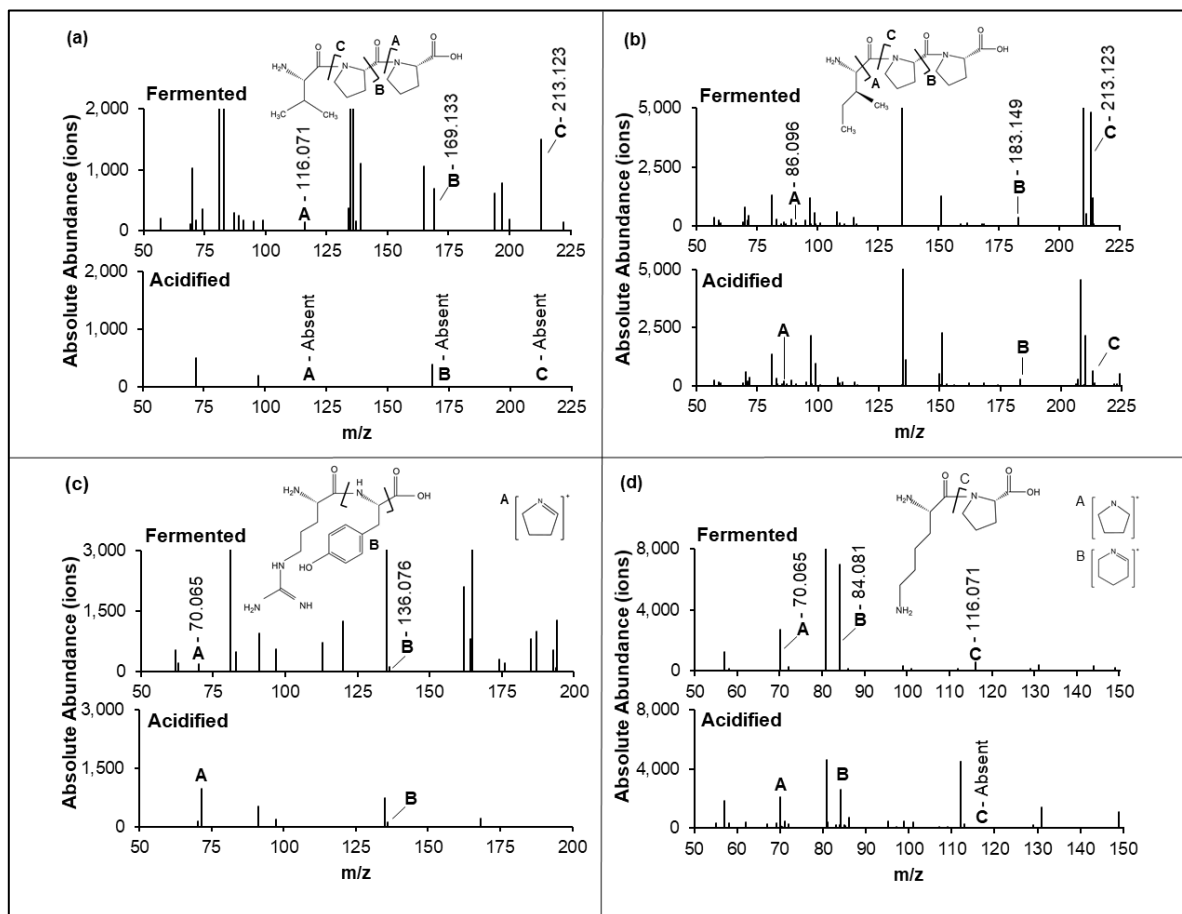
Figure 3. MS/MS spectra for $[M+H^+]^+$ adducts of bioactive peptides in acidified and fermented cucumbers. (a) val-pro-pro m/z 312.2 ± 0.5, 30 NCE, (b) ile/leu-pro-pro m/z 326.2 ± 0.5, 30 NCE, (c) arg-tyr m/z 338.2 ± 0.5, 40 NCE (d) lys-pro m/z 244.2 ± 0.5, 20 NCE.
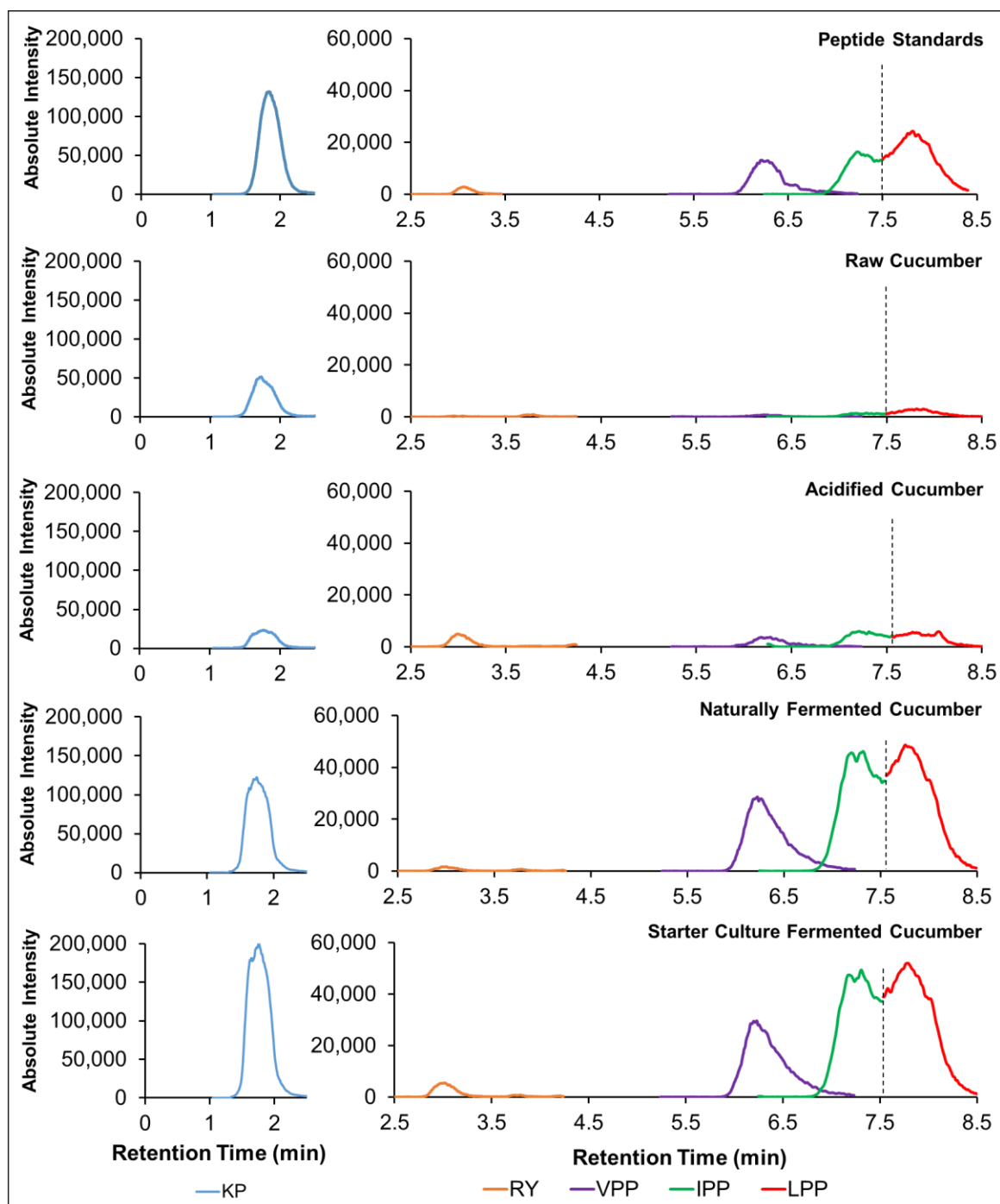
Figure 4. Separation of bioactive peptides in raw, acidified, and fermented cucumbers on a Restek Pinnacle DB biphenyl column (100×2.1 mm, 1.9 μm). Column temperature was held at 45 °C with a segmented gradient of 0.2% formic acid in water and 0.2% formic acid in methanol. MRMs used for quantification were: KP (244.20 > 84.05 m/z), RY (338.15 > 175.10 m/z), VPP (312.15 > 197.15 m/z), IPP (326.15 > 213.10 m/z), and LPP (326.15 > 213.10 m/z).

CHAPTER 3: Changes in Free Amino Acids During Cucumber Fermentation

3.1 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 (1302 ± 102 mg/kg and 635 ± 35 mg/kg, respectively). Neither brine salt level (2%, 3%, 4%, 6% NaCl) nor starter culture addition significantly affected total FAA in fermented cucumber. 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, while 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 FAA 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 is the most complete report of FAA composition of fresh or fermented pickling cucumbers and aids in understanding the impact of fermentation conditions on cucumber amino acid profiles while providing insight for manipulating fermentations for health promotion and consumer acceptance.

3.2 Introduction

Fermented vegetable consumption in the United States is dominated by cucumber pickles. 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 carbon dioxide with minimal amounts of 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, Lb. plantarum, Lb. brevis,*

Weissella spp*., Pediococcus ethanolidurans, Leuconostoc* spp. and *Lactococcus* spp. (Pérez-Díaz et al., 2016).

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. During early fermentation, 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 their environments (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 reported human health benefits, including arginine (arg) and citrulline (cit) for nitric oxide metabolism and cardiovascular health (Schwedhelm et al., 2007), ornithine (orn) for ammonia metabolism and fatigue attenuation (Sugino et al., 2008), gln for gastrointestinal health (Klimberg and Souba, 1990), GABA for antihypertensive effects (Diana et al., 2014b) 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. In line with this information, proteolysis has been demonstrated during cucumber fermentation by an increase in total peptides and generation of four bioactive di- and tri-peptides (Fideler et al., 2019). Additionally, changes in glutamine (gln) content and conversion of free glu to γ-aminobutyric acid (GABA) during LAB fermentation of cucumber has been observed (Moore, DuVivier & Johanningsmeier, 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.

3.3. Materials and Methods

3.3.1 Chemicals and Materials

Vinegar (acetic acid, 20%), pickling salt (sodium chloride (NaCl) $\geq$ 99%) and pickling cucumbers were provided by Mount Olive Pickle Company (Mount Olive, NC, USA). LC-MS grade acetonitrile, calcium hydroxide (Ca(OH)$_2$, $\geq$ 95%), LC-MS grade water and 10 kDa cutoff Amicon Ultra-0.5 filters were purchased from Fisher Scientific (Hampton, NH, USA). Sodium benzoate ($\geq$ 99%) and tyrosine (tyr, 98%) was purchased from Acros Organics (Waltham, MA, USA). Arginine (arg, $\geq$ 98%), aspartic acid (asp, 99%), isoleucine (ile, 99%), leucine (leu, 99%), lysine (lys, $\geq$ 98%), methionine (met, $\geq$ 98%), phenylalanine (phe, $\geq$ 98%) and proline (pro, 99%) were purchased from MP Biomedicals (Santa Ana, CA, USA). Aminoadipic acid (AAA, $\geq$ 98%), ammonium formate ($\geq$ 99%), asparagine (asn, $\geq$ 98%), calcium chloride (CaCl$_2$, $\geq$ 93%), citrulline (cit, $\geq$ 98%), formic acid ($\geq$ 98.5%), γ-aminobutyric acid (GABA, 99%), glutamate (glu, 99.5%), glutamine (gln, 98%) hydrochloric acid (HCl, $\geq$ 37%), lactic acid ($\geq$ 85%), ornithine (orn, 99%) and sulfuric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). The stable isotope labeled 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, his, ile, leu, lys, met, phe, pro, ser, thr, trp, tyr, and val in water (98 atom %) were also purchased from Sigma-Aldrich.

3.3.2 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. The initial experiment included one lot of cucumbers from the United States with each treatment independently prepared in triplicate. The time course experiment included two lots of cucumbers from separate farms in Mexico and the United States with each treatment independently prepared in duplicate for each lot at each sampling time point. For the brining salt study three lots of cucumbers were obtained from separate farms in Mexico and the United States and treatments were independently replicated in duplicate for each lot. 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 analyzed at one time point only, day 0.

3.3.3 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 were sealed with a septum fitted lid for aseptic brine sampling. Starter culture fermented cucumbers were brined and inoculated with 6.4×10^5 CFU/mL *Lactobacillus pentosus* strain LA0445 (Food Science Research Unit Culture Collection, USDA-ARS, Raleigh, NC, USA) prior to jar sealing. 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. Fermented and acidified cucumber for the time course experiment were prepared as described (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).

3.3.4 Preparation of Cucumber Slurries for Analysis

On the indicated sampling days, whole cucumbers were removed from jars, sectioned into 1-inch pieces, and blended with a Waring Model CB15V blender (Waring Commercial, Torrington, CT, USA). Samples 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.

3.3.5 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 x 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, Kyoto, Japan) 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 in an eight-point calibration curves for all analytes (0.5 to 100 mM).

3.3.6 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 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 sec prior to oscillation at 30 Hz for 4 min. Slurries were centrifuged at 11,000 x g for 5 min and supernatant filtered with 10 kDa centrifugal cutoff filters spun at 11,000 x 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 x 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 x g for 8 min at 4°C. Supernatants were filtered using 10 kDa centrifugal cutoff filters spun at 14,000 x g for 10 min at 4°C. Filtrate (323 μL) and 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 in 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 sec and first and third quadrupole resolutions were set to 0.7 dalton (Da) and 1.0 Da, respectively. Transitions, reference ions, collision energies, and calibration curve ranges were optimized for each experiment (Tables 1, 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 every 48 h by combining internal standards, amino acid mixtures, asn, gln and mobile phase A to a final volume of 1 mL in LC-MS vials. 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.

3.3.7 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.

3.4 Results and Discussion

3.4.1 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 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 (Pérez-Díaz et al., 2013; McMurtrie, Johanningsmeier, Breidt & Price, 2019). 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.

3.4.2 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 2442 ± 39 mg/kg in fresh cucumbers (Table 1). FAA content of naturally fermented (1302 ± 102 mg/kg) and starter culture fermented (1250 ± 108 mg/kg) cucumbers were not significantly different and both were twofold greater than that of acidified cucumbers (635 ± 35 mg/kg) (Table 1). The most abundant free amino acid in fresh cucumbers was gln (1491.4 ± 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 3A, B, C). Gln significantly decreased 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 (pGlu) acid 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). 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 and is consistent with the findings of Fideler et al. (2019). 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 would result in greater levels of their constituent amino acids as observed in this study (Savijoki, Ingmer & Varmanen, 2006).

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. While 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.

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, 2007, Teref & 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 branched chain amino acids, 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. As a result, the availability of essential amino acids from cucumbers may increase due to LAB fermentation. 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, Sánchez & De Castro, 2000).

3.4.3 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.

3.4.4 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). Levels of asp, GABA, ile, orn, pro, and tyr were significantly different between the four brining salt treatments suggesting differences in proteolytic activity and utilization of amino acid dependent acid resistance systems (Table 2, Figure 4). The effect of salt on FAA changes in fermented cucumbers differed from that of 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 to 48 hours of fermentation (Moore et al., 2021). Fermentation biochemistry data shows 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 while 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

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had lower levels of arg and orn, suggesting less proteolytic release of arg and less conversion of arg to orn (Figure 4). Examination of the microbial population of fermented cucumbers in varying salt brine levels would help identify which organisms are responsible for pH differences and could reveal why the GAD or ADI pathways are activated under such conditions.

3.4.5 Formation of Ornithine During Cucumber Fermentation

In addition to proteolytic activity, LAB metabolic pathways also contribute to compositional changes in amino acids. The current 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 et al., 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 2). The ADI pathway is commonly distributed among LAB and in addition to its function in pH regulation it also produces 1 mole of ATP for every mole of arg (Fernández et al., 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 which coincides with lactic acid formation and greatest decrease in pH during typical cucumber fermentations (Figure 3G, 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 2). Cit was present at 110.3 ± 8.5 mg/kg in fresh cucumbers and decreased in fermented cucumbers, but did

not decrease 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 greater 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, Loessner & Golden, 2005; Pérez-Díaz et al., 2013) and while both the ADI and GAD systems are widely distributed among LAB (Fernández et al., 2006; Wu, Tun, Law, Khafipour & Shah, 2017), individual species and strains possess differing levels of activity for these two acid resistance systems and selection of these bacteria with different salt 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 3G). 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 (Fernández et al., 2008). No increase in arg was observed in the stored fermented cucumbers (Figure 3A); therefore, orn may have been converted to other compounds such as carbamoyl-phosphate (Figure 2) and/or further metabolized to the polyamine putrescine.

3.4.6 Health Beneficial Amino Acids

Amino acids provide health benefits beyond the fulfillment of nutritional requirements and their availability as FAA in foods has prompted much investigation. The conditionally essential amino acid arg is required by mammalian infants and also 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). NO synthase (NOS, EC 1.14.13.39) converts arg to NO and cit, and the release of NO results in vasodilation of arterial and venous blood vessels (Schwedhelm et al., 2007). Kaore & 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 found to be 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 (Shearer et al., 1997; Flynn et al., 2002; Schwedhelm et al., 2008). In contrast, cit is not degraded by this enzyme and also serves as a precursor for arg formation in human endothelial cells (Waugh, Daeschner, Files, McConnell & Strandjord, 2001). Schwedhelm et al. (2007) found that oral supplementation of pure cit in humans increased plasma arg concentration and NO-dependent signaling in proportion to its dosage and that cit supplementation was effective in raising plasma arg concentrations.

Plants from the Cucurbitaceae family including melons, squash, pumpkins and cucumbers are natural sources of cit and have been investigated for their therapeutic benefits. Watermelon is the most abundant source of citrulline among cucurbits and therefore has been studied most extensively (Fish et al., 2012). Collins et al. (2007) found that long term (3 week) feeding of watermelon juice (1280 mg/kg cit) delivering 1 g cit (780 g juice) or 2 g cit (1560 g juice) 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). Fish (2012) reported 112.4 mg/kg cit in fresh market cucumbers with the peel and seed cavities removed. 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 post-menopausal women, resulting in a significant reduction in aortic systolic blood pressure and arterial stiffness (Figueroa et al., 2012).

Similar to cit, orn is a non-essential 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. A recent critical review of supplements purported to increase strength and muscle mass 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 would be necessary to achieve a higher dosage of orn from fermented cucumbers.

3.4.7 Taste-active Amino Acids

FAA, peptides and amino acid derivatives significantly impact taste and consumer liking in fermented foods (Zhao et al., 2016). Products can be designed to meet consumer desires for a particular flavor profile or aid in the reduction of negatively perceived ingredients such as salt and monosodium glutamate (MSG). Salt reduction is a significant goal for product developers in order to attenuate the impact of sodium on cardiovascular disease in humans. Additionally, pickled vegetable processors seek to limit the release of chlorine into the environment from processing waste streams. 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. Zhao et al. (2015) found that a consumer panel rated sourdough breads produced with 1% NaCl and either GABA- or glu-producing *Lb. reuteri* starter cultures as equivalent in saltiness to a reference bread produced with 1.5% NaCl, demonstrating that

accumulation of glu or GABA allowed for NaCl reduction without compromising taste. In the case of fermented cucumber pickles, chloride reduction in wastewater has been achieved by replacing high levels of NaCl (1.03M) 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. Combining sodium reduction approaches with the technology of starter cultures that produce taste-active amino acids could enhance savory notes and salt perception to bolster consumer liking.

Another way to boost taste-active amino acids in fermented foods is to release them from food proteins utilizing the proteolytic activity of LAB. 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 (1400 - 1800 mg/kg) compared with the control (240 mg/kg). While 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 other 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 perception 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 while fermented cucumbers contained 0.37 mM phe and 0.43 mM tyr, which fall below threshold levels as well, but 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.

3.5 Conclusion

Free amino acid profiles of fresh, acidified, naturally fermented and starter culture fermented cucumbers were compared and revealed that fermentation significantly increased the concentration of several amino acids in cucumbers. Additionally, brine salt level and the use of a

starter culture both affected 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 sensory acceptability standards.

3.6 References

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

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

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

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

*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.
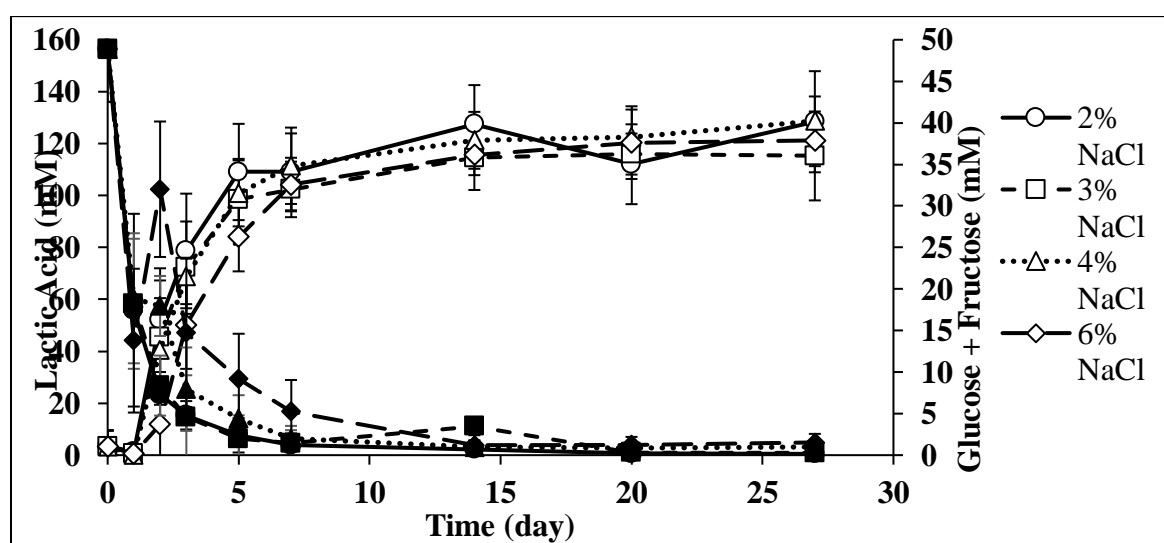
Figure 1. Changes in sugars and lactic acid over time of cucumbers fermented in 2%, 3%, 4% and 6% NaCl brines. Open markers represent lactic acid, closed markers represent glucose + fructose.
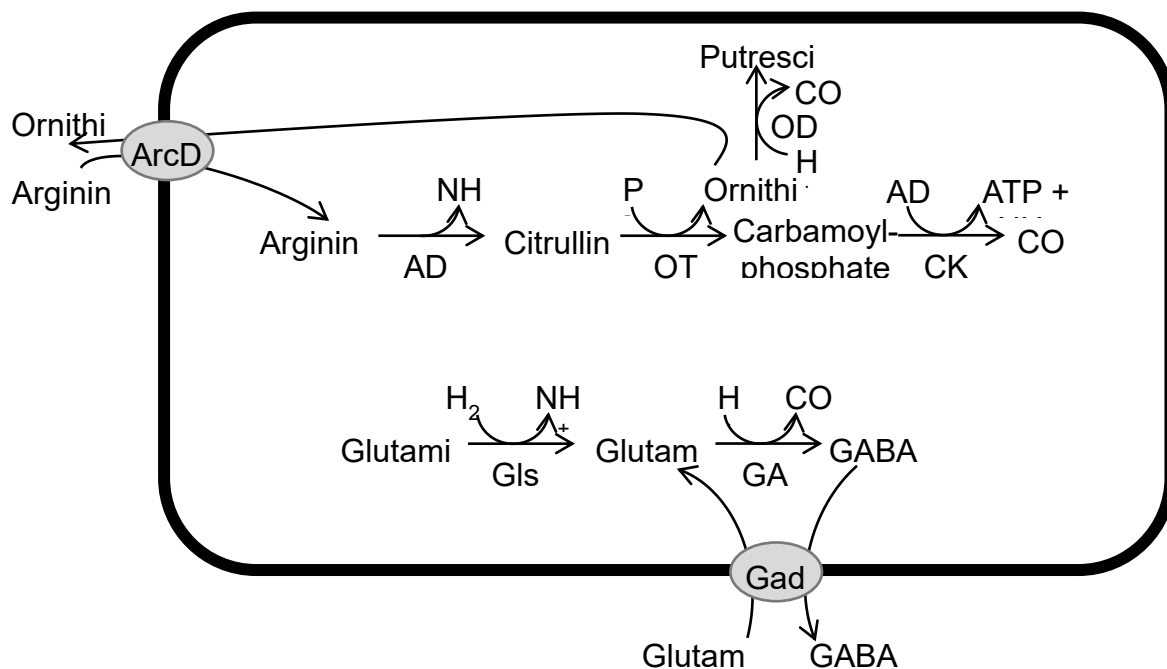
Figure 2. Schematic representation of glutamate decarboxylase and arginine deiminase pathways in lactic acid bacteria. ADI, arginine deiminase; ArcD, arginine/ornithine antiporter; CK, carbamate kinase; Gls, glutaminase; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; GadC, glutamate-GABA antiporter; ODC, ornithine decarboxylase; OTC, ornithine carbamoyl-transferase
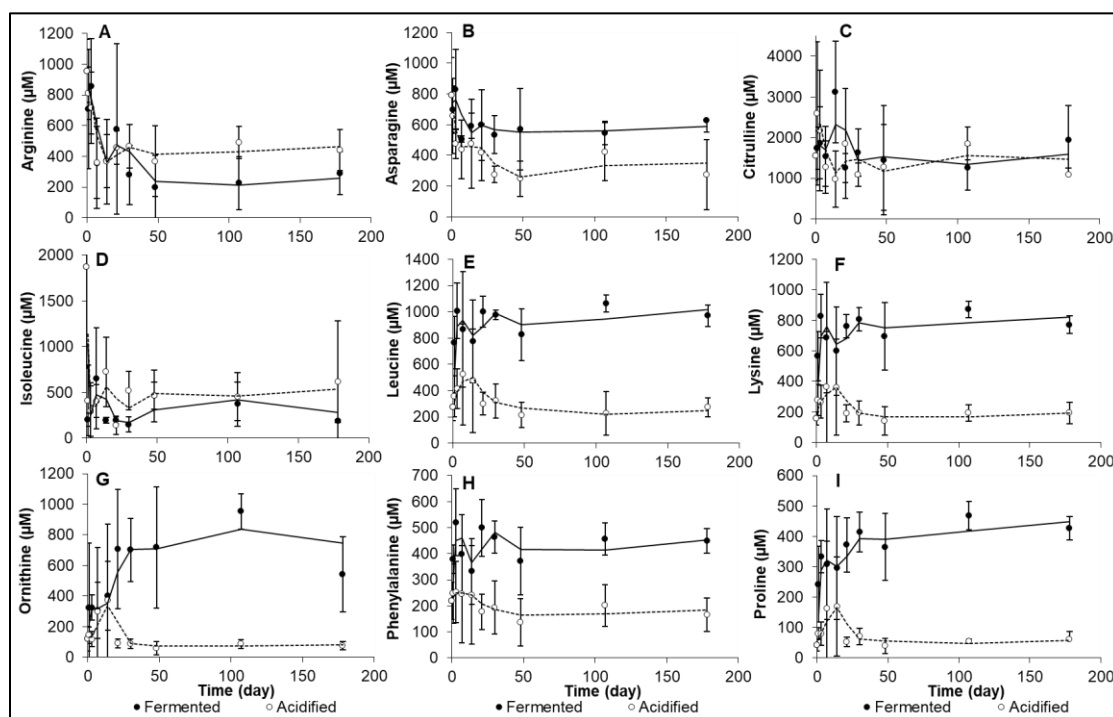
Figure 3. Amino acid content in fermented and acidified cucumbers over time.
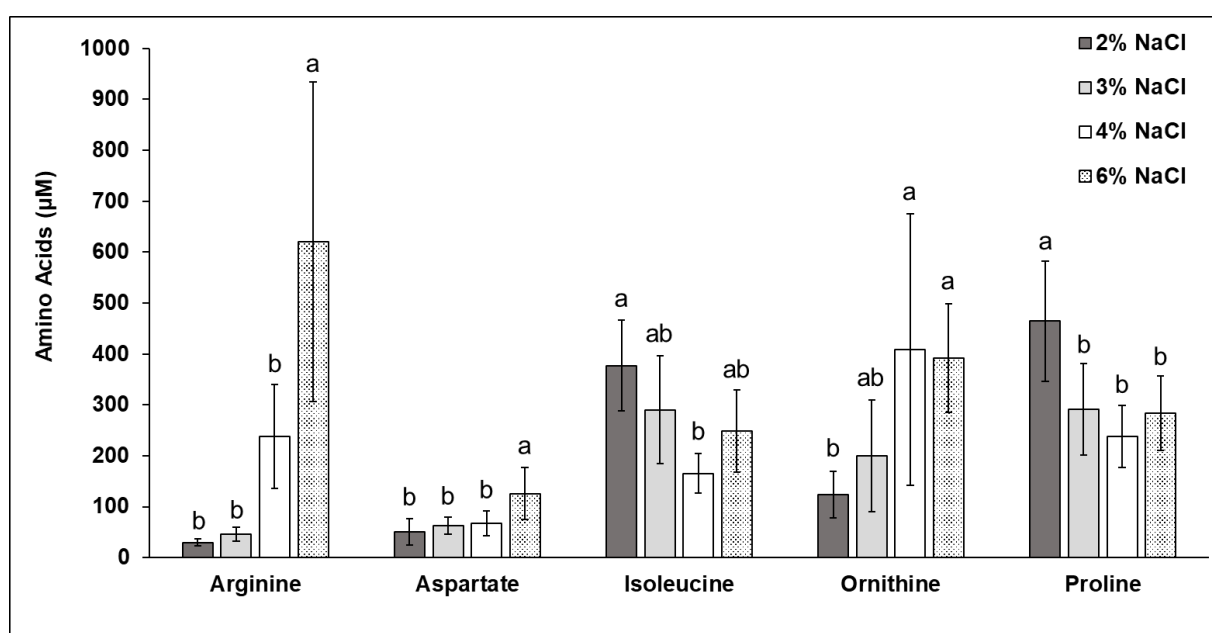
Figure 4. Differences in free amino acid content of fermented cucumber brined in varying salt concentrations.
Letters indicate significant differences between treatments ($p < 0.05$) using a one-way analysis of variance with post-hoc Tukey HSD test.
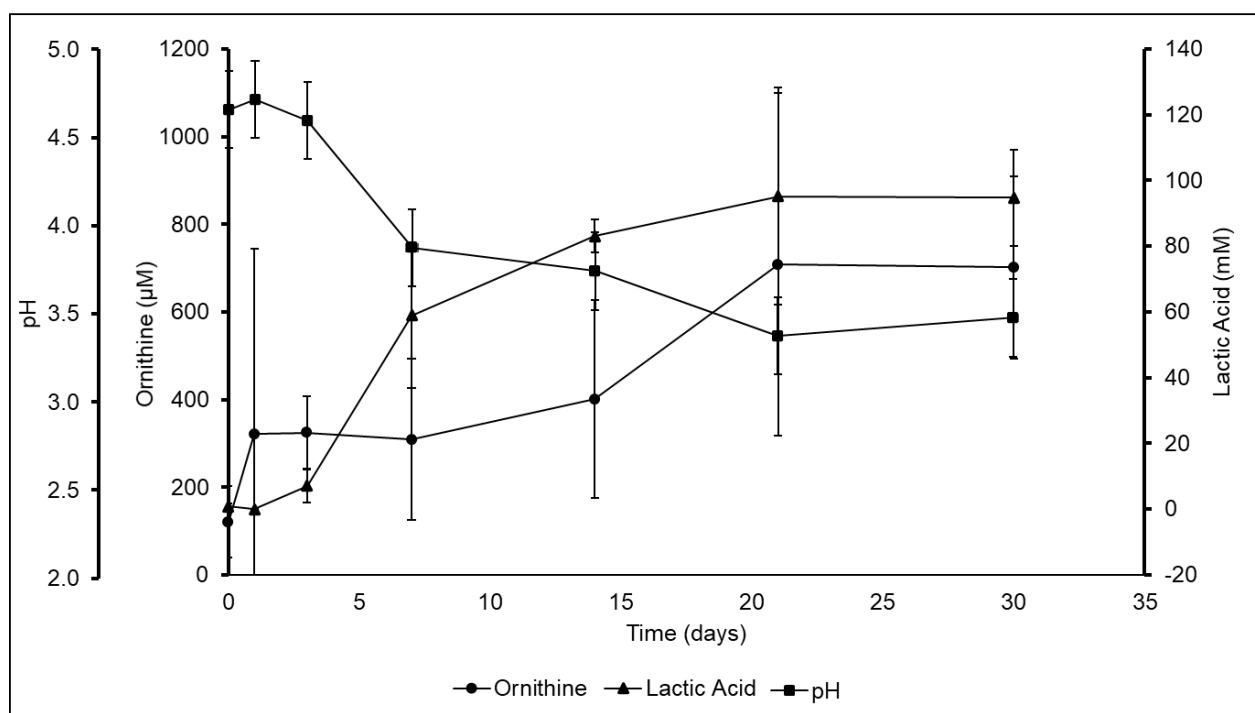
Figure 5. pH and formation of ornithine and lactic acid over time in naturally fermented cucumbers.

CHAPTER 4: Formation of γ-aminobutyric acid (GABA) during the natural lactic acid fermentation of cucumber

4.1 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%, 3%, 4%, 6%) affected GABA formation with greatest GABA formation in cucumber brined in 2% NaCl (1.38 ± 0.31 mM). Commercially available, directly packed, fermented cucumber pickles 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.

4.2 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), 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. Costilow 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 0.17g/100g 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., 2016). 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 & Oh, 2007), as well as a number of other LAB species (Cui et al., 2020). 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 may be 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.

4.3 Materials and Methods

4.3.1 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.

4.3.2. 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%, and 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.

4.3.3. 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.

4.3.3.1. 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 CB15V, 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.

4.3.3.2. 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.

4.3.4 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.

4.3.5 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 x 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-visible 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 to 100 mM) were prepared from external standards for all analytes.

4.3.6. 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 sec 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 $\times$ 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 sec was used and resolution for the first and third quadrupoles was set to 0.7 dalton (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 ≥ 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.

4.3.7. Commercial product survey

Commercial cucumber pickle products were analyzed for amino acid content. Products were chosen from three categories: fermented and directly packed without desalting (n = 7), fermented, desalted and re-packed in fresh cover brine (n = 12), and 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.

4.3.8. Statistical analysis

Analysis of variance (ANOVA) and post-hoc Tukey test with α = 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.

4.4 Results and discussion

4.4.1 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.

4.4.2 Formation and stability of GABA in fermented and acidified cucumbers

GABA was present in fresh 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 & 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., 2016) 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. Additionally, diversity in GAD systems and their organization within LAB genomes was observed, noting 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 hours 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 slurries from day 7 onward (1.50 ± 0.48 mM in slurry, 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's analysis focused on whole cucumber slurry. Opportunity exists for 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 the uses of 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 & Stadhouders, 1971). Costilow 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 et al., 1971) which may explain the low levels of glutamine in both acidified and fermented cucumbers at the end of storage.

Treatments designated 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) which 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.

4.4.3 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% (1M) salt after equilibration with brine which helps select for robust organisms that 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 2-6%, 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.

4.4.4 GABA Content of Commercially Available Cucumber Pickles

Fermented, directly packed cucumber pickles contained significantly greater 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

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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 hours 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%, 1M) 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.

4.5 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 directly packing them for consumption.

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

Amino Acid (precursor *m/z*)	Retention Time (min)	Product Ion	Reference Ions	Standard Curve Range (μM)	Concentration (mM)*				
					Fresh	Acidified	Acidified Processed**	Fermented	Fermented Processed**
GABA [M+H]$^+$ 104.00 *m/z*	8.13	87.10 (-13.0 CE)	69.15 (-17.0 CE)	0.40 - 350.0	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 *m/z*	8.50	83.95 (-15.0 CE)	102.00 (-15.0 CE) 130.15 (-13.0 CE)	0.50 - 60.0	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 *m/z*	8.50	84.05 (-16.0 CE)	56.10 (-29.0 CE) 42.05 (-55.0 CE)	1.50 - 1450.0	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
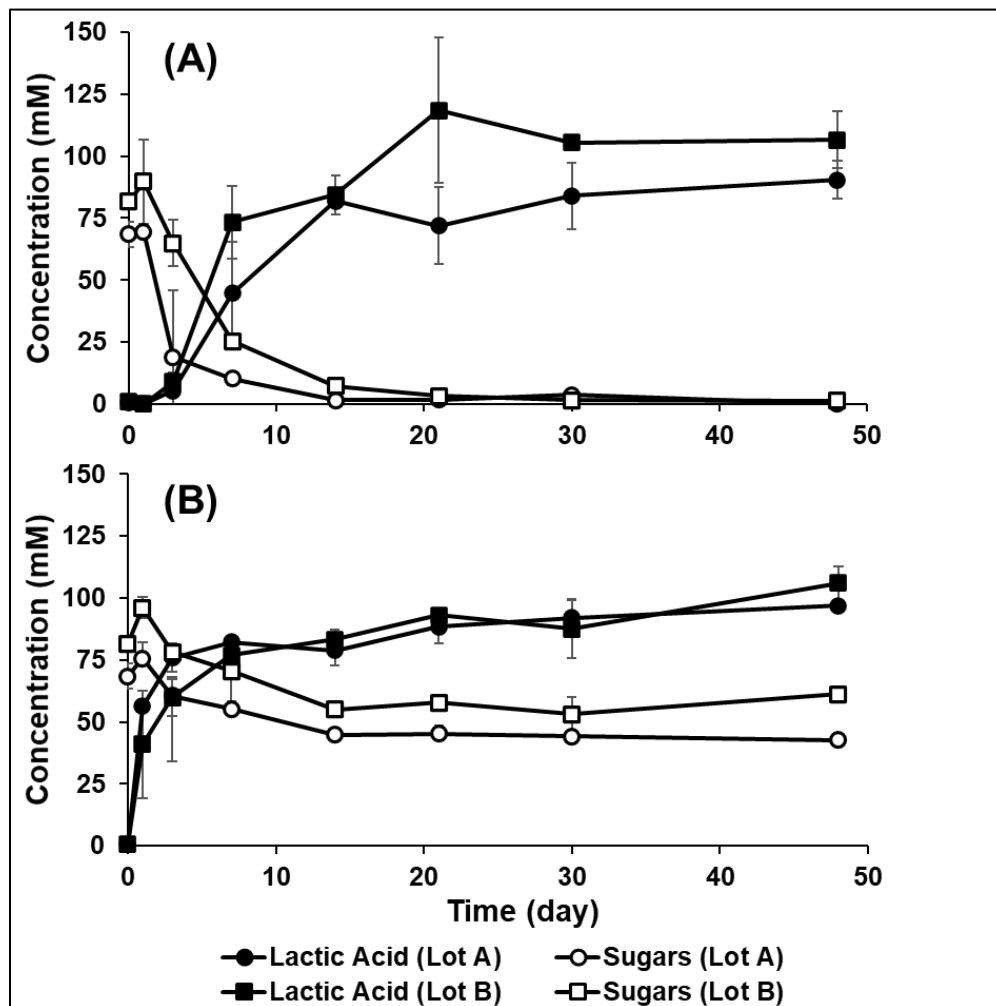
Figure 1. Changes in lactic acid and sugar content in fermented and acidified cucumber slurries. (A) Fermentation biochemistry of fermented cucumbers (B) equilibration of sugars and acid in acidified cucumber over time
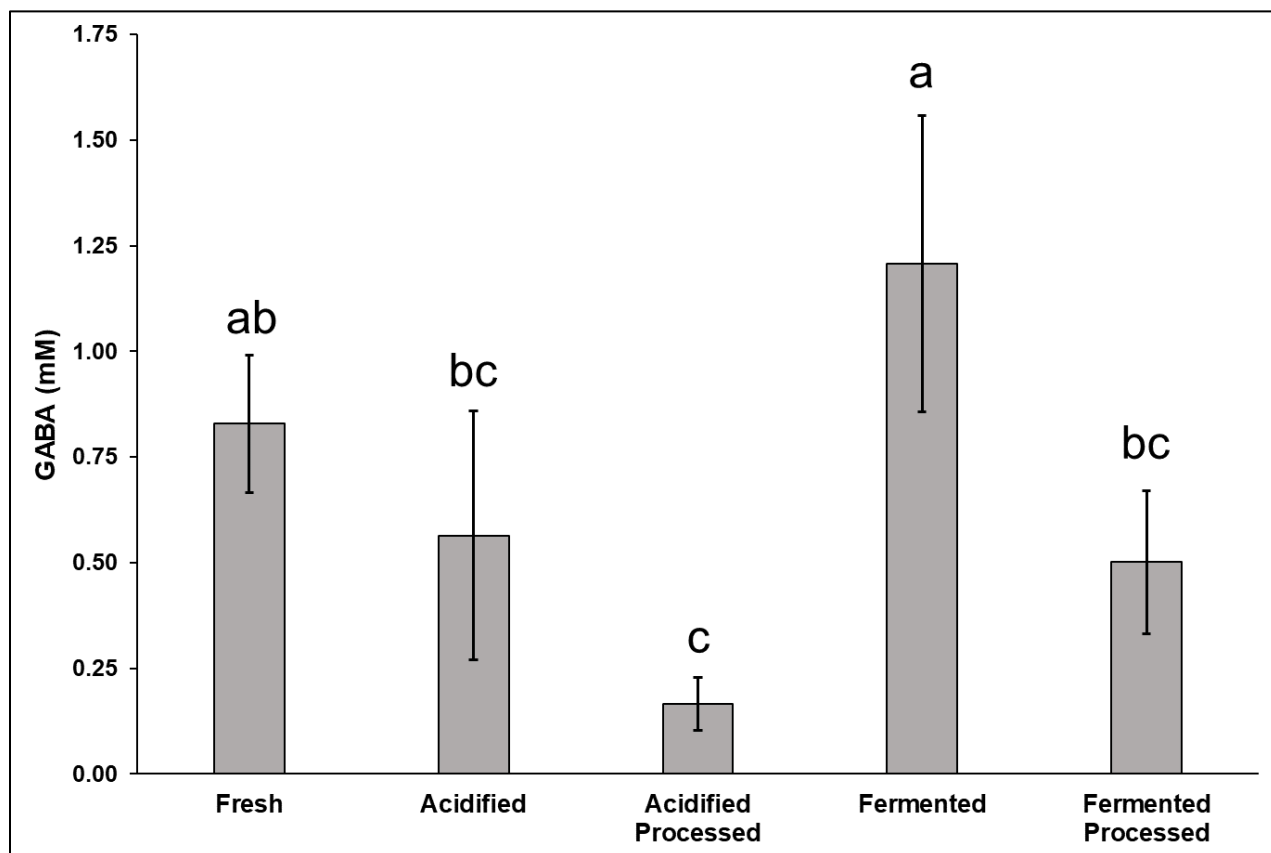
Figure 2. Average GABA content in size 2B cucumbers by treatment. Acidified and fermented treatments were packed with a 55:45 cucumber:brine ratio. Processed treatments were desalted in deionized water, sliced into chips, and re-packed in fresh cover brine at a 58:42 cucumber:brine ratio, then pasteurized 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
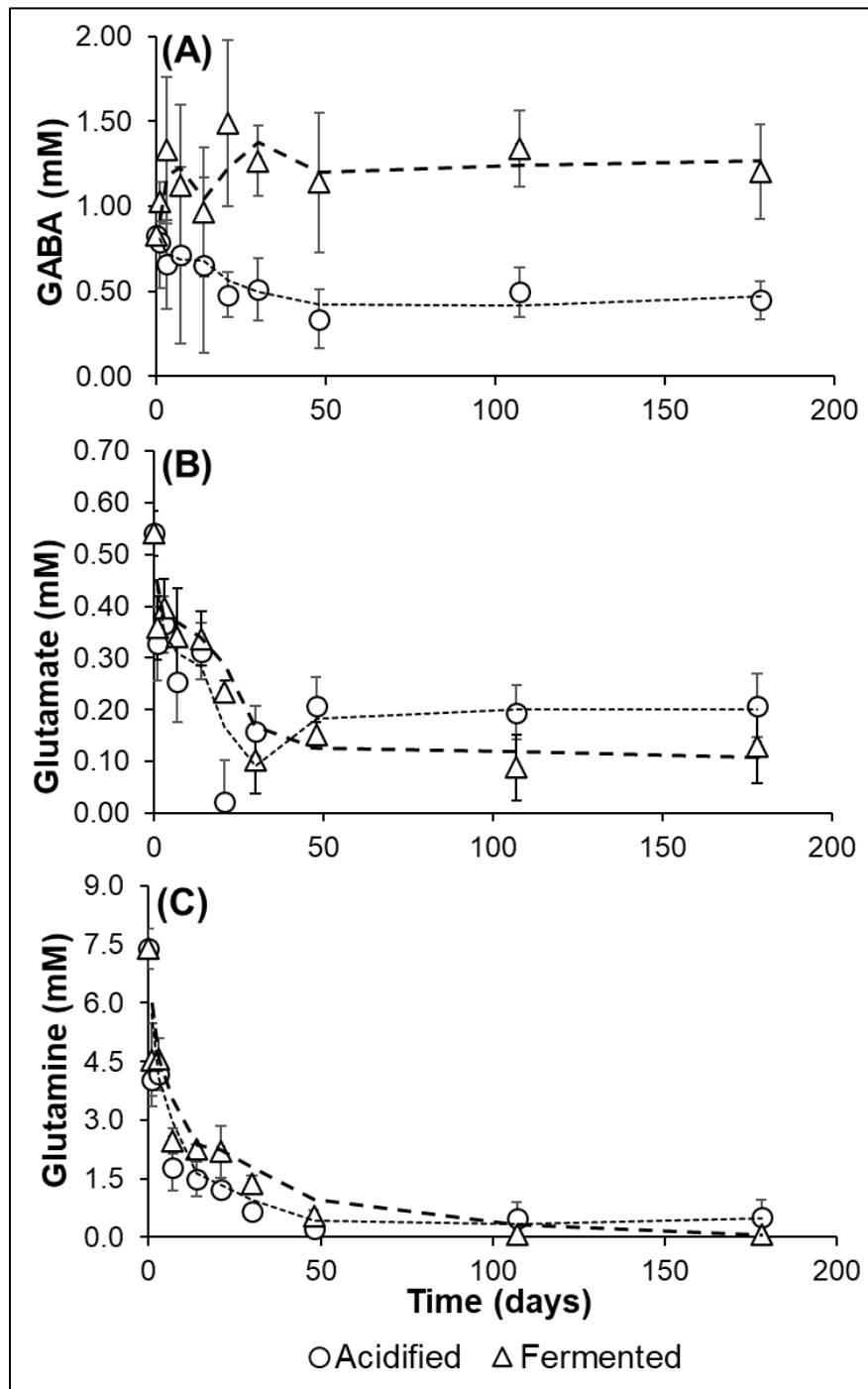
Figure 3. Amino acid content in fermented and acidified cucumbers over time
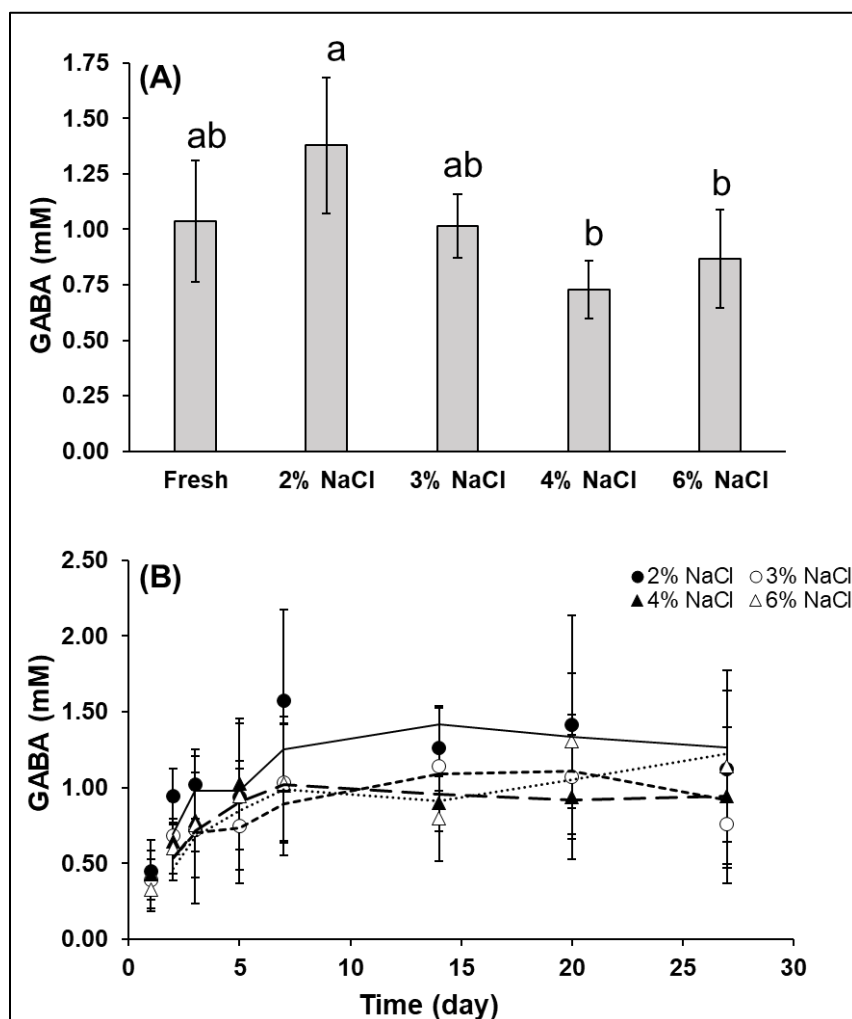
Figure 4. (A) Average GABA in fresh and fermented cucumber slurries of varying salt concentrations. Letters indicate significant differences between treatments ($p < 0.05$) using a one-way analysis of variance with post-hoc Tukey HSD test. (B) GABA generation over time in fermented cucumber brines of varying salt concentrations
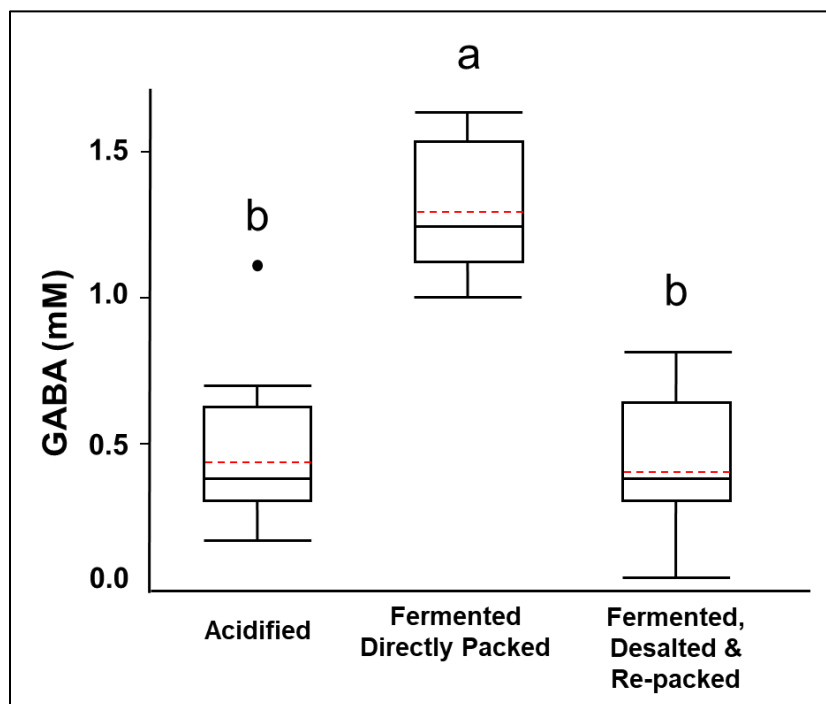
Figure 5. GABA content in commercial cucumber pickle products by product type. Letters indicate significant differences between treatments ($p < 0.05$) using a one-way analysis of variance with post-hoc Tukey HSD test. Red dotted line indicates average

CHAPTER 5: Enhancement of γ-aminobutyric Acid in Fermented Cucumbers

5.1 Abstract

The effect of initial brine acidification, glutamate addition, and starter culture addition on γ-aminobutyric acid (GABA) production during lactic acid fermentation of cucumbers in 2% NaCl brine was investigated. Cucumbers were fermented in triplicate in acidified brines or non-acidified brines with and without added glutamate (10 mM). Both natural and starter culture fermentations were completed for each treatment. Starter cultures included lactic acid bacteria with genes encoding for glutaminase (*Lactobacillus gasseri* ATCC 33323) and glutamate decarboxylase (*Lactiplantibacillus plantarum* WCFS1 ATCC BAA-793). Addition of glutamate resulted in significantly higher GABA production during fermentation for all treatments (acidified + glutamate, 10.39 ± 2.23 mM; acidified + glutamate, inoculated, 14.86 ± 0.72 mM; non-acidified + glutamate, 14.78 ± 1.15 mM; non-acidified + glutamate, inoculated, 13.65 ± 0.97 mM) compared with naturally fermented cucumber (1.12 ± 0.16 mM). Approximately 100% conversion efficiency of glutamate to GABA was observed for all treatments. No significant increases in glutamate or downstream formation of GABA were observed in any treatment, indicating that glutaminase production by *Lb. gasseri* ATCC 33323 and naturally present microbiota was minimal or absent under these conditions. Interestingly, the addition of a starter culture only improved GABA content when glutamate was added and brine was acidified. Further optimization of fermentation conditions and microbiota may allow for additional enhancement of GABA via glutaminase production in starter culture fermented cucumbers. Glutamate addition to natural cucumber fermentations in 2% NaCl brines resulted in the formation of pickles ready for direct consumption that can deliver clinically relevant levels of GABA in less than one typical US serving size.

5.2 Introduction

Consumption of vegetables is a priority for Americans, and in 2018, 38% of consumers indicated they were adding more vegetables to their diets (Mintel, 2018). Furthermore, 44% of consumers claimed they were interested in fermented condiments – including pickles – for their health benefits (Mintel, 2019). Lactic acid bacteria (LAB) fermented foods, including vegetables, are commonly considered healthful, and the scientific community has recognized them as sources of non-nutritive bioactive compounds (recently reviewed by Marco et al., 2017 and

Şanlier et al., 2019). Among LAB fermented vegetables, cucumber pickles are the most commonly consumed product in the United States. According to market reports, 2/3 of the American population consumes various types of pickles (Statista, 2019) and 58% of consumers bought relish or pickles every six months (Mintel, 2019). Interest in pickles and relishes has increased due to the perception that they promote healthfulness due to the antioxidants, vitamins, minerals and probiotics they contain (IMARC, 2018). Widespread interest in and consumption of fermented vegetable products indicates that research into the content of health beneficial compounds of these types of foods has potential for far-reaching impact.

Traditional fermented foods are perceived to be healthful due to the presence of live microbes, assumed to be probiotic. However, the low final pH (3.0-3.5) and common commercial practice of heat treatment for extending shelf stability (i.e., pasteurization, baking) of LAB fermented foods greatly compromises microbial viability and these products seldom contain live microbes. Health promoting benefits of LAB fermented foods are therefore primarily associated with compounds such as vitamins, bioactive peptides, antioxidants, neurotransmitters, and organic acids generated or released from foods as a result of microbial activity during fermentation.

Our previous research demonstrated the production of one such compound, γ-aminobutyric acid (GABA), during lactic acid bacteria (LAB) fermentation of pickling cucumbers (Moore, DuVivier & Johanningsmeier, 2021). From a microbiological standpoint, GABA is the product of an amino acid dependent acid resistance system that many LAB employ to protect themselves from intracellular pH decline. In this system, glutamate is decarboxylated by glutamic acid decarboxylase (GAD, EC 4.1.1.15) forming GABA and consuming a proton (Wu et al., 2017; Pennacchietti et al., 2018). GAD is widely distributed in LAB that are naturally present on fresh pickling cucumbers and responsible for fermentation, including *Lactobacillus plantarum (Lactiplantibacillus plantarum), Lactobacillus pentosus* (*Lactiplantibacillus pentosus*) and *Pediococcus acidilactici,* (Tang and others, 2018; Kim & Kim, 2012; Siragusa et al., 2007; Feehily et al., 2013, Pérez-Díaz et al., 2016).

While GABA is naturally present in some fresh fruits and vegetables such as spinach, cabbage, carrots, tomatoes, sweetpotatoes and peas (Ito et al., 2019; Akihiro et al., 2008), fermentation has been utilized to increase GABA content of certain foods in order to enhance their health promoting potential. Examples of these foods include buckwheat sprouts (Koyama et

al., 2013), sourdough bread (Rizzello et al., 2008; Venturi et al., 2019), dairy products (Inoue et al., 2003; Wu et al., 2017), soy sauce (Yamakoshi et al., 2007), and Pu-erh tea (Zhu et al., 2016). Our previous research found that the GABA content of cucumbers is also enhanced by LA fermentation, with fresh, acidified, and fermented cucumbers containing 0.85 ± 0.15 mM, 0.33 ± 0.14 mM and 1.16 ± 0.32 mM GABA, respectively (Moore et al., 2021). Additionally, GABA remained stable over time and was not affected by pasteurization, demonstrating its suitability for enhancement in shelf-stable fermented pickles. Commercially, cucumbers are often times fermented in high salt (6% NaCl) brines, desalted, and packed in fresh cover brine to achieve a final salt content that is acceptable for consumers (~2% NaCl). This desalting and repacking process also depletes other water-soluble compounds, such as GABA. In our study, we found that desalting, re-packing, and pasteurizing fermented pickles reduced GABA content by 62% (0.44 ± 0.16 mM) which was consistent with the expected dilution from desalting and re-packing with fresh cover liquor. Reduction of brine salt concentration may reduce or eliminate the need for desalting and conserve GABA in the finished product.

GABA's health benefits include lowering blood pressure (Hayakawa et al., 2004; Inoue et al., 2003; Pouliot-Mathieu et al., 2013), boosting immunity (Abdou et al., 2006), lowering anxiety and stress (Abdou et al., 2006; Hinton, Jelinek, Viengkhou, Johnston & Slade, 2019; Nakamura, Takishima, Kometani & Yokogoshi, 2009) and improving decision making (Steenbergen et al., 2015). Several human trials have been conducted to test GABA's effects on blood pressure, stress, anxiety and fatigue with effective doses of GABA ranging from 2.01 mg to 100 mg (Abdou et al., 2006; Hinton et al., 2019; Inoue et al., 2003; Pouliot-Mathieu et al., 2013; Kanehira et al., 2011; Nakamura et al., 2009; Nishimura et al., 2016; Yamatsu, Yamashita, Pandharipande, Maru & Kim, 2016; Yoto et al., 2011). In one study, human subjects consuming 50 g of cheese containing 16 mg GABA experienced a decrease in systolic blood pressure by 3.5 mmHg (Pouliot-Mathieu and et al., 2013). Appel, Brands, Daniels, Karanja, Elmer & Sacks (2006) determined that a decrease in blood pressure by just 2 to 5 mmHg is clinically significant and able to impact public health. Consumers would need to eat approximately 3.3, 4" fermented pickle spears (126 g) that were not desalted in order to consume 16 mg GABA. However, many commercially produced cucumber pickles are fermented in high salt (6% NaCl) brines, desalted, and packed in fresh cover brine to achieve a final salt content that is acceptable for consumers (~2% NaCl). This desalting and repacking process depletes other water-soluble compounds, such

as GABA, leading to a 62% reduction in GABA content (Moore et al., 2021) and the need to consume 8.5, 4" fermented, desalted pickle spears (332 g). Both serving sizes are relatively large compared to typical pickle consumption in the US, and pickles that have not been desalted are typically not acceptable to consumers due to their overly salty taste. Increasing the amount of GABA produced during fermentation has the potential to deliver clinically relevant levels of GABA to consumers, and further development of processes for producing fermented pickles with less salt would further preserve GABA in the finished product. Eliminating the desalting step would also aid commercial processors in reducing high salt waste streams to meet discharge limits set by the Environmental Protection Agency (McFeeters & Pérez-Díaz, 2010).

GABA enhancement in foods has been accomplished through fermentation using LAB with high GABA producing abilities (Di Cagno et al., 2010; Kim et al., 2012; Peñas et al., 2015; Rizzello et al., 2008; Tang et al., 2018; Zareian et al., 2015). However, the amount of GABA produced in these fermentations is limited by the glutamate content of the starting material. The addition of exogenous glutamate to fermentations, typically in the form of monosodium glutamate (MSG) is one way of boosting glutamate concentration for GABA conversion. This method has been applied to fermentations of water dropwort (Kwon et al., 2016) and black raspberry juice (Kim, Lee, Ji, Lee & Hwang, 2009). In the former, researchers found that glutamate was converted to GABA with 76% efficiency while in the latter approximately 130% conversion efficiency was reported. Our previous research showed that GABA is produced during natural lactic acid fermentation of cucumbers with approximately 160% conversion efficiency (Moore et al., 2021), suggesting that additional glutamate was provided for conversion to GABA during fermentation, likely through proteolysis or the conversion of glutamine to glutamate. We hypothesize that adding exogenous glutamate to cucumber fermentations will result in higher GABA content as a result of glutamate decarboxylation by the naturally occurring microbiota.

Here we propose a second method for increasing glutamate content for conversion to GABA. Inclusion of glutaminase expressing starter cultures in fermented foods could potentially convert naturally present glutamine to glutamate for further conversion to GABA. Identification of glutaminase producing LAB from fermented foods has been primarily driven by the desire to enhance umami taste by increasing glutamate content (Nandakumar, Yoshimune, Wakayama & Moriguchi, 2003; Thongsanit, Tanikawa, Yano, Tachiki & Wakayama, 2009). Glutaminase

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converts glutamine to glutamate and ammonia in the presence of water (Binod and others, 2017). Fresh pickling cucumbers contain 7.40 ± 1.04 mM glutamine in the free amino acid form (Moore et al., 2021), which is significantly higher than the free glutamate content (0.54 ± 0.09 mM). Conversion of glutamine naturally present in cucumbers has the potential to significantly increase glutamate availability for conversion to GABA during natural lactic acid fermentation.

While the microorganism most commonly associated with cucumber fermentation has been *Lb. plantarum*, others including *Lactobacillus brevis (Levilactobacillus brevis), Lb. pentosus, Enterococcus faecalis, Leuconostoc mesenteroides*, and *Pediococcus cerevisiae* (likely *P. pentosaceus* and/or *P. acidilactici* after reclassification) are also highly involved (Pérez-Díaz and others, 2016). Glutaminase expression in LAB is strain specific and has been observed *in Lb. brevis, Lb. fermentum, Lb. casei,* and *Lb. paracasei* (Kieronczyk and others, 2011; Weingand-Ziade et al., 2003). A study of 338 LAB isolated from fermented Thai foods found glutaminase expression in only 22 isolates (Woraharn et al., 2014). Notably, a high glutaminase producing strain known as G507/1 (2.88 ± 0.19 U/mg protein) was identified and found to be a close relative of *Lactobacillus brevis* ATCC 14869. In addition to LAB, enteric microorganisms are present on fresh cucumbers, including but not limited to: *Citrobacter freundii, Enterobacter cloacae, E. kobei, Kluyvera cryocrescens, Ochrobactrum pseudogringonense,* and *Pantoea agglomerans* (Pérez-Díaz et al., 2019). These species commonly possess glutaminase; however, their growth may be inhibited in cucumber fermentations due to the addition of acetic acid or use of acidic recycled brines (Pérez-Díaz et al., 2013; McMurtrie, Johanningsmeier, Breidt & Price, 2019). We hypothesize that (1) adding exogenous glutamate to whole cucumber fermentations will result in increased GABA production by the naturally occurring LAB and that (2) adjusting the initial pH of cucumber fermentations will allow enteric microbiota to be metabolically active for a limited period of time, which may enable conversion of glutamine to glutamate and provide higher levels of glutamate for conversion to GABA. The objective of this study was to determine if GABA could be increased sufficiently in whole cucumber fermentations to achieve a clinically relevant concentration of GABA in a small serving of the finished pickle product.

5.3 Methods

5.3.1 Experimental Design

Eight whole cucumber fermentation treatments that varied in acetic acid, $Ca(OH)_2$, glutamate content and starter culture addition were prepared in triplicate. The concentrations representative of the brines after equilibration with cucumbers were as follows: (1) acidified brine containing 12 mM $CaCl_2$, 342.2 mM NaCl, 18 mM $Ca(OH)_2$ and 53 mM acetic acid; (2) acidified brine with 10 mM glutamate added; (3) non-acidified brine containing 12 mM $CaCl_2$ and 342.2 mM NaCl; (4) non-acidified brine with 10 mM glutamate added; (5) control brine inoculated with *Lb. gasseri* ATCC 33323 and *Lb. plantarum* ATCC BAA-793; (6) control brine with 10 mM glutamate and inoculated with *Lb. gasseri* ATCC 33323 and *Lb. plantarum* ATCC BAA-793; (7) non-acidified brine inoculated with *Lb. gasseri* ATCC 33323 and *Lb. plantarum* ATCC BAA-793; and (8) non-acidified brine with 10 mM glutamate and inoculated with *Lb. gasseri* ATCC 33323 and *Lb. plantarum* ATCC BAA-793. The initial pH values of the acidified brines were 4.80 (without glutamate) and 4.68 (with glutamate, adjusted with 1 N NaOH). Initial pH values of the non-acidified brines were 6.61 (without glutamate) and 6.61 (with glutamate, adjusted with 1 N NaOH).

5.3.2 Chemicals and materials

Pickling cucumbers, pickling salt (sodium chloride (NaCl) ≥ 99%) and vinegar (acetic acid, 20%) were sourced from Mount Olive Pickle Company (Mount Olive, NC, USA). Calcium hydroxide ($Ca(OH)_2$, ≥ 95%), LC-MS grade water and LC-MS grade acetonitrile were purchased from Fisher Scientific (Hampton, NH, USA). Formic acid (≥ 98.5%), ammonium formate (≥ 99%), lactic acid (≥ 85%), calcium chloride ($CaCl2$, ≥ 93%), glutamate (glu, 99.5%), glutamine (gln, 98%) and γ-aminobutyric acid (GABA, 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stable isotope labeled standards were also purchased from Sigma-Aldrich including 4-aminobutyric acid-2,2,3,3,4,4-d6 (97 atom %) and a universally labelled ^{13}C, ^{15}N mixture containing glu and gln. Amicon Ultra-0.5, 10 kDa filters were purchased from Fisher Scientific.

5.3.3 Starter culture selection

Control microorganisms with GAD or glutaminase were identified using a bioinformatic approach. The National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov, accessed December 2019) database, GenBank, was used to search fully annotated genomes for genes encoding glutaminase (key words "glsA", "glsB" and "glutaminase") or GAD (key words "gadA", "gadB", "gadC" and "glutamate decarboxylase"). Three genera commonly associated with vegetable fermentations were surveyed: *Lactobacillus* (now reclassified into 23 genera by Zheng et al., 2020)*, Leuconostoc,* and *Pediococcus*. Two organisms, *Lactobacillus gasseri* ATCC 33323 and *Lactiplantibacillus plantarum* WCFS1 ATCC BAA -793 possessing genes for glutaminase and GAD, respectively, were selected. These organisms were obtained from the USDA Food Science and Market Quality & Handling Research Unit (FSMQHRU) culture collection with internal strain identification numbers of LA0257 and LA1196, respectively.

5.3.4 Inoculum preparation

A preliminary experiment was performed to verify survival and growth of *Lb. plantarum* WCFS1, an isolate from human saliva, and *Lb. gasseri*, an organism not typically found in cucumber fermentations, in simulated cucumber fermentation conditions. Six brines were prepared: low acid/low salt (27 mM $CaCl_2$, 760 mM NaCl, 40 mM $Ca(OH)_2$, 117.8 mM acetic acid), medium salt/medium acid (43 mM $CaCl_2$, 1225 mM NaCl, 64 mM $Ca(OH)_2$, 190 mM acetic acid), high salt/high acid (59 mM $CaCl_2$, 1690 mM NaCl, 89 mM $Ca(OH)_2$, 262 mM acetic acid), low salt/no acid (27 mM $CaCl_2$, 760 mM NaCl, 40 mM $Ca(OH)_2$), medium salt/no acid (43 mM $CaCl_2$, 1225 mM NaCl, 64 mM $Ca(OH)_2$) and high salt/no acid (59 mM $CaCl_2$, 1690 mM NaCl, 89 mM $Ca(OH)_2$). Brines were sterile filtered (0.2 µm) and mixed with sterile filtered cucumber juice (CJ) in a 55:45 CJ to brine ratio to create cucumber juice media (CJM). Starter cultures were streaked onto deMan, Rogosa and Sharpe (MRS) plates and incubated for 24 hr at 30°C. Colonies (2 – 3) were selected from the plate using a sterile loop, inoculated into 1 mL CJ, and incubated for 24 hr at 30°C. The prepared culture (10 µL) was inoculated into 990 µL CJM for each treatment, incubated at 30°C and visually assessed with MacFarland standards at 12, 24, 36, and 48 hrs. Both organisms grew to ~10^{7-8} in all treatments within 48 hours, confirming their ability to proliferate in whole cucumber fermentations.

Inocula for whole cucumber fermentations were prepared as follows: stock starter cultures were removed from -80°C storage, thawed briefly and 40 µL was aliquoted into 4 mL sterile filtered CJ and incubated at 30°C for 36 hours or until density was ~10^{8-9} according to visual assessment with MacFarland standards. CJM was prepared as described above for the high acid/high salt treatment, resulting in an equilibrated concentration of 27 mM $CaCl_2$, 760 mM NaCl, 40 mM $Ca(OH)_2$, and 117.8 mM acetic acid. The inocula in CJ (1.75 mL) were aliquoted into 33.25 mL CJM (1:20 dilution factor) and incubated at 30°C for 36 hours or until cell density was ~10^{8-9} according to visual assessment with MacFarland standards. Cell density was measured by OD_{600} using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA) and starter culture volumes adjusted for each organism.

5.3.5 Brining and fermentation of cucumbers

Size 2B (3.5-3.8 cm diameter) pickling cucumbers were packed into glass jars (1.36 L, 46 oz), covered with brine (55:45 cucumber:brine ratio). Starter cultures were added to four of the treatments (acidified, acidified + glu, non-acidified, and non-acidified + glu) to achieve cell densities of ~3.1 x 10^6 CFU/mL each *Lb. gasseri* and *Lb. plantarum*. Jars were closed with heat-sealing lids fitted with rubber septa for aseptic brine sampling. Brined cucumbers were incubated for 7 days at 28°C. This incubation length was chosen based on previous data showing that GABA is formed within the first 48-72 hours of fermentation (Moore et al., 2021*).* For aseptic brine sampling for pH measurement, jars were inverted to mix contents thoroughly and 1 mL brine was sampled using a needle and syringe on days 1, 2, 3, 5, and 7.

5.3.6 Preparation of cucumber slurries for amino acid and fermentation biochemistry analysis

Each jar was processed as follows after 7 days: cucumbers were removed from brine and sectioned into 1-inch pieces, placed in a blender (Waring, Model CB15V, Waring Commercial, Torrington, CT, USA) and ground at speed 3 for 1 min and at speed 6 for 2 min to form a homogenous cucumber slurry for amino acid analysis. Fresh cucumbers were prepared in a similar manner on day 0. Samples were stored at -80°C until the time of analysis.

5.3.7 Amino acid quantification by LC-QQQ-MS

Amino acid quantification was performed using liquid chromatography triple quadrupole mass spectrometry (LC-QQQ-MS) per Moore et al. (2021). Briefly, frozen cucumber slurries were thawed and 2.5 g was aliquoted into 25 mL stainless steel capsules with three 9 mm stainless steel balls. Capsules were submerged in liquid nitrogen for 30 sec and oscillated at 30 Hz for 4 min by a Retsch mixer mill MM301 (Cole-Parmer, Vernon Hills, IL, USA). Ground cucumber samples were centrifuged at 12,000 x g for 5 min at 4°C. Insoluble material was precipitated from the samples by mixing supernatant (90 µL) with mobile phase A (565 µL, 85% acetonitrile, 10 mM ammonium formate, 0.15% formic acid) and storing for 1 h at 4°C. Samples were centrifuged at 12,000 x g for 8 min at 4°C and 500 µL supernatant aliquoted into 10 kDa centrifugal cutoff filters. Filters were centrifuged at 14,000 x g for 10 min at 4°C and 323 µL filtrate was combined with 10 µL internal standard mixture in LC-MS vials. Vials were stored in a chilled autosampler (4°C) and analyzed within 24 h.

Amino acid quantification was performed on a Shimadzu LCMS-8040 triple quadrupole LC-MS/MS (Shimadzu Corporation) using an Atlantis HILIC column (100 × 4.6 mm, 3.0 µm) (Waters Corporation, Milford, MA, USA) for separation. The column was held at 37°C and separation achieved 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 was carried out as follows at 0.6 mL/min: 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). Flow was diverted to waste during the first 2 min to reduce salt accumulation on the ion source. Multiple reaction monitoring in positive ion mode was used to detect all amino acids. Drying and nebulizing gas (nitrogen) flow rates were 15 L/min and 3 L/min, respectively. Heat block temperature was 400°C. Resolution for the first and third quadrupoles was 0.7 dalton (Da) and 1.0 Da, respectively and loop time of 1 sec was used. Transitions and references ions for the amino acids quantified were as follows (m/z): GABA, $[M+H]^+$ 104.00 > 87.10, ref. 69.15; glutamate, $[M+H]^+$ 148.25 > 83.95, ref. 102.10, 130.05; glutamine $[M+H]^+$ 147.20 > 84.04, ref. 56.00, 41.95.

Standard curves were prepared using a mixture of amino acids at 8 different levels with concentrations ranging from 0.5 to 45 µM for glutamate, 1.5 to 1250 µM for glutamine, and 0.4 to 250 µM for GABA. GABA and glutamate were prepared in 0.1 N HCl, mixed together, and

stored at -19°C. Glutamine was solubilized in LC-MS grade water and stored at -19°C. Final standard mixes used for quantification were prepared by combining amino acids, internal standards and mobile phase A in LC-MS vials to a final volume of 1 mL. LabSolutions software ver. 5.8 (Shimadzu) was used for LC-MS analyses and data processing. Quantification was performed using peak height.

5.3.8 Fermentation biochemistry

Quantification of glucose, fructose and lactic acid was carried out as described by McFeeters & Barish (2003). Briefly, cucumber slurries that had been prepared and stored at −80°C were thawed and centrifuged at 9000 × g for 10 min at 4°C. Supernatants were injected onto an Aminex HPX-87H resin column (300 × 7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA) held at 37 °C with a 0.03 N H2SO4 mobile phase flowing at 0.6 mL/min. Lactic acid was detected by an Agilent 1260 Infinity diode array detector (Agilent Technologies Inc., Santa Clara, CA, USA) set to 210 nm. Sugars were detected by an Agilent Infinity refractive index detector (Agilent Technologies, Santa Clara, CA, USA) connected in series to the diode array detector. External standard curves with eight levels of concentration ranging from 0.5 to 100 mM were used for quantification of all analytes.

5.3.9 Statistical analysis

Analysis of variance (ANOVA) was performed using JMP Pro v. 14 (SAS Institute Inc., Cary, NC, USA). The dependent variable was GABA content. The ANOVA model included brine acidification, glutamate addition, starter culture addition, two-way and three-way interactions. Means separation after ANOVA was carried out using post-hoc Tukey analysis (α = 0.05).

5.4 Results and discussion

5.4.1 Fermentation biochemistry

Fermentations proceeded normally as evidenced by decreases in glucose and fructose and increases in lactic acid. The addition of glutamate or starter cultures did not impact the amount of lactic acid formed; however, significantly more lactic acid was generated in acidified, buffered

fermentations compared to non-acidified brined cucumbers(Figure 1). Correspondingly, residual fructose, and therefore total sugar, was lower in acidified fermentations than non-acidified fermentations. Typically, cucumber fermentations take 3 to 6 weeks to convert all sugars to organic acids; however, the fermentations in this experiment were terminated after 7 days leaving 10 – 25 mM sugar unfermented. Initial brine acidification aids in selecting for LAB growth and inhibits acid-sensitive bacteria (Pérez-Díaz et al., 2013) and the fermentation biochemistry data suggest that LAB were more metabolically active in the acidified treatments.

5.4.2 GABA formation during cucumber fermentation

GABA was present in fresh cucumbers and formed in all cucumber fermentations (Table 1) as expected. The highest levels of GABA were produced in the fermented cucumbers to which glutamate was added, including both acidified and non-acidified brines as well as inoculated and non-inoculated treatments. Fermentations were designed with lower salt content (2% NaCl) compared to commercial processes (6% NaCl) in order to eliminate the need for desalting which results in GABA dilution. If cucumbers were fermented at 2% NaCl with added glutamate (10 mM) and re-packed in fresh brine at a typical 58% pickle 42% brine ratio, the final GABA content of the product would be ~6 mM (748 mg/kg). A single one-ounce serving of pickle (3/4 of a spear or 28 g chips) manufactured in this manner would deliver ~21 mg of GABA. Reported clinically relevant doses of GABA range from 16 mg for antihypertensive (Pouliot-Mathieu and et al., 2013) to 26.4 mg for tranquilizer and anti-depressive effects (Okada et al., 2000) to 100 mg for antianxiety effects (Abdou et al., 2006). Given these levels as a guide, consumers would need to eat only approximately ½ of a pickle spear to obtain a clinically relevant quantity of GABA. This serving size is typical for consumers and much improved compared to the more than 8 spears (332 g) required for cucumber pickles fermented and stored in high salt brines. Alternatively, several smaller producers offer fermented cucumbers packed in their fermentation brine. If the GABA-enhanced pickles developed in this study were consumed directly without re-packing in fresh cover brine, a 1 oz serving would deliver 36.5 mg GABA.

Conversion efficiency was calculated by comparing the final GABA content of fermented cucumbers to the sum of glutamate and GABA in fresh cucumbers after correcting for the 55:45 cucumber:brine pack ratio. In this study, the conversion efficiency of the treatment most closely resembling commercially processed fermented cucumbers was 115%. When glutamate was

added to this treatment, conversion efficiency was 95% (acidified, non-inoculated with added glutamate) (Table 1). These data demonstrated that glutamate addition was effective for increasing GABA content during natural cucumber fermentations and provides a basis for the development of commercial fermented cucumber products with enhanced GABA content. GABA is not an approved food additive in the United States nor has it been given Generally Recognized as Safe (GRAS) status. Pharma Foods International (Kyoto, Japan) submitted GABA produced by *L. hilgardii* K-3 for GRAS status on 8/3/2015 (GRAS Notice No. GRN 000595) to be used in food at no more than 100 mg/serving (FDA, 2015a). They sent a follow-up letter to the Food and Drug Administration (FDA) on 11/10/2015 withdrawing their GRAS notice and the FDA ceased to evaluate it (FDA, 2015b). Due to GABA's lack of GRAS status, it must be naturally present or generated in food through fermentation. Glutamate, however, is approved as an additive in food (21 CFR 182.1045, 182.1047, 182.1500, 182.1516, 182.1); therefore, commercialization of fermented cucumber pickles with enhanced GABA via glutamate addition is feasible.

5.4.3 Effect of starter culture on GABA formation in fermented cucumbers

Glutamate and glutamine levels did not significantly differ between fermented cucumber treatments (Table 1, Figure 2), suggesting that significant levels of glutamine were not converted to glutamate and that glutaminase was not noticeably expressed under these conditions. Vermeulen et al., (2007) investigated glutamine deamidation by cereal-associated LAB and found that *Lb. reuteri* and *Lb. sanfranciscensis* produced only 0.5 mM glu from 40 mM gln (1.25% efficiency). It is possible that while the genome of the *Lb. gasseri* used in this study encodes for glutaminase, the conditions were not ideal for its expression or that it was only weakly expressed. Weingand-Ziade et al., (2003) observed glutaminase production and activity by *Lb. rhamnosus* at pH 5.8 while Thongsanit et al., (2009) determined that the optimal pH for glutaminase activity expressed by *Weissella cibaria* was 6.5. According to these authors' findings, the starting pH of the non-acidified cucumber fermentations (~6.6) were very near the optimal pH range for glutaminase activity. Further study of this organism as a pure culture in model cucumber fermentations would provide insight into whether it can produce glutaminase *in vivo* and whether it may have been outcompeted by the *Lb. plantarum* starter culture in this system.

Significantly more GABA was formed in the acidified, starter culture assisted fermentations with added glutamate than in the corresponding natural fermentations (Figure 2), indicating that starter culture addition may have a positive effect on GABA production when glutamate supplementation is combined with acidification. Genomic analysis of *Lb. gasseri* ATCC 33323 showed that this organism possesses a gene encoding for glutaminase; however, glutaminase activity was not directly measured in this study. Expression of glutaminase by *Lb. gasseri* ATCC 33323 in fermentations with starter cultures may result in the conversion of glutamine to glutamate and subsequent decarboxylation to GABA, however, these treatments did not have significantly higher GABA than the non-acidified, naturally fermented treatment (Figure 2). A statistical model fitting GABA content with the three independent variables and their interactions (glutamate addition, starter culture addition, acidification) found a significant three-way interaction ($p < 0.0001$), indicating that the effects of each variable depend upon the other two variables. Notably, the model indicated that starter culture addition did not significantly affect GABA production on its own ($p > 0.05$), but that it formed part of significant three-way interaction with acidification and glutamate addition ($p = 0.003$), showing that starter culture use becomes significant when initial brine pH is lower and glutamate is added. This interaction suggests that fermentation conditions may be manipulated in order optimize glutaminase expression and/or glutamate to GABA conversion in starter culture assisted cucumber fermentation.

5.4.4 Effect of initial brine pH on GABA formation

Initial pH of brines had a significant effect on the pH of 7 day cucumber fermentations; however, no correlation was found between GABA production and brine pH. Initial brine pH values were 4.68 - 4.80 (acidified treatments) and 6.61 (non-acidified treatments). Wu et al., (2017) determined that GadA and GadB from *Lb. brevis* and *Lb. plantarum* were active between pH 3.25 and 5.5 with an optimal pH range of 4.25 – 5.0. Production of lactic acid during fermentation and resultant pH decline to below pH 5.0 was accomplished within the first 24 hours of fermentation (Figure 3), bringing the fermentation pH into the optimal range for GABA production in all brining treatments.

The brine pH on day 7 was between 3.60 and 3.94 for all fermentation treatments Despite not being acidified at the beginning of fermentation, the pH of the non-acidified

fermented cucumber treatments were significantly lower than their acidified & buffered counterparts (Figure 4). Additionally, more lactic acid was generated in acidified compared to non-acidified treatments, yet did not result in lower pH values (Figure 1). The non-acidified treatments lacked buffering agents (acetic acid and $Ca(OH)_2$) that mediate pH changes, which is consistent with day 7 pH values that were lower than their acidified counterparts.

The aim of performing non-acidified fermentations was to promote survival of naturally present, glutaminase-expressing enteric organisms so that they could potentially convert glutamine to glutamate for enhanced GABA production. However, the non-acidified fermentations did not have higher GABA or glutamate than the acidified fermentations. The rapid decline in pH to < 4.25 observed within the first 48 hours of fermentation (Figure 3) likely inhibited these organisms' survival and growth (Pérez-Díaz et al., 2019). Starter cultures have been utilized by commercial processors to increase fermentation rate and consistency (Pérez-Díaz and McFeeters, 2011). Consistent with this practice, the non-acidified, inoculated cucumbers had the lowest pH values indicating that the starter cultures contributed to rapid pH decline. Furthermore, observation of pH change over time showed that addition of starter cultures resulted in greater pH decreases within 48 hours (Figure 3b) compared with natural fermentations (Figure 3a) in both acidified and non-acidified treatments. When similar treatments were compared, the addition of glutamate did not result in significantly different pH values. For example, pH of the acidified treatment with added glutamate (4.00 ± 0.05) compared to the non-acidified treatment (3.94 ± 0.05) was not different, however, significantly higher GABA was produced in the former (Table 1), demonstrating that despite this increased conversion of glutamate to GABA, extracellular pH was not significantly different compared to the acidified treatment. The GAD system serves to maintain intracellular pH homeostasis, therefore internal pH measurements of these fermentation microbes may provide greater insight into the impact of glutamate addition on fermentations. Wu et al. (2017) found that internal pH of GAD-expressing *Lb. brevis* grown in Lactobacilli MRS with added glutamate (1 g/L, 5.91 mM) decreased from pH 6.5 to 6.0 over 37 hrs while external pH declined from 6.5 to 5.0. However, the internal pH of *Lb. brevis* cells lacking GAD genes decreased from 6.5 to 5.5 with external pH dropping to 4.5 over the same period of time, demonstrating that external pH was significantly impacted by GAD expression. Our study differs from that of Wu et al. (2017) in that our fermentations were not pure culture nor are cucumber fermentations typically dominated

by *Lb. brevis*. In our study, metabolic activity from autochthonous and starter culture organisms influenced extracellular biochemistry and resulting pH. Conducting pure culture fermentations using cucumber associated starter cultures with and without GAD-expressing capabilities would provide information about the specific effect of GAD expression on pH in this matrix.

5.5 Conclusion

Addition of glutamate to natural cucumber fermentations resulted in enhanced GABA concentration with approximately 100% conversion efficiency. Cucumber fermentation using non-acidified brines did not result in increased GABA concentration compared to a buffered brine system containing 53 mM acetic acid, indicating that naturally present enteric microorganisms did not express significant glutaminase activity. A naturally fermented cucumber pickle with 10.4 mM (1289 mg/kg) GABA was produced, resulting in a directly consumable product that would deliver ~36 mg of GABA per 28 g serving of pickles. This study provides evidence that the development of a commercial fermented cucumber product with enhanced GABA is feasible via glutamate addition.

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Table 1. GABA, glutamate and glutamine content of cucumbers fermented under varying conditions for 7 days at 28°C

Treatment	Concentration (mM)*							Conversion Efficiency
	$CaCl_2$	$Ca(OH)_2$	Acetic acid	Added Glutamate	GABA	Residual Glutamate	Glutamine	
Fresh Cucumber	-	-	-	-	1.62 ± 1.21^c	0.31 ± 0.04^a	7.13 ± 1.29^a	N/A
Acidified	12	18	53	-	1.21 ± 0.16^c	0.42 ± 0.22^a	2.50 ± 0.70^b	114.6%
Acidified + glutamate	12	18	53	10	10.39 ± 2.23^b	0.36 ± 0.19^a	3.92 ± 0.95^{ab}	95.6%
Non-acidified	12	-	-	-	1.63 ± 0.20^c	0.03 ± 0.02^a	3.67 ± 0.91^{ab}	93.9%
Non-acidified + glutamate	12	-	-	10	14.78 ± 1.15^a	0.04 ± 0.04^a	3.54 ± 1.18^b	134.4%
Acidified, with starter culture	12	18	53	-	1.01 ± 0.60^c	0.36 ± 0.29^a	3.93 ± 1.62^{ab}	153.6%
Acidified + glutamate, with starter culture	12	18	53	10	14.86 ± 0.72^a	0.05 ± 0.05^a	3.79 ± 0.97^{ab}	117.8%
Non-acidified, with starter culture	12	-	-	-	1.25 ± 0.24^c	0.12 ± 0.02^a	2.67 ± 0.78^b	133.6%
Non-acidified + glutamate, with starter culture	12	-	-	10	13.65 ± 0.97^a	0.18 ± 0.28^a	3.07 ± 1.16^b	123.4%

All treatments contained 342 mM NaCl. Starter cultures included *Lb. gasseri* ATCC 33323 and *Lb. plantarum* ATCC BAA-793.

*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.
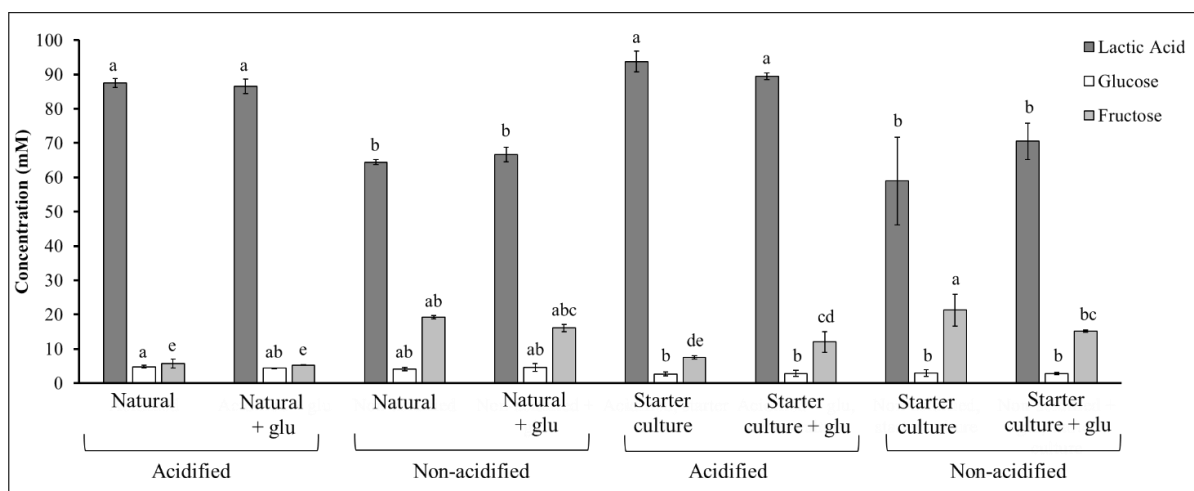
Figure 1. Fermentation biochemistry of acidified, non-acidified, natural and starter culture fermented cucumber slurries after 7 days at 28°C. Different lowercase letters indicate significant differences between treatments ($p < 0.05$) within individual metabolites using a one-way analysis of variance with post-hoc Tukey HSD.
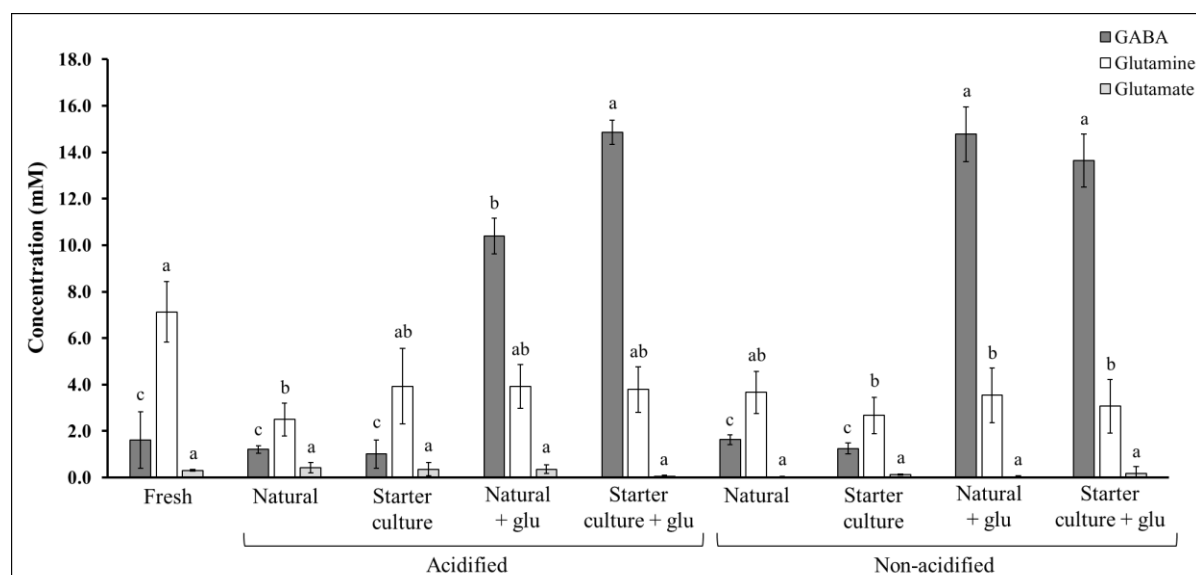
Figure 2. GABA, glutamate and glutamine content of fresh, acidified, non-acidified, natural and starter culture fermented cucumber slurries on day 7. Different lowercase letters indicate significant differences between treatments ($p < 0.05$) within individual metabolites using a one-way analysis of variance with post-hoc Tukey HSD.
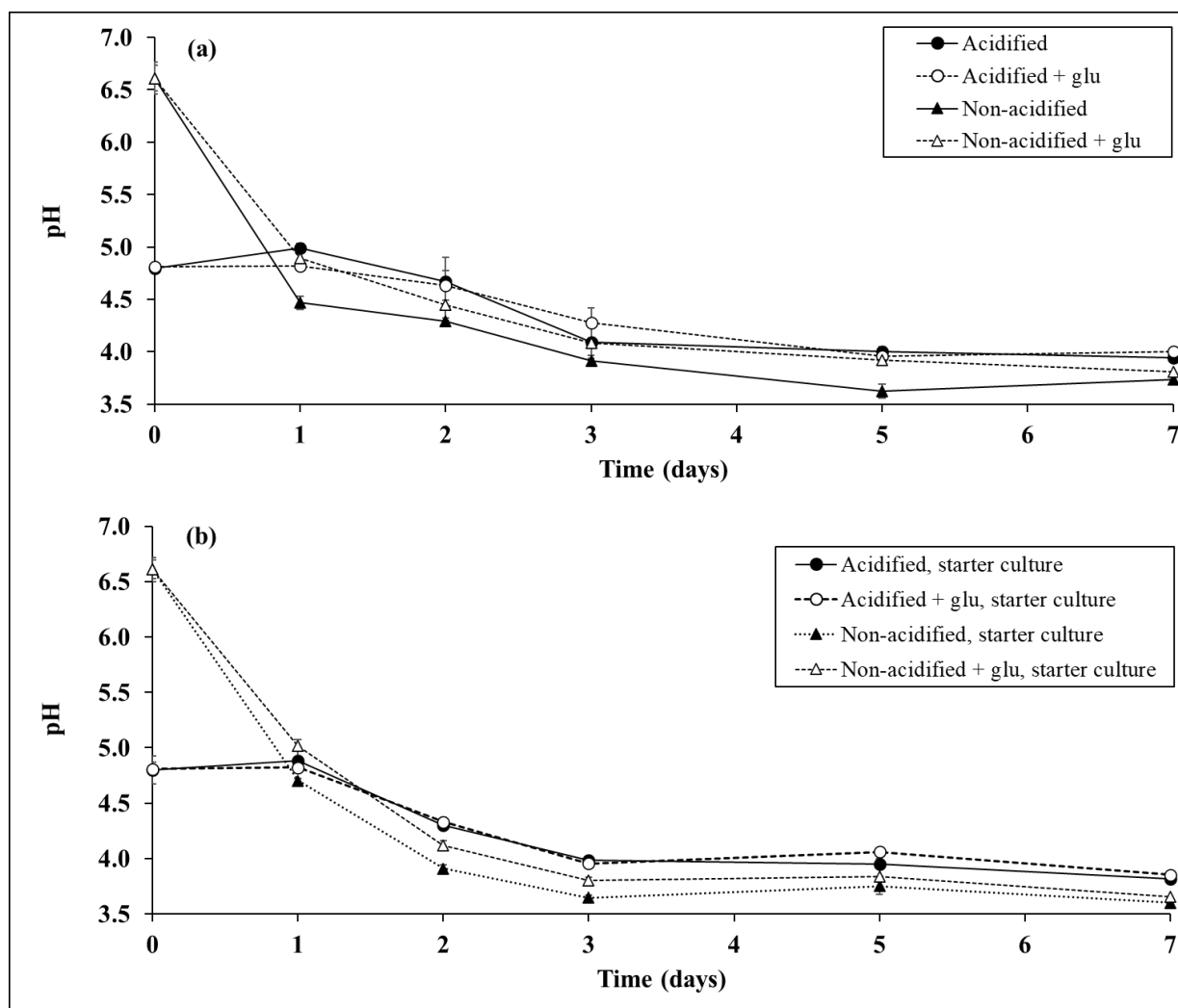
Figure 3. pH of acidified, non-acidified, natural and starter culture assisted cucumber fermentation brines over time.
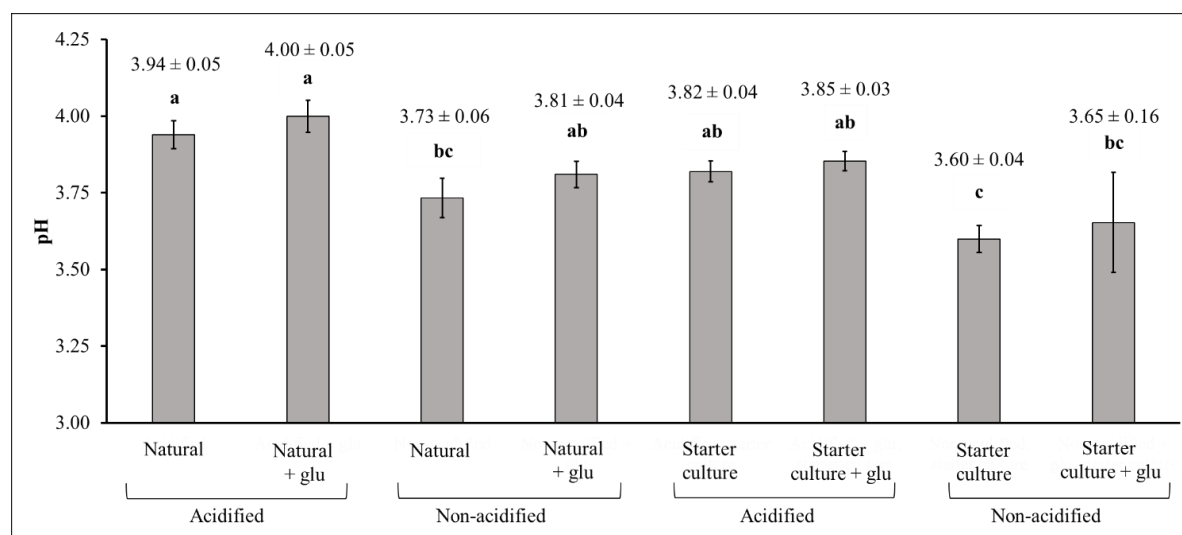
Figure 4. pH of acidified, non-acidified, natural and starter culture fermented cucumber slurries on day 7. Different lowercase letters indicate significant differences between treatments (p < 0.05) using a one-way analysis of variance with post-hoc Tukey HSD.

CHAPTER 6: Comprehensive Review: Health-Promoting Compounds Generated by Lactic Acid Fermentation of Fruits and Vegetables

6.1 Abstract

Fermentation by lactic acid bacteria (LAB) has been used for centuries to preserve perishable foods and transform them into products with improved organoleptic properties and health benefits. A variety of foods including dairy, fruits, grains, legumes, meat, and vegetables are fermented by LAB. Often these fermented foods are considered healthful due to their potential containment of live, probiotic microorganisms. However, due to low pH, extended storage times, and processing methods applied for shelf stability, the viability of these organisms is compromised. During fermentation, LAB incur compositional changes in foods including reducing antinutrients, hydrolyzing carbohydrates, fats and proteins to release nutrients, and generating new bioactive compounds. Fruits and vegetables such as beets, cabbage, cocoa, carrots, cucumbers, garlic, olives and onions are considered healthful in their fresh state, and fermentation may further increase their healthfulness as a result of LAB metabolic activity. Changes in fruits and vegetables during fermentation include but are not limited to the release of bound vitamins and phenolics from plant cells, conversion of glucosinolates to active anticancer compounds, release of bioactive peptides from proteins, and the formation of γ-aminobutyric acid, conjugated linoleic acid and exopolysaccharides. Furthermore, lactic acid fermentation is a promising technology for developing value-added products and reducing food waste from commercial fruit and vegetable processing operations. Mixing of multiple fruits and/or vegetables in shredded, diced, juiced, and pureed formats is a growing area of research, with a goal of combining various foods and naturally present microbiota to optimize health beneficial compounds generated during fermentation. This review summarizes the health-promoting properties of lactic acid fermented fruits and vegetables including current advances in fermentation technology and areas of future research for LAB fermentations.

6.2 Introduction

Food fermentation has been widely employed as a means of food preparation and has been defined as "a technology in which the growth and metabolic activities of microorganisms are used to preserve foods" (Şanlier, Gökcen & Sezgin 2019). Lactic acid (LA) fermentation is

an approachable, affordable technology that allows for the conversion of perishable fruits and vegetables into shelf-stable products due to the generation of acids by lactic acid bacteria (LAB). It is believed that Littoral foragers in Asia were some of the earliest adopters of LA vegetable fermentation, dating back to between 8000 and 3000 B.C. (Lee, 2009) and that cucumber fermentation can be traced back to 2000 B.C. in the Middle East (Breidt, McFeeters, Pérez-Díaz & Lee, 2013). Currently, LA fermented fruits and vegetables are produced in numerous cultures ranging from regionally crafted products such as dakguadong (mustard leaves; Thailand), khalpi (cucumber; Eastern Himalayas) and hardaliye (grape, mustard seed, sour cherry leaves; Turkey) to foods known and produced across the globe such as sauerkraut, kimchi, and cucumber pickles. LA fermented foods often remain stable without high energy input thermal processing technologies such as pasteurization or sterilization. In addition to enhancing stability, LA fermentation also transforms the organoleptic properties of fresh fruits and vegetables through the generation of acids and volatile compounds and the release of taste-active amino acids and peptides (Zhao, Schieber & Ganzle 2016). Finally, the acidic environment of LA fermented foods also serves as a means to increase food safety by inhibiting pathogen survival (Breidt, 2006).

Fresh fruits and vegetables contain a variety of health-promoting nutritional compounds including fiber, vitamins, and minerals as well as numerous non-nutritive bioactive compounds such as phenolics, flavonoids, antioxidants, enzymes, bioactive peptides, and polysaccharides that serve as prebiotics. The United States Department of Agriculture recommends that adults consume 2.5 – 3 cups of vegetables and 1.5 – 2 cups of fruit daily (https://www.choosemyplate.gov/ Accessed 10.07.20), highlighting the importance of these food groups in a balanced diet. While these foods are already considered healthy, fermentation has the potential to further enhance their healthfulness through compositional changes and the generation of bioactive compounds. A variety of reviews on the healthfulness of fermented foods have been published (Annunziata, Ciampaglia, Tenore & Novellino 2020; Borresen, Henderson, Kumar, Weir & Ryan, 2012; FitzGerald & Murray, 2006; Marco et al., 2017; Marsh, Hill, Ross & Cotter, 2014; Melini, Melini, Luziatelli, Ficca & Ruzzi, 2019; Rizzello, Tagliazucchi, Babini, Rutella, Saa & Gianotti 2016; Selhub, Logan & Bested, 2014; Van Hylckama Vlient, Veiga, Zhang, Derrien & Zhao, 2011). The majority of these articles examine products of animal (dairy, meat), grain (wheat, rice), and legume (soy) origin with minor mention of products derived from

vegetables or fruits. While reviews by Şanlier et al. (2019) and Septembre-Malaterre, Remize & Poucheret (2018) contain significant sections focused on LA fermented fruits and vegetables, a comprehensive review of the compositional changes and health benefits conferred through LA fermentation of fruits and vegetables has not been published.

The probiotic potential of LA fermented foods has been extensively researched and summarized in dairy products (Granato, Branco, Cruz, Faria & Shah, 2010; Turkmen, Akal & Özer., 2019) and to a much lesser extent in fruit and vegetable products (Patel, 2017; Swain, Anandharaj, Ray & Rani, 2014). Due to the acidic nature of LA fermented fruits and vegetables, these foods are typically unsuitable for long term probiotic survival (Fan, Breidt, Price, Pérez-Díaz, 2017). Additionally, commercial processing of LA fermented products such as pickles, kimchi and sauerkraut often involves pasteurization to ensure shelf stability, resulting in the death of probiotic microorganisms. While LAB do not survive thermal processing, they generate new health-promoting compounds such as vitamins, bioactive peptides, conjugated linoleic acid (CLA), short-chain fatty acids, neurotransmitters, and exopolysaccharides which remain intact in the final product. Furthermore, LAB can convert unstable precursors to stable compounds (e.g., glutamate to γ-aminobutyric acid (GABA)), increase the digestibility of food, and eliminate antinutrients. LAB are fastidious microorganisms that require nutrients from their environments. As such, they possess a variety of enzymes that assist in the breaking down of plant tissues including amylases, proteases, peptidases, pectinases, esterases, glucosidases, decarboxylases, and dehydroxylases (Selma, Espín & Tomás-Barberán, 2009; Savijoki, Ingmer & Varmanen 2006; Champ, Szylit, Raibaud & Nadra, 1983; Karam & Belarbi 1995). This review summarizes the health benefits conferred by metabolic activity of LAB during fruit and vegetable fermentation and discusses fermentation technologies for the generation of novel health-promoting products.

6.3 LA fermentation of fruits and vegetables

During fermentation, LAB metabolize sugars to organic acids and carbon dioxide (CO_2). Fermentation microbiota can be divided into two major metabolic categories: homofermenters and heterofermenters. Homofermentation produces two moles of lactic acid from one mole of glucose, and heterofermentation produces one mole each of lactic acid, acetic acid, and CO_2 from one mole of glucose. Additionally, some LAB are considered facultative heterofermenters

and will produce CO_2 under certain conditions or from specific substrates. LA fermentation of fruits and vegetables is primarily due to LAB naturally present on the starting material, however other bacteria and yeasts are also involved and lead to the development of small amounts of other metabolic products including ethanol and acetic acid (Pérez-Díaz et al., 2013).

LAB genera primarily responsible for fruit and vegetable fermentations include *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella* (Breidt, 2013; Di Cagno, Coda, De Angelis & Gobetti, 2013; Vinicius De Melo Pereira et al., 2020). Due to the success and consistency of natural fruit and vegetable fermentations, the use of starter cultures has been explored academically but not widely adopted commercially (Vinicius De Melo Pereira et al., 2020; Pérez-Díaz et al., 2013). However, due to the high economic value of chocolate, starter culture use has gained traction in commercial cocoa production for improved fermentation efficiency, consistent quality and enhanced sensory attributes of finished chocolate (Lefeber, Papalexandratou, Gobert, Camu & De Vuyst, 2012; Figueroa-Hernández et al., 2019).

Fruit and vegetable fermentations are often aided by the addition of salt, primarily in the form of sodium chloride (NaCl) but also calcium chloride ($CaCl_2$). Salt may be incorporated into fermentations through dry salting or liquid brining. Dry salting, used in kimchi and sauerkraut fermentation, involves the mixing or layering of the food material with salt to draw water from the plant cells, resulting in a small amount of brine. In liquid brining, the food material is submerged in brines containing salt or salt and acid to adjust the initial pH of the fermentation. In both of these processes, salt aids in selecting for and controlling microbial activity, preventing softening, influencing taste of the final product, and rupturing plant tissue membranes, resulting in the release of cellular components into brine for access by microorganisms (Pérez-Díaz et al., 2013).

LA fermented fruits and vegetables may be fermented individually (i.e., cucumber pickles) or combined in mixed fermentations (i.e., kimchi). Spices, sweeteners and flavoring agents such as chili powder, turmeric, mustard seeds, garlic, fruit syrup, honey, or dill oil may be incorporated to individual or mixed fermentations to produce a desirable organoleptic profile. These added components may also introduce substrates for microbial metabolism that result in the production of bioactive compounds. For example, fermented fish sauce is commonly added to kimchi fermentations and contains a significant amount of glutamate that can be utilized by LAB to produce GABA (Jung et al., 2018).

6.4 Cabbage

Cabbage is fermented in various cultures with the most well-known products being sauerkraut and kimchi. Sauerkraut is produced from the fermentation of white cabbage (*Brassica oleracea* var. *capitata*) by *Leuconostoc mesenteroides, Lb. brevis and Lb. plantarum* (Breidt, 2013). Traditionally, sauerkraut is produced and consumed as a side dish or condiment in Europe, Asia, and the United States (Peñas, Martinez-Villaluenga & Frias, 2017). Cabbage is prepared for fermentation by removing the outer leaves and core and finely chopping or shredding the inner leaves. Shredded leaves are layered in fermentation vessels with salt (0.7-2.5% NaCl, Peñas et al., 2017) which draws moisture from the leaves to generate a liquid brine. Leaves are pressed to eliminate oxygen and anaerobic fermentation commences, lasting from a week to several months. Sauerkraut can be packaged and refrigerated for fresh consumption or thermally processed (pasteurized or canned) for shelf stability.

Glucosinolates (GLS) are a class of nitrogen- and sulfur-containing compounds present in cabbage and other *Brassica* species such as broccoli, brussels sprouts, kohlrabi, turnips and mustard greens. Breakdown of GLS by the action of myrosinase, an enzyme contained within plant cells, produces indoles and isothiocyanates that have been associated with anti-cancer properties (Bonnesen, Eggleston & Hayes, 2001; Peñas, Frias, Martínez-Villaluenga & Vidal-Valverde, 2011). Glucobrassicin is a GLS present in cabbage that is first hydrolyzed by myrosinase to form indole-3-carbinol and then spontaneously reacts with ascorbic acid under mildly acidic pH to yield ascorbigen (Wagner & Rimbach, 2009). Several *in vitro* studies involving ascorbigen have been published in both human and animal models. In mouse hepatoma cells, ascorbigen administered at levels between 1-1000 µmol/L increased detoxification enzyme activity (cytochrome P450 1A1) in a dose-dependent manner (Stephensen, Bonnesen, Schaldach, Andersen, Bjeldanes and Vang, 2000). Bonnesen et al. (2001) found that treating human colon cells with 700 µmol/L ascorbigen enhanced cytochrome P450 activity and stimulated apoptosis. While, both indole-3-carbinol and ascorbigen have demonstrated chemoprotective effects, the former is an unstable intermediate and conversion to ascorbigen is preferable for stability (Wagner et al., 2009).

A key factor in the conversion of GLS to bioactive compounds is the disruption of plant tissues to allow contact with myrosinase which is physically separated from GLS in tissue compartments (Peñas et al., 2017). Disruption of plant tissue is accomplished through culinary

procedures (i.e., chopping, shredding) (Wagner & Rimbach, 2009) and may also be accomplished through salting as is practiced in sauerkraut and kimchi manufacturing. Martinez-Villaluenga et al., (2009) found that small amounts of ascorbigen were present in raw cabbage (5.71 - 8.12 µmol/100g dry weight) and were enhanced 10 to 20-fold after fermentation. In that study, cabbages grown in winter had higher starting glucobrassicin levels than those grown in summer. Accordingly, after fermentation, winter-grown cabbages had higher final ascorbigen content (104.56 - 108.97 µmol/100g dw, 0.5% NaCl) compared to summer-grown cabbages (91.98 - 97.06 µmol/100g dw, 0.5% NaCl). No significant difference in ascorbigen formation was found in 0.5% NaCl sauerkrauts fermented naturally or inoculated with starter cultures of *Lb. plantarum* or *L. mesenteroides*. Interestingly, when 1.5 % NaCl was used, sauerkrauts fermented with starter cultures had significantly greater ascorbigen content than the naturally fermented sauerkraut, indicating that starter culture addition enhances ascorbigen formation. Ascorbic acid content of sauerkraut was also measured in that study and found to remain stable in starter culture fermented treatments across a range of salt levels and between both cultivation seasons. A significant loss of ascorbic acid was observed in naturally fermented sauerkrauts from summer cabbages packed with 1.5% NaCl, which may explain the lower final ascorbigen content in this treatment. Ascorbic acid loss during fermentation is thought to be a result of its involvement in ascorbigen formation and utilization as an antioxidant, while further degradation occurs in downstream processing due to oxidation and pasteurization (Hrncirik, Valusek & Velisek, 2001). Since ascorbic acid is abundant in *Brassica* vegetables, glucobrassicin content is the limiting factor in ascorbigen formation (Hrnciki et al., 2001). Horticultural selection of cabbage varietals with high glucobrassicin levels is one means for crafting sauerkraut with improved chemoprotective effects through enhanced ascorbigen content.

A second method for increasing ascorbigen content in sauerkraut is to manipulate the fermentation conditions. The reaction of indole-3-carbinol with ascorbic acid has an optimal pH range of 4.5-5 (Wagner & Rimbach, 2009), and near the pKa of ascorbic acid (4.2) water competes for reaction with indole-3-carbinol (Martinez-Villaluenga et al., 2009). Delaying the accumulation of acid during cabbage fermentation would allow for a longer period of time at the pH optimal for ascorbigen formation. Interestingly, Johanningsmeier, Fleming, Thompson & McFeeters (2005) found that preparing sauerkraut by mixing cabbage with brine (60:40 cabbage-brine) to equilibrate to 0.5% NaCl resulted in slower pH decline than in the traditional method of

dry salting cabbage with 2% NaCl. This delayed pH decrease may help explain why Martinez-Villaluenga et al., 2009 observed higher ascorbigen formed in fermentations with 0.5% NaCl than 1.5% NaCl. However, development of low salt sauerkraut fermentations for high ascorbigen production must also take into consideration the resulting impacts on product stability and sensory quality. Johanningsmeier et al., 2005 reported that off-flavors were developed and unpredictable softening occurred in sauerkraut fermented with 0.5 % or 1% NaCl compared to a control (2% NaCl). The addition of a *L. mesenteroides* starter culture ameliorated these issues by ensuring a swift and complete fermentation, meaning that its pH decline rate was similar to that of the control. Selection of an alternative starter culture with a slower metabolism, adjusting the inoculation load or controlling fermentation temperature represent three areas of opportunity to optimize ascorbigen production while maintaining product quality in sauerkraut.

Kimchi is a traditional Korean product consumed both within Korea and widely abroad. The global kimchi market is valued at $4.5 billion with a projected increase of $743 million by 2024 with consumption for health benefits projected to be a significant driver of growth (Technavio, 2019). Kimchi is made from a salted mixture of vegetables and spices, most prominently featuring Chinese cabbage (*Brassica rapa*). Spices and seasonings including red and black pepper, garlic, ginger, mustard, fish sauce, soy sauce, sesame seeds and corn syrup. Additional plant materials may be added such as celery, cucumber, carrot, radish, leek, green onion, barley, rice, pear or apple (Cheigh, Park & Lee, 1994). Manufacturing of kimchi involves the cutting of cabbage into halves or quarters and soaking in brine (5-10% NaCl) to wilt. The cabbage is then washed, drained, and mixed with spices and seasonings. Kimchi is packed into jars or pouches and fermented at 18°C for a few days then refrigerated (1-2 °C). This process allows for heterolactic fermentation during the beginning of incubation and delays homolactic fermentation to prevent kimchi from becoming excessively sour (Pérez-Díaz et al, 2013). The most common LAB involved in kimchi fermentation are *Leuconostoc* spp., *Lactobacillus* spp., and *Weisella* spp. (Noh, Seo, Park & Oh, 2016).

Consumption of kimchi has been associated with anti-obesogenic (An et al., 2013; Cui et al., 2015; Kim et al., 2011; Kim & Park, 2018; Park, Tirupathi Pichiah, Yu, Oh, Daily & Cha, 2012; Park et al., 2020), antidiabetic (Choi, Noh, Han, Kim, Han & Song, 2013; Islam & Choi, 2009), antioxidant (Ryu, Yang, Lee, Kim, Seo & Jung, 2019), and probiotic (Park et al., 2014; Son, Jeon, Yang, Lee & Paik, 2017) health effects. Similar to sauerkraut, LAB in kimchi perform

bioconversion of inactive plant precursors to bioactive compounds. The variety of vegetables, spices, and seasonings in kimchi provide a wide range of potentially biologically active plant metabolites. Quan, Piao, Min, Yang, Lee & Yang (2011) isolated a strain of *Leuconostoc citreum* from kimchi and evaluated its ability to convert ginseng-derived ginsenoside RB1 to compound K, which is known to suppress tumors and prevent metastasis. *L. citreum* LH1 possessed β-glucosidase which was effective in converting pure RB1 to compound K with an optimum pH of 6. This study shows promise for the inclusion of ginseng in kimchi to boost chemoprotective effects; however, further investigation is needed to observe the effects of pH decline on bioconversion of RB1 to compound K. The form of ginseng (sliced, powdered) should be carefully chosen to ensure accessibility of RB1 by β-glucosidase producing LAB during fermentation. Additionally, selection of LAB with high β-glucosidase activity may increase bioconversion of RB1 to compound K.

Kimchi may also provide health benefits to consumers due to the formation of γ-aminobutyric acid (GABA). GABA is a non-proteinogenic amino acid and is the primary inhibitory neurotransmitter in the mammalian brain (McCormick, 1989). Consumption of GABA either as a supplement or within foods has been shown to lower blood pressure (Hayakawa, Kimura, Kasaha, Matsumoto, Sansawa & Yamori, 2004; Inoue et al., 2003; Pouliot-Mathieu, Gardner-Fortier, Lemieux, St-Gelais, Champagne & Vuillemard, 2013), reduce anxiety (Abdou, Higashiguchi, Horie, Kim, Hatta & Yokogoshi, 2006; Yongjian et al., 2019), improve immune system function (Abdou et al., 2006) and aid in decision making (Steenbergen, Sellaro, Stock, Beste & Colzato, 2015). During fermentation, LAB decarboxylate glutamate using glutamic acid decarboxylase (GAD) to form GABA, consuming a proton and resulting in an increase in intracellular pH (Wu, Tun, Law, Khafipour & Shah 2017). GABA production by LAB is strain specific, leading researchers to select LAB with high GABA producing capabilities (Cho, Park, Kim, Ryu & Park, 2011; Kim & Kim, 2012; Lee, Shim, Yao, Kim & Kim, 2018;).

Mukeunjee, or "sour" overripened kimchi is fermented longer than regular, "optimally fermented" kimchi to achieve a softer, more acidic product ideal for use as an ingredient in side dishes (Cheigh et al., 1994). A commercial product survey of regular and mukeunjee kimchi revealed that mukeunjee samples contained 182 – 990 mg/kg GABA which was 3.1 – 16.9 times greater than GABA in regular kimchi (58.5 mg/kg) (Cho et al., 2011). This commercial survey shows that longer fermentations may be used to produce kimchis with naturally enhanced

GABA. Conversely, in cucumber fermentations GABA is known to be formed within the first 48 hours and that extended incubation times do not increase its concentration (Moore, DuVivier & Johanningsmeier, 2021). The cucumber fermentations in this study contained significantly more brine than kimchi (48:52 brine-cucumber) and were conducted at a higher temperature (28°C), allowing for quicker equilibration of glutamate into the brine. Delayed glutamate equilibration due to lower fermentation temperature (18°C) and lower brine-vegetable ratios may explain the increased GABA production over time. Cho et al., (2011) also isolated a high GABA producing strain of *Lb. buchneri* from commercial mukeunjee and inoculated it into laboratory prepared kimchi. After three days of fermentation at room temperature, the GABA content of naturally fermented and *Lb. buchneri* inoculated kimchis were 81 ± 5 mg/kg and 617 ± 90 mg/kg, respectively. Sensory analysis revealed no significant difference in acceptability between control and *Lb. buchneri* kimchis, demonstrating that this starter culture may be utilized to enhance GABA production in both optimally fermented and mukeunjee kimchis.

Kimchi's composition of mixed vegetables, spices, and seasonings makes it an ideal product for adjusting raw ingredients to provide enhanced content of bioactive compounds. Inclusion of 1% black raspberry juice powder to kimchi fermentations yielded products with phenolic compounds and resulting antioxidant effects when fed to rats with liver cirrhosis (Ryu et al., 2019). Lee et al. (2018) found that addition of either monosodium glutamate (MSG) or kelp extract increased GABA in kimchi fermented with *Lb. zymae* GU240 compared to a control ($1,119 \pm 9$ mg/kg, 812 ± 35 mg/kg, and 555 ± 27 mg/kg GABA, respectively). Inclusion of fermented fish sauce (jeotgal) in kimchi is common and contributes glutamate for GABA conversion (Jung et al., 2018). Kimchi made with jeotgal from anchovy and shrimp had greater GABA (5 mM) than those made without jeotgal (3 mM). Additionally, the type of jeotgal impacted the succession of bacteria during kimchi fermentation which may also impact the amount of GABA produced. Formulation of kimchi with mustard leaf, Chinese pepper, pear, sea tangle (*Lentinus edodes*) juice, and mistletoe extract resulted in a functional product with anticarcinogenic activity (Kim et al., 2018). These ingredients contributed terpenylated coumarins, 4-quinolone alkaloid, lectins, GLS, alkaloids, flavonoids, polysaccharides and triterpenes. Upon consumption of the functional kimchi, healthy young adults had significantly improved body fat and skeletal mass compared to subjects consuming standard kimchi. The inflammation-related marker interleukin 6 and serum lipids (total cholesterol and triglycerides)

also significantly decreased in subjects consuming functional kimchi, demonstrating that kimchi with added phytochemicals is capable of enhancing human health to a greater extent than traditionally made kimchi. Kimchi serves as an example for the opportunities that exist in mixed fruit and vegetable fermentations for optimal natural production of bioactive compounds.

6.5 Cucumber

Cucumber pickles are produced throughout the world through either acidification with acetic acid (vinegar) or fermentation with LAB. Despite widespread consumption of cucumber pickles, limited research has been conducted on their potential health promoting properties. In commercial fermentations, fresh cucumbers (*Cucumis sativus* L.) are submerged in brine (0.6–1.7M NaCl) in large capacity vats and are fermented by naturally present LAB (Franco, Johanningsmeier, Lu, Demo, Wilson & Moeller, 2016; Pérez-Díaz et al., 2013). After fermentation, cucumbers may be stored in fermentation tanks for one year or more (Breidt et al., 2013). In addition to large-scale commercially fermented cucumbers, smaller scale, regional fermented cucumber pickles are produced in Nepal (khalpi), Taiwan (jiang-gua), China (paocai) and Turkey (turşu) (Tamang, Tamange, Schillinger, Franz, Gores, & Holzapfel, 2005; Chen et al., 2011; Yan, Xue, Tan, Zhang, & Chang, 2008; Kabak & Dobson, 2011). Khalpi is made by slicing ripe cucumbers, drying them in the sun for 48 hours, placing them in a bamboo vessel, covering the cucumbers with dried leaves, and allowing them to ferment for 3-5 days. After fermentation, salt, mustard oil, and powdered chilis are added (Swain et al., 2014). Jiang-gua is made by cutting cucumbers and layering them with salt in a bucket then sealing the bucket with stones for 4-5 hours to expel water from the cucumbers. Cucumbers are drained, mixed with sugar and vinegar and potentially soy sauce depending on the formulation (Chen et al., 2011). Paocai is produced in a similar manner to American and European cucumbers in that sliced cucumbers and other vegetables are submerged in brine containing 6-8% NaCl and fermented for 6-10 days (Yan et al., 2008). To prepare turşu, whole or sliced cucumbers are combined with flavoring agents in glass or plastic containers, pressed, covered with brine containing 10-15% NaCl and grape vinegar, and fermented for 4 weeks (Kabak et al., 2011). Typical microbiota involved in cucumber fermentation include *Lb. plantarum* or *Lb. pentosus, Lb. brevis, Enterococcus faecalis, L. mesenteroides*, and *Pediococcus cerevisiae* (likely *Pediococcus pentosaceus* and/or *Pediococcus acidilactici* after recent reclassification) (Pérez-Díaz et al.,

2013). Additional LAB identified in khalpi and jiang-gua include *Leuconostoc fallax* (Di Cagno et al., 2013), *W. cibaria* and *Leuconostoc lactis* (Chen et al., 2011).

Probiotic benefits have been attributed to fermented cucumber products due to the involvement of LAB such as *Lb. plantarum* in the fermentation process. However, these autochthonous LAB are not verified probiotic strains, nor has their survival been verified in fermented cucumbers. A study involving the inoculation of acidified, refrigerated pickles with 10 probiotic strains of *Pediococcus* or *Lactobacillus* found that the most acid resistant strain (*Lb. casei*) survived for 63 days at 4°C at an acceptable probiotic concentration of 10^8 CFU/mL (Fan et al., 2017). Thus, delivery of fermented cucumbers containing live microorganisms or probiotics would require quick, refrigerated distribution to consumers.

Cucumbers are a natural source of antioxidant phenolic compounds including flavonols and proanthocyanidins (Melo, Lima, Maciel, Caetano & Leal, 2006). Phenolics are one of the most widely studied classes of bioactive compounds found in fruits and vegetables. The main classes of phenolic compounds include flavonoids, phenolic acids, and tannins. Phenolic compounds are secondary metabolites that are structurally distinguished by containing an aromatic ring with one or more hydroxyl groups and varying in composition from simple monomers to complex polymers (Balasundram, Sundram & Samman, 2006). Due to their structure, phenolic compounds are especially adept at radical scavenging and the antioxidant potential of fruits and vegetables is largely attributed to them. The bioactive potential of phenolic compounds is affected by their bioavailability within plant tissue as well as their structural format. Phenolics exist in both free/soluble (~ 76%) and bound/insoluble (~24%) forms and become insoluble and lack bioavailability when bound to structural components of cell walls including lignan, pectin, cellulose, hemicellulose and proteins (Acosta-Estrada, Gutiérrez-Uribe & Serna-Saldívar, 2014). Structurally, phenolics that occur as polymers or in a glycosidic form have reduced antioxidant activity (Hur, Lee, Kim, Choi & Kim, 2014). Liberation of bound phenolics as well as the breakdown of polymeric and glycosidic compounds is accomplished by thermal and mechanical food processing, malting, and fermentation.

Both the phenolic and the vitamin C content of cucumbers may be responsible for their antioxidant activity, and Murcia, Jiménez & Martínez-Tomé (2009) determined that cucumbers stored for 7 days had a 24.2% reduction in antioxidant activity. In this study only cucumber flesh was examined, whereas cucumbers are typically consumed with the peel, therefore the

antioxidant capacity of the entire consumable portion was not determined. Investigation of antioxidant activity of whole fresh and fermented cucumbers is needed, including the biotransformation of phenolic compounds by LAB as has been observed in olives (Benincasa, Muccilli, Amenta, Perri & Romeo, 2015). Pickling cucumbers used for fermentation are a different varietal than eating cucumbers and are most often consumed with the peel on, therefore the whole cucumber's antioxidant potential should be examined.

Proteolysis has been demonstrated during cucumber fermentations leading to the development or enhancement of bioactive peptides and release of free amino acids. Five bioactive peptides, isoleucine-proline-proline (IPP), leucine-proline-proline (LPP), valine-proline-proline (VPP), lysine-proline (KP) and arginine-tyrosine (RY) were identified in fermented cucumbers (Fideler, Johanningsmeier, Ekelöf & Muddiman, 2019). KP and RY were also found in acidified cucumbers and KP in raw cucumber. These bioactive peptides have antihypertensive properties and IPP, LPP and VPP have been extensively studied in fermented dairy products (Fitzgerald et al., 2006). While these peptides were formed at subclinical levels individually, their combined effect *in vivo* is unknown. In addition to increases in peptides, greater levels of proline, tyrosine and the essential amino acids histidine, isoleucine, leucine, lysine, methionine and phenylalanine were reported in fermented cucumbers compared to fresh cucumbers, suggesting their liberation from proteins and peptides (Moore, DuVivier & Johanningsmeier, 2021).

GABA formation has also been demonstrated during cucumber fermentation. Moore et al., (2021) found that GABA was present in fresh cucumbers at 0.83 ± 0.16 mM (105 ± 21 mg/kg) and generated during cucumber fermentation to reach a total of 1.21 ± 0.35 mM (150 ± 44 mg/kg) GABA. GABA was formed within the first 24-48 hours of fermentation, equilibrated between the brine and cucumbers after 7 days, and remained stable over 9 months of storage. Early formation of GABA suggests that products with brief fermentations such as khalpi could be suitable for GABA production. Furthermore, the drying of cucumber slices during khalpi preparation could effectively increase GABA concentration in the final product. That study demonstrated the potential for the development of enhanced GABA cucumber pickles using similar methods as applied to kimchi including the addition of exogenous glutamate.

6.6 Olives

Olives (*Olea europaea* L.) are a fruit rich in phenolics, carotenoids, tocopherols, monounsaturated fatty acids, and phospholipids (Covas, 2012). The known phenolic compounds in processed table olives include caffeic acid, ferulic acid, hydroxytyrosol, oleuropein, *p*-coumaric acid, rutin, syringic acid, quercetin, tyrosol and vanillic acid (Hashmi, Khan, Hanif, Farooq & Perveen, 2015; Charoenprasert & Mitchell, 2012). These compounds and their derivatives have been associated with the number of health-promoting properties, including antioxidant, anticancer, antimicrobial, anti-inflammatory and antihypertensive effects, among others (Ghanbari, Anwar, Alkharfy, Gilani & Saari, 2012). Olives and olive oil are the main source of fat consumed in Mediterranean style diets, which is associated with reduced risk of cardiovascular disease and certain cancers (Knoops et al., 2004). It is commonly reported that olives possess health promoting antioxidants; however, the impact of LA fermentation on these protective compounds is less understood.

Olives may be divided into three classes based on the type of final product: treated green olives, treated black olives and untreated "California-style" black olives (Fernandez, Garcia & Balbuena, 1995). Treated olives have been washed in lye which performs three functions: it degrades oleuropein to attenuate bitterness (Borbollo y Alcala & Rejano Navarro, 1981), increases permeability of the skin to allow sugars to diffuse during fermentation, and reduces the natural antimicrobial activity of olive phenolics to increase fermentation speed (Fleming, Walter & Etchells, 1973). Fermentation is primarily carried out by *Lb. plantarum* and to a lesser extent *Lb. delbrueckii* (Fernandez et al, 1995). The use of starter cultures is not common in olive fermentation, but various studies have demonstrated their use for optimized debittering, phenolic biotransformation, and fermentation efficiency (Tataridou & Kotzekidou, 2015; Montaño, Sánchez & Castro, 2000). Debittering of olives through oleuropein degradation is accomplished by lye treatment as fermentation by LAB with oleuropeinolytic capabilities. Kaltsa, Papaliaga, Papaioannou & Kotzekidou (2015) isolated *Lb. plantarum* strains from olives and tested their ability to degrade oleuropein, finding that isolates with the greatest oleuropeinolytic capabilities also had the highest β-glucosidase activity. β-glucosidase is an important enzyme in oleuropein hydrolysis as it cleaves the glycosidic bond of oleuropein leading to the formation of two antioxidant compounds, tyrosol and hydroxytyrosol (Aponte et al., 2010; Charoenprasert et al., 2012). A comparison between raw, naturally fermented and starter culture (*Lb. pentosus* 1MO)

fermented olives showed that the starter culture fermented olives contained the highest amounts of hydroxytyrosol (Servili et al., 2008). Similarly, β-glucosidase can hydrolyze sugars from other bound phenols, transforming them into bioactive compounds. Benincasa et al., (2015) determined that the antioxidants, caffeic acid, ferulic acid, and hydroxytyrosol, continuously increased during fermentation of olives with *Lb. plantarum* DSM 20205, while other phenolics including apigenin, oleuropein and verbascoside decreased after 90-120 days. While LA fermentation promotes the conversion of inactive phenolics in olives to active compounds, it also promotes phenolic release into olive brines through tissue modification. In a study by Servili et al. (2008), untreated olives were brined in NaCl and naturally fermented for 5 days. Thereafter, half of the olives were inoculated with *Lb. pentosus* 1MO with the addition of glucose (3 g/L) and yeast extract (0.5 g/L) and fermented for 3 more days. Naturally fermented olives released low amounts of phenolic compounds into brine while *Lb. pentosus* 1MO olive brines contained much greater amounts. Scanning electron microscope images revealed that the external surface and internal tissue of naturally fermented olives were perfectly intact while *Lb. pentosus* 1MO fermented olives' epicarp was completely degraded and internal tissues was significantly damaged. Understanding the structural impact of LAB on olive tissue and the resulting phenolic release is useful for developing olives and olive products with retained phenolics. Additionally, criteria for starter culture selection should include their impact on the phenolic content of the finished product in addition to their value in ensuring a consistent and efficient fermentation.

Post-fermentation processing can greatly impact the phenolic profile and content of olives. California-style olives undergo extensive oxidation and lye treatments in which phenolic compounds are oxidized to blacken the fruit skin and pulp (Fernandez et al., 1995). pH rises during this treatment and the resulting product requires commercial sterilization for shelf stability. Heat processing further degrades olive phenolics, with California-style canned olives containing little to no hydroxytyrosol (Charoenprasert et al., 2012).

Olive oil is considered healthy due to its high content of monounsaturated fatty acids as well as its antioxidant potential. Table olives contain tenfold greater phenolics than olive oil, approximately 10 – 40 mg/kg (Lanza & Ninfali, 2012). This large difference in phenolic content may be due to the fact that olive oil is produced from untreated, unfermented olives which have not undergone chemical or biological transformation of phenolics. Additionally, many olive-derived phenolic compounds are water soluble and remain in olive pomace after pressing.

Consumption of LA fermented olives may provide greater protection against oxidative stress than olive oil.

An active area of research in is the development of value-added LA fermented products and the reclamation of processing waste by LA fermentation. Numerous studies highlight the utilization of waste-water from olive processing due to its high content of bioactive compounds. Brines from olive fermentation tanks are commonly discarded and known as table olive wastewater (TOW) (Belaqziz et al., 2017) and many of the phenolic compounds in olives are water soluble due to their multiple sites of hydroxylation and sugar moieties (Charoenprasert et al., 2012). This waste stream is of environmental concern due to its high content of polyphenols and resulting phytotoxicity, therefore repurposing of TOW for use in value-added foods is of both economic and environmental benefit. The most prominent phenolic compounds in TOW are hydroxytyrosol and tyrosol (Belaqziz et al., 2017) however wastewaters are not microbially stable at their natural pH. Brenes, Romero & de Castro (2004) found that TOW had an initial alkaline pH of 11.8 due to lye treatment and that it may undergo spoilage by putrefactive bacteria. Adjustment to pH 3.4 inhibited putrefactive bacteria but allowed yeast growth, ethanol production, and resulted in a significant decrease in hydroxytyrosol concentration. TOW adjusted to pH 5 and inoculated with the autochthonous starter cultures (*Lactobacillus pentosus* and *Enterococcus casseliflavus*) prevented growth of putrefactive bacteria and yeast and preserved the hydroxytyrosol content of the wastewater. This product can be evaporated to concentrate bioactives for use in other foods or for further processing for use in food supplements, cosmetics and pharmaceuticals (Brenes et al., 2004).

Olive waste water is also known to have strong antimicrobial activity and partially inhibits the growth of certain enterics and LAB (Caporaso, Formisano & Genovese, 2018). Antimicrobial compounds in olives include hydroxytyrosol, oleuropein, oleoside 11-methyl ester, and dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA), (Medina, Brenes, Romero, García & de Castro, 2007). Hydroxytyrosol has been shown to be effective against non-LAB including *E. coli, Enterococcus faecalis, E. aerogenes, Streptococcus pyogenes, Staphylococcus aureus,* and *Klebsiella pneumoniae* (Tafesh, Najami, Jadoun, Halahlih, Riepl, & Azaizeh, 2011; Medina et al., 2007), HyEDA was extremely effective in inhibiting *Lb. pentosus* (Medina et al., 2007). Furthermore, the concentrations of these antimicrobial compounds in TOW varied depending on the type of olive fermented. For

example, Manzanilla olives had extremely high hydroxytyrosol and HyEDA concentrations (4.25 mM and 0.5 mM, respectively) compared to Gordal olives (1.5 mM and 0 mM, respectively) (Medina et al., 2007). TOW in its native or concentrated form could be utilized as a natural antimicrobial preservative in other foods including LA fermented fruits and vegetables while also providing antioxidative benefits to consumers.

6.7 Cocoa

Cocoa beans are the fermented fruit of the *Theobroma cacao* tree. Spontaneous fermentation is typically employed and involves a succession of yeast (*Saccharomyces* spp., *Pichia* spp., *Kluyveromyces* spp.), LAB (*Lactobacillus fermentum, Leuconostoc* spp.) and acetic acid bacteria (*Acetobacter* spp., *Gluconobacter* spp.) (Figueroa-Hernández et al., 2019). After fermentation, cocoa beans are dried, roasted, and winnowed to remove their shells prior to grinding into cocoa mass for cocoa powder or chocolate production. Cocoa beans are rarely consumed unfermented or raw (unroasted); therefore, the majority of research into health promoting properties of cocoa involves the impact of both of these processes. Proteolytic enzymes produced by bacteria release bioactive peptides during cocoa fermentation. Cocoa bean protein hydrolysates producing antioxidant, antihypertensive, antidiabetic, anti-Alzheimer, anti-obesogenic and anti-tumor activities (Domínguez-Pérez, Beltrán-Barrientos, González-Córdova, Hernández-Mendoza & Vallejo-Cordoba, 2020). Additional antidiabetic properties of cocoa may be related to its α-glucosidase inhibitory activity. Racine et al. (2019) found that cool fermentation and cool roasting of cocoa beans produced the greatest α-glucosidase inhibitory activity compared with raw cocoa beans or hot fermented/hot roasted beans. GABA is also formed during cocoa fermentation and has been found at levels ranging from 317 to 1,020 mg/kg depending on the region in which it is grown (Marseglia, Palla & Caligiani, 2014).Cocoa is known for its polyphenol content, specifically flavanols and epicatechin, and touted as a potent antioxidative food beneficial especially for cardiovascular health (Vlachojannis, Erne, Zimmermann & Chrubasik-Hausmann, 2016). However, both fermentation and roasting result in decreased total polyphenol content and antioxidant capacity of cocoa beans (Albertini et al., 2015; Jolić, Redovniković, Marković, Šipušić & Delonga, 2011). Furthermore, the roasting process results in melanoidin formation due to Maillard browning, a class of compounds that have reported antioxidant, antimicrobial and antihypertensive activity (Wang, Qian & Yao;

2016). Despite its decreased polyphenol content post-fermentation and roasting, cocoa is a valuable LA fermented food with health promoting properties. The fermentation and roasting processes are essential for preserving cocoa and transforming it into a sensorily appealing product for consumers.

6.8 Capers

Capers are a promising area of growing research in health promoting properties of fermented fruits. The *Capparis spinosa* plant is cultivated in the Mediterranean basin with both its flower buds (capers) and fruits (caper berries) harvested for processing and consumption (Pérez Pulido, Ben Omar, Abriouel, López, Cañamero & Gálvez, 2005). Capers undergo natural LA fermentation in either plain tap water, salt brine, or a mixture of salt brine and vinegar (Pérez Pulido et al., 2005; Jiménez-López, Ruiz-Medina, Ortega-Barrales, & Llorent-Martínez, 2018). Health beneficial components studied in fresh and fermented capers include phenolic compounds and glucosinolates. Jiménez-López et al., (2018) quantified phenolics in fresh and fermented capers and found that while there was a decrease in certain phenolics (i.e., epicatechin, *p*-coumaric acid, and ferulic acid) and glucosinolates, the total phenolic content and resulting antioxidant activity of fermented capers was higher than that of fresh capers. Capers are one of the richest food sources of quercetin, a free-radical scavenging flavonoid, however most of it is present in the glycoside form in fresh capers which has low bioavailability (Aksay, Sellib & Kelebek, 2021). Multiple studies confirm that LA fermentation of capers hydrolyzes quercetin glycosides to their active aglycone form while also decreasing bitterness, thereby enhancing both healthfulness and palatability (Francesca et al., 2016; Aksay et al., 2021). Fermentation method also impacts the phenolic profile of capers. Aksay et al. (2021) found that dry-salting capers resulted in higher antioxidant potential than fermenting them in liquid brine, potentially due to the dilution of water-soluble antioxidant compounds in liquid brine. In addition to antioxidant activity, fermented capers have been known to lower blood glucose and improve the blood lipid profile of diabetic patients (Eddouks, Lemhadri & Michel, 2005; Eddouks et al., 2017). In a randomized clinical trial, Khavasi et al. (2017) found that daily consumption of 40-50 g of capers with meals over 12 weeks significantly decreased the severity of non-alcoholic fatty liver disease. Determination of the component(s) responsible for this decrease in liver disease may aid

researchers in optimizing its concentration during fermentation and processing to achieve a more manageable, consumable dose for consumers.

6.9 Carrots

Carrots (*Daucus carota* L.) are naturally rich in antioxidant carotenoids and provide a source of the provitamin A β-carotene. While commonly consumed raw or cooked, carrots may also be included in mixed vegetable fermentations (i.e., kimchi, turşu) or may be cut into sticks or rounds and naturally fermented in brine (Montaño, Sánchez, Rejano & de Castro, 1997; Gardner, Savard, Obermeier, Caldwell & Champagne, 2001). Carrot fermentation is primarily practiced on a small scale in homes, and research on LA fermentation of carrots has primarily focused on fermentation of carrot juice or purees.

Bioavailability of β-carotene in plant foods including carrots is affected by physical accessibility to the β-carotene within plant cells. Particle size reduction in order to liberate β-carotene can be achieved mechanically and Edwards, Nguyen, You, Swanson, Emenhiser & Parker (2002) found that β-carotene absorption in human subjects was twofold greater from pureed carrots than from boiled, mashed carrots. Vegetal cell walls can also be broken down enzymatically to release nutritive and bioactive components. During juice manufacturing, fruits and vegetables are ground into a mash and pressed to expel juice. Juice yield is often increased by adding pectolytic enzymes to the mash to further degrade cell wall components. Select strains of LAB produce pectolytic enzymes including pectinesterase and polygalacturonase (Sakellaris, Nikolaropoulos & Evangelopoulos, 1988; Karam et al., 1995). Demir, Bacheci & Acar (2006) included RSKK 1602 *Lb. plantarum* in carrot mash for this purpose, noting that a higher inoculum of 3×10^7 CFU/g resulted in higher juice yield (86.55%) than lower inoculums (3×10^6 CFU/g, 77.77% yield; 3×10^5 CFU/g, 74.88% yield). Not only did LAB increase juice yield, but fermentation by *Lb. plantarum* increased bioavailability of α- and β-carotene in carrot juice by 30% after 16 hours of incubation (Díaz, Hedrén, Ruales & Svanberg, 2004). These studies have shown that in addition to health benefits provided by LAB in carrot fermentation, fermentation serves a technological function by increasing juicing yield.

Various studies support the antidiabetic effects of LA fermented carrot mash and its resulting juice. Carrot juice fermented with the probiotic *Lb. rhamnosus* GG had increased free phenol and short chain fatty acid (SCFA) content and the consumption of this juice produced

antidiabetic effects in rats including significantly lower insulin resistance and improved chronic hyperglycemia and renal function (Hu et al., 2019). Li, Ding, Nie, Zhang, Xiong & Xie (2014) also found that carrot juice fermented with *Lb. plantarum* NCU116 had increased SCFA and regulated blood glucose, lipid metabolism and digestive hormones in an animal model. The presence of SCFA including acetate, propionate and butyrate in the gut influence host glucose metabolism by inducing hormone secretion to increase glucose sensitivity, inhibit gastric emptying, and stimulate insulin release (Gupta, Jelinek, Al-Aubaidy & Hayder, 2016). Furthermore, diabetic patients are known to have a significant imbalance in gut microbiota (He, Shan & Song, 2015) and studies have shown that LA fermented foods have the potential to combat diabetes by positively impacting microbiome composition. Increases in the abundance of functionally beneficial enterotypes including *Akkermansia*, *Christensenellaceae*_R-7_group, *Lachnospiraceae*_NK4A136_ group, *Oscillibacter*, and *Ruminococcaceae*_UCG-013 were observed in rats fed *Lb. rhamnosus* GG carrot juice (Hu et al., 2019). In addition to improvement in gut microbiota, oxidative stress in the gut decreased in these rats and was attributed to the activity of *Lb. rhamnosus* GG produced phenol acid esterases on bound phenols. Investigation of the hypoglycemic effects of a carrot pulp fermented by *Lb. plantarum* revealed that the water-soluble polysaccharides resulting from the LAB induced pectinolytic activity had better hypoglycemic effects than polysaccharides in non-fermented carrot pulp (Wan et al., 2019). In addition to traditional orange fleshed carrots, carrots of varying colors have also been fermented to improve their health beneficial properties. Black carrots fermented by *Lb. plantarum* prevented hippocampal insulin resistance in a diabetic animal model, resulting in improved cognitive function (Park, Kang, Jeong, Jeong & Yun 2016). Consumers are familiar with carrot juice and juice blends containing carrot juice, therefore the development of LA fermented carrot juices with antidiabetic properties may receive positive consumer acceptance.

While the majority of studies on fermented carrots involve carrot juice rather than whole or diced carrots, the latter form is still fermented in brine. Carrot contains less water (86-89%; Sharma, Karki, Thakur & Attri, 2012) than cabbage (93%; Canet, 1988) or cucumber (95% (USDA, 2016)) which delays equilibration of solutes into brine. Size reduction may not only be useful to create products that are easily consumable (i.e., carrots sticks or chips) but also to accelerate fermentation by decreasing solute equilibration time. However, LA fermented carrots lack storage stability due to their sucrose content. Carrots contain glucose, fructose and sucrose,

and while glucose and fructose are metabolized by LAB, sucrose is only metabolized by certain strains of LAB. For example. *L. mesenteroides* is able to produce exopolysaccharides from sucrose (Dols, Chraibi, Remaud-Simeon, Lindley & Monsan, 1997). LAB fermented carrots therefore contain high levels of residual sugars available for spoilage microbiota (Castro, Rejano, Sánchez & Montaño, 1995; Fleming, McFeeters, Thompson & Sanders, 1983). Montaño et al. (1997) used of a mixed culture of *Lb. plantarum* and *Saccharomyces cerevisiae* to reduce residual sugar concentration in carrot fermentations, but observed only 54% sucrose utilization. The authors noted that pasteurized, fermented carrots were stable over a 9-month storage period while fermented carrots preserved with sorbic and benzoic acid allowed for microbial growth. Therefore, careful consideration should be taken in developing LA fermented carrot products to ensure long term stability.

6.10 Other Fermented Fruits & Vegetables

A small number of studies have observed changes in health promoting compounds in various less well-studied fermented fruits and vegetables ranging from tomatoes and berries to tropical fruits and cactus. The majority of these studies emphasize changes in plant secondary metabolites and increases in antioxidant potential achieved through fermentation. In one study, total phenolic compounds decreased to near zero levels over a four-week tomato fermentation while lycopene concentration and antioxidant activity significantly increased (Bah et al., 2019). Conversely, fermentation of tomato juice demonstrated slight increases in total phenolic compounds and β-carotene over 48 hours with a slight loss of lycopene (Liu, Chen, Chen, Zhong, Zhang & Chen, 2018). Kim, Choi, Park & Kim (2019) naturally fermented a diverse mixture of 18 sliced fruits and vegetables including aloe, cucumber, tomato, apple, water parsley, carrot, onion, tangerine, grape, jujube, chives, celery, onion, burdock, kale, cabbage, spinach, and perilla leaves finding that the fermented mixture had significantly greater anti-inflammatory and anti-oxidative activities than the raw mixture. On the other hand, LA fermentation of fruits and vegetables may also decrease their antioxidant activity. Chinese quince (xuan mu gua, *Chaenomeles speciosa*) was fermented to ameliorate its harsh astringency by removing tannins, but the antioxidant properties were significantly decreased compared to non-fermented fruit (Shang et al., 2019).

A few studies have been conducted on broccoli fermentation, one of which explored the effects of thermal processing and LA fermentation on glucosinolate and total phenolic content of broccoli (Ye, Huang, Terefe & Augustin, 2019). The authors performed thermal processing (autoclave) alone and thermal processing combined with LA fermentation by autochthonous LAB finding that glucosinolate and phenolic content of broccoli purees significantly increased after fermentation, however the alterations in chemical composition were strain specific. A principal component analysis grouped the broccoli fermented by *L. mesenteroides* strains with the control, non-fermented broccoli while differentiating it from broccoli fermented by *Lb. plantarum* strains. It is hypothesized that the metabolic activity of LAB aided in the release of these two classes of compounds from the broccoli matrix. This study emphasizes the impact of starter cultures on the accessibility of bioactive compounds in vegetables and the opportunity that exists for development of products with enhanced bioactives.

Production of GABA in a variety of fermented fruits and vegetables is also attractive, given GABA's notoriety as a bioactive compound. Fermentation of prickly pear cactus (*Opuntia ficus-indica* L.) with *Lb. brevis* resulted in GABA production (38.9 - 45.7 mg/kg), preservation of vitamin C and carotenoids, and the development of two radical scavenging flavonoid derivatives (isorhamnetin and kaemferol) compared with an acidified control (Filannino et al., 2016). A consecutive fermentation of water dropwort (*Oenanthe javanica* DC) with *L. mesenteroides* SM, and *Lb. plantarum* K154 with 3% added MSG resulted in the production of dextran and GABA, respectively (Kwon, Garcia, Song & Lee, 2016). Hasegawa, Yamane, Funato, Yoshida & Sambongi (2018) fermented leftover date (*Phoenix dactylifera*) processing pomace with *Lb. brevis* and 0.5% added MSG, resulting in the generation of a novel date residue with enhanced GABA. Date paste is already utilized heavily in nutrition bar manufacturing and confectionary production; therefore, incorporation of this GABA-enhanced product as an ingredient may be feasible.

6.11 Mixed Fermentations

Combining botanically diverse plants and/or foods of animal origin with fruits and vegetables is a promising area of innovation for LA fermentation. These mixed fermentations have demonstrated synergistic effects on the generation and retention of bioactive compounds.

Kimchi is the most popularly consumed mixed fermented vegetable; however, others from a diverse array of cultures exist with equally enticing bioactive potential.

Hardaliye is a LA fermented beverage consumed in Turkey made from grapes (*Vitis vinifera*) and sour cherry leaves (*Prunus cerasus*) with the addition of either crushed mustard seeds (*Sinapis alba*) or benzoic acid to prevent yeast growth (Amoutzopoulos et al., 2013). Hardaliye's proposed healthfulness is derived from its phenolic content and resulting antioxidant activity. In a human trial, subjects consuming 500 mL or 250 mL hardaliye daily for 40 days experienced a significant reduction in biomarkers of oxidative stress, including dien conjugate, malondialdehyde, and serum homocysteine levels (Amoutzopoulos et al., 2013). In addition to the phenolics provided by grapes, glucosinolates from mustard seeds may also contribute to hardaliye's bioactive potential.

Similar to hardaliye's combination of leaves and fruit, legumes and fruit have also been merged to create novel LA fermented products. A combined fermentation of powdered camu-camu (*Myriciaria dubia* Mc. Vaugh) and soymilk with either *Lb. plantarum* or *Lb. helveticus* resulted in a 3-fold greater *in vitro* antioxidant activity than fermented soymilk alone (Fujita, Sarkar, Genovese & Shetty, 2017). Soymilk and camu-camu co-fermentations also had higher α-amylase and α-glucosidase inhibition than fermented soymilk alone, boosting their potential antidiabetic properties. When fermented with *Lb. plantarum*, the camu-camu soymilk mixture also exhibited improved angiotensin converting enzyme (ACE) inhibitory activity. Consumers familiar with soy milk or fermented soy products (i.e., soy-based yogurt) may find a co-fermented soy product more approachable than one with a completely novel flavor or texture profile. Sensory analysis is essential in the development of novel fermented products in order to determine if consumer markets are open to these innovations.

Shalgam (*şalgam*), a LA fermented beverage native to Turkey, is primarily composed of black carrots, bulgur flour, turnip, salt, water and sourdough starter (Sanlier et al., 2019). Manufacturing of shalgam involves a primary fermentation (3-5 days) of a bread dough made with sourdough, salt, bulgur flour and water (Altay, Karbancıoglu-Güler, Daskaya-Dikmen & Heperkan, 2013). Next, chopped black carrots, turnips and water are added to begin a secondary fermentation typically lasting up to 10 days. The mixture is then filtered to produce a juice called shalgam. Shalgam is of interest due to the anthocyanins contained in black carrot, primarily cyanidin-3-glucoside and cyanidin-3-galactoside (Ekinci et al., 2016). When compared to pure

black carrot juice, shalgam has a lower total phenol content and lower antioxidant capacity. Toktaş Bildika & Özçelikaet (2017) found that shalgam had lower phenolic compounds (81.5 ± 3 mg/kg gallic acid equivalents (GAE) dry basis) than black carrot (112.7 ± 11 mg/kg GAE dry basis). Nonetheless, total flavonoids, phenolic compounds and anthocyanins were greater at the end of fermentation (day 24) than at day 12 or day 1, showing that fermentation length is related to phenolic extraction. It should be noted that shalgam typically contains only 10-20% black carrot juice, therefore antioxidant capacity is expected to fall below 20% of that of black carrot juice, however much greater antioxidant levels have been reported. *In vitro* digestion of black carrot and shalgam revealed no significant difference in total antioxidant capacity or total flavonoid, phenolic or anthocyanin content indicating that fermentation enhanced bioaccessibility of these compounds in black carrot (Toktas et al., 2017). Due to the inclusion of black carrot as chopped pieces rather than juice, LAB may be important in degrading plant cell wall polysaccharides to release phenolic compounds and hydrolyze glycosidic linkages to enhance their bioactivities. A similar phenomenon was observed with increases in the bioavailability of β- and α-carotene in fermented, orange-fleshed carrots (Díaz et al., 2004).

The main species of LAB responsible for shalgam fermentation include *Lb. plantarum, Lb. casei, Lb. paracasei,* and *Lb. brevis,* with *Lb. sharpeae, Lb. delbrueckii, Lb. acidophilus, Lb. helveticus and Lb. reuteri* which are also present in commercial shalgam juices (Ekinci et al., 2016). This microbial diversity may be attributed to the variety of raw materials ranging from roots to cruciferous vegetables to grains. Additionally, yeast from sourdough will have an impact on the nature of the product and the proliferation of bacteria in the product. Sparse research exists for the use of starter cultures for shalgam production, however one study found that shalgam produced with the traditional spontaneous fermentation used in commercial production resulted in greater total anthocyanins (299.2 ± .002 mg/mL) than shalgams produced with the addition of autochthonous starter cultures isolated from previous batches (227.48 +/- 0.01 - 295.95 ± 0.04 mg/mL) (Tanguler, Bozdogan, Aksay, Kelebek & Erten, 2020). These data demonstrate that while starter cultures may be attractive to ensure complete fermentations, they may also negatively affect the bioactive compounds present in fermentations. Conversely, Avila, Hidalgo, Sánchez-Moreno, Pelaez, Requena & Pascual-Teresa (2009) studied bacterial anthocyanin bioconversion in malvidin and delphinidin glycosides, concluding that *Lb. plantarum, Lactococcus lactis,* and *Bifidobacterium lactis* were effective in cleaving sugars from

malvidin glycoside and further converting it to new metabolites with greater bioactivity including syringic and gallic acids. Selection of starter cultures for increasing the bioavailability of anthocyanins requires the pairing with fruit and vegetable products containing specific anthocyanins upon which these organisms can act.

Interest in food waste reduction through fermentation has prompted research into LA fermentation of produce nearing the end of its shelf life and by-products of fruit and vegetable processing. Flavonoids are abundant in citrus peels; however, many citrus varietals are juiced and the resulting peels and flesh discarded. Conversion of citrus pomace into a functional food via fermentation has been explored. Pomace from *Citrus depressa* Hayata (shiikuwasha), a fruit native to Japan and Taiwan, was fermented for five days with probiotic *Lb. plantarum* E58 resulting in a product with 108 cfu/ml cell density, reduced bitterness and full retention of the flavonoid nobiletin (Kimoto-Nira, Moriya, Nogata, Sekiyama & Toguchi, 2019). In addition to citrus, apple processing produces a significant amount of fiber and phenolic-rich waste from coring and peeling. Fermentation of apple by-product by *Weissella cibaria* PEP23F and *Saccharomyces cerevisiae* AN6Y19 significantly increased its hydration properties and, when incorporated into wheat bread, resulted in a product with enhanced phenolics and fiber with no discernible effect on textural properties or color (Cantatore et al., 2019). Fermentation of apple by-product acted as a pre-treatment by hydrolyzing fiber, including pectin, which increased its hydrophilicity and water retention capacity thus improving the viscosity and textural quality of the dough system. Additionally, the resulting bread also resisted mold contamination and firming to a greater extent than bread alone or bread with unfermented apple by-product. Generation of LA fermented, value-added foods from the byproducts of fruit and vegetable processing has potential to benefit both consumers and promote sustainable manufacturing.

6.12 Juices

Differences in the starting material, either whole fruit, pieces, puree or juice, will significantly impact a final products' composition. Juicing excludes the pulp that is rich in pigments, phenolic compounds, vitamins, and lycopene; therefore, whole fruit fermentations are optimal for delivering the highest concentration of bioactive compounds. However, juices are an ideal format for combining raw products for optimized fermentations and utilizing produce near the end of its shelf life or with an undesirable, soft texture. Fruit and vegetable juices are

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naturally lactose-free, offering an alternative to dairy-based beverages for those with lactose intolerance and dairy allergies. The unique physical form of juices allows for efficient blending of bioactive compound precursors from multiple foods with the necessary natural microbiota to transform the precursors into the desired bioactives.

LA fermented fruit and vegetable juices and their associated functional properties were well summarized in multiple recent reviews with health benefits cited for most juices being bioconversion of phenolics to bioactive compounds and increases in antioxidant potential (Szutowska, 2020; Garcia, Guerin, Souidi & Remize, 2020). Most often, LA fermented fruits and vegetables are produced via natural fermentation, however in a systematic review by Szutowska (2020), a total of 42 studies were examined with only one study involving spontaneous fermentation (Kazimierczak et al., 2014). As was seen in shalgam fermentation, traditional natural fermentations and modern inoculated methods result in products with varying levels of bioactive compounds and are not always augmented by the use of a starter culture (Tanguler et al., 2020). One of the benefits of natural LA fermentation is its low cost, whereas starter culture addition poses an added cost for producers. In order for starter culture use to be helpful, the enhancement of bioactive compounds in the final product or efficiency of production must outweigh the cost to the producer.

The natural acidity of fruit juices necessitates verification that autochthonous LAB or starter cultures survive, proliferate, and complete the fermentation to create a stable product safe from spoilage and pathogenic organisms. Maldonado et al., (2017) investigated fermentation of fruit juice from carambola (*Averrhoa carambola* L.), guava (*Psidium guajava*), mango (*Magifera indica* L.) and pitaya (*Hylocereus undatus*) with microbial cultures typically employed in dairy fermentations: *Lb. casei, Lb. bulgaricus*, and *Streptococcus thermophilus*. Carambola and pitaya juices reduced LAB survival, likely due to natural antimicrobial compounds within the fruit. The final pH of all juices was stable between 3.0 and 4.0, ensuring safety from most foodborne pathogens, however they may remain susceptible to acid resistant pathogens such as *Escherichia coli*.

Fresh juices are microbiologically unstable and subject to spoilage unless processed for shelf stability. In addition to spoilage organisms, pathogenic and spore-forming bacteria have been a food safety concern and are the basis for the requirement of commercially produced juices in the United States to receive treatment accomplishing a 5-log reduction of *E. coli* O157:H7

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(FDA, 2004). In addition to food safety concerns, fresh juices contain spoilage organisms. LA fermentation of fruit and vegetable juices preserves them through acid accumulation and competition with pathogenic and spoilage microbiota. Thermal preservation of acidified foods is more easily achieved (i.e., pasteurization) than non-acidified foods (i.e., commercial sterilization) with less severe impacts on nutritional and organoleptic properties. Development of fermented, pasteurized juices from fruits and vegetables that are commonly consumed after commercial canning (i.e., beets, carrots, corn, lima beans and peas) may better preserve their nutritional components and deliver health promoting compounds to consumers. Creating novel fermented juice products will require consumer acceptance testing and market analysis. Maldonado et al. (2017) found that consumer acceptance of fruits commonly consumed in Brazil (guava and mango) was greater than less commonly consumed fruits (pitaya and carambola). Therefore, LA fermented juices may receive greater sensory acceptance if made from commonly consumed fruits and vegetables rather than novel or foreign produce.

6.13 Conjugated linoleic acid

Conjugated linoleic acids (CLAs) are isomers of linoleic acid that have been shown to combat cancer, prevent atherosclerosis, improve lean mass and reduce body fat, and modulate inflammatory responses (Kim, Kim, Kim & Park, 2016). Humans are unable to produce CLA and must obtain it from dietary sources, the most prominent being milk and meat from ruminants such as cows, sheep and goats. The microbiota in the rumen of these animals hydrogenate linoleic (C18:2) and linolenic (C18:3) fatty acids to form CLA, most notably the *cis*-9,*trans*-11 isomer (Dhiman, Nam & Ure, 2007). CLA may be generated through chemical synthesis or microbial fermentation including biologically engineered and natural organisms (Gong, Hu, Wei, Jin & Want, 2019). While CLA has achieved GRAS (Generally Recognized as Safe) status as a food additive in the United States, natural generation of CLA within a food matrix would be optimal to meet consumer demands of foods with "natural" appeal and minimal added ingredients. *In situ* production of CLA in fermented foods by non-genetically modified cultures would provide both health benefits and be attractive from a consumer marketing standpoint.

Interest in the formation of CLA in foods of non-animal origin has prompted researchers to investigate CLA production by LAB. Autochthonous bacteria may be suitable for CLA generation during fruit and vegetable fermentation or starter cultures with high CLA producing

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capabilities may be utilized. Liu, Shen, Ruan, Zhou, Ma & He (2011) isolated 43 strains with CLA producing capabilities from naturally fermented Chinese pickle brines. Of these strains, *Lb. plantarum* lp15 converted linoleic acid to CLA with 25% efficiency in microbiological media. In addition to pure cultures, co-culture fermentations may be used to optimize CLA production. Renes, Linares, González, Fresno, Tornadijo & Stanton (2017) found that co-culture fermentations of autochthonous *Lactococcus lactis* strains and *Lb. plantarum* TAUL 1588 demonstrated higher CLA production than either of the strains individually. The former strain was a non-CLA producing strain used primarily for acidification while the latter had proven CLA-producing capabilities.

Due to the naturally low lipid content of fruits and vegetables, a primary consideration of CLA generation during LA fermentation is the inclusion of a substrate suitable for biohydrogenation by LAB. Pure linoleic acid or vegetable oils containing specific unsaturated fatty acids may be used. Kishino, Ogawa, Ando, Omura & Shimizu (2002) reported that ricinoleic acid from castor oil was an effective substrate for CLA formation by *Lb. plantarum* AKU 1009a; however, castor oil itself did not function well as a substrate unless first treated with lipase. The amount of fat added to fruit and vegetable fermentations must also be considered so as not to gratuitously increase their caloric value. Given that most fruit and vegetable fermentations have a high concentration of water, added oil should be pretreated by a detergent in order to disperse it and increase accessibility by LAB (Ogawa, Kishino, Ando, Sugimoto, Mihara & Shimizu, 2005). Currently, flavorings including dill oil are dispersed in some cucumber pickle brines using emulsifiers such as polysorbate 80, therefore inclusion of oils for CLA production is an approachable process. Giardiniera, a non-fermented, pickled relish of Italian origin made from cauliflower, bell pepper, celery, carrots and gherkins, is packed in a brine composed of olive oil, red or white wine vinegar, spices and herbs. The oil in this product may provide substrates for CLA production if giardiniera were inoculated with LAB to perform the acidification rather than adding vinegar. Natural sources of linoleic acid from plants include microalgae (Guedes, Amaro, Barbosa, Pereira & Malcata, 2011) and lupin (García-López et al., 2001); both of which have been investigated as substrates for LAB fermentation to generate functional foods (Khan, Shin & Kim, 2018; Bartkiene et al., 2013). Design of mixed fermentations of commonly fermented fruits and vegetables with added sources of linoleic acid-containing plants or plant extracts is a promising alternative to adding pure linoleic acid or

vegetable oils. Fruit and vegetable fermentations with enhanced bioactive fatty acids can be made possible with microbial and technological advancements.

6.14 Non-Probiotic Benefits of Lactic Acid Bacteria

Fermented foods are often touted for their probiotic effect despite lacking evidence that strains responsible for fermentation meet the criteria for consideration as "probiotic". That is, probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill, 2014). Few fermented fruits or vegetables are prepared with verified probiotic strains, and their low pH and thermal processing for shelf stability compromises live organisms. However, LAB both living and non-living have the potential to positively impact consumer health. It has been shown that administration of heat-killed bacteria or their purified fractions confer health benefits including immunomodulatory effects, reduction of gastrointestinal diseases and diarrhea, and protection against enteropathogens (Piqué, Berlanga & Miñana-Galbis, 2019). Upon death, bacterial cells release metabolites and components such as EPS, lipoteichoic acids and peptidoglycans with anti-inflammatory or antipathogen properties. Garcia-Castillo et al. (2020) isolated EPS produced by *Lb. fermentum* UCO-979C and found that it significantly reduced *Helicobacter pylori* colonization in mice by modulating the innate gastric immune response. Peptidoglycan derived from *Lb. acidophilus* has been found to reduce nitric oxide synthase and cyclooxygenase-2 levels in human intestinal epithelial cells, effectively reducing inflammation (Wu, Pan, Guo & Zheng, 2013). Similarly, lipoteichoic acid derived from *Lb. plantarum* suppressed interleukin-8 production and conferred an anti-inflammatory response in porcine intestinal epithelial cells (Kim et al., 2017). Many studies on the impact of non-viable LAB cells on gastrointestinal health involve clinically proven probiotic strains, leaving much opportunity to study the effects of non-probiotic, autochthonous bacteria associated with natural food fermentations.

Limited studies comparing fermented foods containing live or dead organisms have been conducted, however they are integral to better understand fermented foods' impact on health. A novel study examining this difference in sauerkrauts was conducted by Nielsen et al. (2018). The authors administered naturally fermented sauerkraut that was either pasteurized or unpasteurized to patients with irritable bowel syndrome (IBS) for 6 weeks (75 g/day) and found that both groups' IBS-symptom severity scores significantly improved compared to baseline ratings and

that the improvements were not significantly different based on the type of sauerkraut consumed. Furthermore, significant compositional changes in gut microbiota were observed whether sauerkraut contained live organisms (unpasteurized) or not (pasteurized). This study demonstrates that gut health promoting properties of fermented sauerkraut may not be entirely attributed to live microorganisms but also to dead organisms, prebiotics, or other bioactive compounds present in sauerkraut. Applying this type of research to other commonly consumed fermented foods such as kimchi and pickles would provide significant insight into long-held anecdotal claims about the healthfulness of fermented foods.

6.15 Stability and Safety of Novel Fermented Fruit & Vegetable Products

Two important considerations in the commercialization of novel fermented fruits and vegetables are stability and safety. LA fermented products should be able to effectively inhibit the growth of pathogenic microorganisms or be processed in a manner to sufficiently reduce this risk. The products must also be stable and not subject to secondary fermentation by spoilage organisms that can change both the organoleptic and health promoting properties of the product. Residual sugars and organic acids in LA fermented products can allow for continued fermentation in which undesirable organisms proliferate. Secondary fermentation of fermented fruits and vegetables may result in the production of spoilage metabolites including butyric and propionic acid, compromising product quality. Additionally, consumption of lactic acid by spoilage organisms (i.e., acetic acid bacteria) can result in increased pH which impairs the safety of the product against pathogenic organisms (as reviewed by Medina et al., 2016). Furthermore, bioactive compounds produced or enhanced during fermentation should remain intact through processing and storage prior to consumption.

Bah et al., (2019) found that during spontaneous fermentation of tomatoes, LAB exhibited antibacterial activity against foodborne pathogens. Similarly, inhibition of *Escherichia coli* substantially increased in tomato juice fermented with *Lb. plantarum* and *Lb. casei* compared to fresh tomato juice (Liu et al., 2018). Conversely, apricot (*Prunus armeniaca*) juice fermented in monoculture or co-culture by probiotic strains of *Lactobacillus* and *Bifidobacterium* had increased antioxidant capacity yet none of the fermentations reached a pH below 4.6, which poses a food safety risk even if stored refrigerated (Bujna, Farkas, Tran, Dam & Nguyen, 2018). Additionally, significant levels of glucose (2.74 - 3.15%) and fructose (4.84 - 5.55%) remained

in the final fermented juice indicating an incomplete fermentation and increased risk of secondary fermentation by spoilage microbiota.

Fermentation of fruits and vegetables naturally colonized by yeast or that incorporate yeast in the starter cultures should also be closely monitored for ethanol production. Alcohol, produced primarily by yeast but also by heterofermentative LAB, poses an issue for commercial processors. In the United States, food products are regulated by the Food and Drug Administration (FDA) and must contain less than 0.5% ethanol. If greater than 0.5% ethanol is present, consumers must be over the age of 21 to purchase the product and regulation falls under the Alcohol and Tobacco Tax and Trade Bureau. This complication has been seen in commercially produced kombucha, a beverage made by fermenting sweetened brewed tea with a symbiotic culture of bacteria and yeast. LAB, acetic acid bacteria, and yeast perform various metabolic activities resulting in a product that is both acidified and carbonated, and often contains a small percentage of ethanol. Yeast growth can be prevented by the addition of potassium sorbate as is common in cucumber and olive fermentations (Pérez-Díaz et al., 2013), but added preservatives may be incongruent with the current consumer trends for natural, health-promoting fermented fruits and vegetables. Assessment of regulatory compliance of novel fermented fruit and vegetable products should be initiated during the conceptualization stage and reassessed regularly throughout development and commercialization.

It is also necessary to consider how further processing such as prolonged refrigeration or pasteurization for shelf stability effects bioactive compounds in novel fermented products. Turker, Aksay & Ekiz (2004) stored fermented shalgam made from black carrots at 4°C, 25°C and 40°C for 90 days, finding that the lowest temperature storage preserved monomeric anthocyanin content the most. Furthermore, pasteurization did not significantly affect total anthocyanin content or the anthocyanin profile of shalgam. Moore et al., (2021) found that in addition to GABA's stability over a 9-month storage period in fermented cucumbers, it also remained stable during pasteurization (15 min at 75°C). Palani et al., (2016) measured ascorbigen and indole-3-carbinol content in sauerkraut during fermentation and throughout storage and found that maximum levels of these compounds were formed between days 5 and 9 of fermentation. Ascorbigen was relatively stable through 8 weeks of storage but indole-3-carbinol slowly degraded immediately following fermentation (7-9 days onward). The number of studies that investigate the stability of bioactive compounds on a practical level, incorporating

both post-fermentation processing and long-term storage is lacking. This area of research is necessary in order to advance technology in LA fermented fruits and vegetables and to deliver products with optimal levels of bioactives.

6.16 Areas of Future Research & Potential for LAB Fermentations

Development of novel fermented foods to optimize delivery of bioactive compounds to consumers to combat cancer, cardiovascular disease, diabetes, Alzheimer's and other diseases is an important area of growth for the food industry. LA fermentation also has the potential to play a role in decreasing food waste by preserving perishable fruits and vegetables. Furthermore, novel LA fermented foods can serve as important sources of micronutrients to combat nutritional deficiencies that effect more than 2 billion people worldwide (World Health Organization, 2007). These deficiencies are prolific in the developing world and lead to diseases such as Beri-Beri, pellagra, anemia and night blindness. Oguntoyinbo et al., (2016) propose that development of fermentation protocols, scaling up production and training personnel are essential steps in accelerating the adoption of LA fermented foods in developing nations.

LA fermentation of fruits and vegetables that are typically consumed pickled (acidified) is an attractive area for growth. Commonly consumed pickled vegetables in the United States include asparagus, beets, cucumber, ginger, green beans, onions, peppers and watermelon rind as well as mixtures such as chow-chow, coleslaw, and giardiniera. These products are especially apt for conversion to LA fermented products as they're already prepared in acidified salt brines and have received wide consumer acceptance. The assortment of LA fermented fruits and vegetables produced and their diverse formats (whole, sliced, shredded, pureed, juiced) creates options for their consumption in a variety of cuisines and cultures and therefore widens their potential impact on human health. Opportunities exist for the development of novel mixed fermentations from non-traditionally fermented foods, investigation of health promoting compounds of less common or regionally consumed fermented products, and completion of *in vivo* human studies with fermented foods containing bioactive compounds to determine their impact and clinically relevant dosages.

6.16.1 Development of Novel Mixed Fermentations

The development of mixed fermentations with consumer familiarity is a promising area of growth for LA fermented fruits and vegetables. Products can be formulated from raw materials with bioactive compounds that have a synergistic effect on illness or disease. For example, the commonly consumed fermented cucumber pickle could be optimized for health promoting compound generation by adding onions and garlic high in glucosinolates along with a source of linoleic acid (i.e., microalgae) to generate indoles, isothiocyanates and CLA with cancer-fighting activities. Alternatively, potent extracts and powders with non-glycosylated anthocyanins or carotenoids could be derived from LA fermented waste products such as carrot peels or grape pomace. Mixed fermentations such as shalgam and hardaliye should inspire producers across the globe to develop beverages from roots and tubers in addition to traditionally fermented fruits and vegetables.

Mixed fermentations may be well suited for pureed, shredded, or chopped products. Relishes are wonderful formats for mixed fermentations due to their consistency and common uses. Typically, relishes consist of chopped or shredded fruits and vegetables, often with a thick sauce or syrup which makes texture a minor concern and easily adjustable with thickening agents. Addition of salt, spices, vinegar and sugar adjust the organoleptic properties of relish and make it an ideal product for use as a condiment or flavoring agent. Furthermore, relishes often combine multiple fruits or vegetables which have the potential for the incorporation of bioactive compounds from a variety of sources. Notably, pickle relish is most often made from LA fermented cucumbers and utilizes fruits that are not suitable for whole, spears, or slices.

Chow-chow is a relish thought to originate from Canada but is now found in the United States and comprised of either mostly cabbage (southern U.S.) or a mixture of green and red tomatoes, carrots, onions, beans, cauliflower, asparagus and peas (northern U.S.). Similarly, coleslaw is a cabbage-based relish with numerous formulations from around the world ranging from white to red or purple cabbage mixed with a dressing of buttermilk, sour cream, mayonnaise or vinaigrette. Additional vegetables such as carrots, bell peppers, onions and pickles may be included. Potential bioactive compounds generated during LA fermentation of chow-chow and coleslaw may be similar to those in sauerkraut and kimchi given their shared main ingredient: cabbage. Addition of buttermilk and sour cream to coleslaw also introduces protein for proteolytic formation of bioactive peptides and free amino acids including glutamic

acid for conversion to GABA. Seldom are vegetables and dairy products combined in fermentations, therefore coleslaw provides a unique opportunity for development of a novel functional food. Both chow-chow and giardiniera contain cauliflower (*Brassica oleracea* var. botrytis) which is rich in glucosinolates which may be converted to bioactive, anti-cancer indoles and isothiocyanates as has been demonstrated in sauerkraut and broccoli fermentations.

Fortification of relishes with exopolysaccharide (EPS) producing LAB could also increase their health promoting potential, textural properties and consumer appeal. A diverse range of LAB are known to produce EPS resulting in products with a "ropy", viscous consistency (Ruas-Madiedo, Hugenholz & Zoon, 2002). EPS are involved in gut colonization and attachment to the gastrointestinal tract and play a role in immune modulation (Caggianiello, Kleerebezem & Spano, 2016; Fanning et al., 2012). For example, Das, Baruah & Goyal (2014) demonstrated that an α-D-glucan produced by *Lb. plantarum* DM5 had low digestibility in gastric juice and increased *in vitro* probiotic bacterial growth but did not aid in non-probiotic Enterobacteriaceae growth. EPS production has been utilized in dairy products such as yogurts and milk drinks which are suitable for natural thickening.

In addition to relishes, fermented foods in the format of purees, mashes and pastes may be leveraged for delivery of health promoting compounds (Demir, et al., 2006; Ye et al., 2019). Advantages of preparing purees for fermentation include increasing access of LAB to fermentable sugars and bioactive precursors as well as easily combining a variety of foods to optimize bioactivity. Purees have applications in popular foods such as sauces, smoothies, soups and baby food.

Mixed fermentations should not be limited to fresh produce but should also be considered as means for incorporating waste streams rich in bioactives. For example, Servili et al., (2011) created a functional milk beverage fortified with phenolic compounds (100 - 200 mg/L) extracted from olive processing wastewater and fermented with GABA-producing *Lb. plantarum* C48 and probiotic *Lb. paracasei* 15N. Despite the concentrated level of phenolics, fermentation rate and final acid levels were similar to a control without olive phenolics. The fermentation did not decrease phenolic composition and GABA was generated at 63 - 67 mg/L. These authors successfully merged a commonly consumed LA fermented food (milk, i.e., kefir, yogurt) with a bioactive-rich waste product of LA fermentation and further enhanced it with LAB generation of GABA

6.16.2 Investigation of Health Promoting Compounds in Less Commonly Consumed or Fermented Products

Common, worldwide consumed LA fermented fruits and vegetables are the focus of a plethora of research while much remains to be explored regarding less common, regionally consumed foods. For example, kimchi's health promoting properties are well studied, however there is little to no information on the health benefits of dhamuoi (*dua muoi*), a Vietnamese dish also made from fermented cabbage (Breidt, 2013), gundruk, a Himalayan mixture of cabbage, mustard leaves and cauliflower leaves (Di Cagno et al., 2013), or dakguadong, a Thai fermented mustard leaf (Di Cagno et al., 2013). Compositional analyses of these regionally consumed foods could reveal previously unknown health promoting properties, prompting process optimization as well as a surge in consumer interest.

Foods that are not commonly fermented also serve as areas of potential investigation. For example, sweetpotato holds significant promise for enhancement via LA fermentation. Sweetpotatoes are grown in Asia, the Americas and Africa and are rich in antioxidants and vitamins including β-carotene and vitamin C (Wang, Nie & Zhu, 2016). Furthermore, purple-fleshed sweetpotato varietals also contain anthocyanins. Interest in sweetpotato fermentation has centered around alcoholic fermentation into products similar to beer due to its high starch content, or wine due to its intense pigmentation (Panda, 2019). Opportunity to utilize sweetpotato in LA fermentation should not be eclipsed by its potential in alcoholic beverages. LA fermentation relies on the availability of sugars like glucose and fructose to fuel LAB metabolism and produce lactic acid. At harvest, sweetpotatoes contain approximately 4% free sugars (Johnson, Padmaja & Moorthy, 2009) with the majority of sugars being sucrose, glucose and fructose (Truong, Avula, Pecota & Yencho, 2018). During storage and curing, sweetpotato starch is converted to sucrose and natural invertase enzymes hydrolyze sucrose to fructose and glucose, increasing substrates available for LA fermentation (Nabubuya, Namutebi, Byaruhanga, Naryhus & Wicklund, 2017). Panda, Naskar, Sivakumar & Ray (2009) fermented raw, grated, purple-fleshed sweetpotatoes with *Lb. plantarum* MTCC 1407 and found that the culture was able to convert starch to sugar for high production of lactic acid with a final pH of 1.91. Enzymatic conversion of sweetpotato starch to its monomer, glucose, would provide more sugar for fermentation and the development of lactic acid and resulting acidic taste could be controlled by degree of hydrolysis. Knowledge of the effect of fermentation on sweetpotato bioactives is

lacking, but one study examined the effect of boiling, steaming, and fermenting sweetpotato tips on phenolic composition. The authors found that fermentation decreased total phenolics in sweetpotato tips, yet resulted in the development of quercetin which was absent in raw, boiled and steamed sweetpotatoes, indicating that quercetin was released from its bound form during fermentation (Cui, Liu, Li & Song, 2011)

Cauliflower is another vegetable with significant potential for improvement through LA fermentation. Few studies on cauliflower fermentation have been published, however, one study explored LA fermentation as a means to utilize stalks from cauliflower and broccoli harvesting that typically would have been discarded. Bekhit, Lingming, Mason, Zhou & Sedcole (2013) performed a natural fermentation of whole stalks of broccoli (*Brassica oleracea* var. Italica) and cauliflower (*Brassica oleracea* var. botrytis) in a 6% NaCl brine solution with a 4:1 stalk:brine ratio. Texture analysis showed that cauliflower stalks softened slightly while broccoli stalks hardened slightly during fermentation. The brittleness/crispiness of the stalks post-fermentation was not drastically different than that of fresh stalks, demonstrating that fermentation in salt brine is a feasible method for preserving waste stalks in a consumer acceptable manner. Total phenolics and vitamin C were measured and shown to decrease in both broccoli and cauliflower during fermentation. GLS and their biotransformation products were not measured in this study. It is known that the action of salt and LAB in shredded cabbage enhances GLS conversion to bioactive indoles and isothiocyanates during fermentation, therefore these reactions should be studied in broccoli and cauliflower fermentation as well. Furthermore, shredding of waste stalks from these vegetables may increase GLS bioconversion as is evident in sauerkraut. Inclusion of these stalks in fermented chow-chow or coleslaw would merge two goals of food processors: waste reduction and value addition.

Valorization of fruits and vegetables not typically consumed without the addition of other ingredients, such as sugar, is another opportunity for LA fermentation. For example, bitter melon (*Momordica charantia*) is rarely consumed fresh in the whole form due to its bitterness. An autochthonous isolate (*Lb. plantarum* BET003) from bitter melon fruit was used to ferment its juice, resulting in a significantly less bitter product (Mazlan, Annuar & Sharifuddin, 2015). During fermentation, several health promoting properties of the juice were also enhanced. Glycosidic momordicosides were converted to their aglycone forms, and β-glucosidase activity was measured during fermentation and was associated with the growth phase of this organism.

Additionally, antidiabetic properties have been attributed to *M. charantia*, specifically the ability to inhibit α-glucosidase and prevent the breakdown of maltose into glucose. In the same study, fermented *M. charantia* juice was significantly more potent in inhibiting α-glucosidase activity than fresh juice. Fermentation may be an option for creating a palatable, functional product from *M. charantia*, and this example may be applied to other typically unpalatable fruits or vegetables.

6.16.3 Completion of *in vivo* Human Studies

It is important that as research in LA fermented fruits and vegetables advances, it also moves our understanding of these foods' healthfulness from an anecdotal level to an objective, scientific level. Well-designed human trials to test bioaccessibility, bioavailability, and impact of these bioactive compounds within their native food matrix are required to provide a basis for genuine health claims. Often, human trials involve consumption of isolated, concentrated compounds to invoke a health-promoting response while consumption of the food itself is not studied. These studies have exploratory value but further research must be conducted to account for the synergistic effects of multiple bioactives within a food and to understand the effects of the food matrix itself (i.e., delayed digestion due to fiber content). Clinical trials involving administration of fermented fruits and vegetables to human subjects are limited but have been performed for capers (Khavasi et al., 2017), hardaliye (Amoutzopoulos et al., 2013), kimchi (Choi et al., 2013; Kim & Park, 2018) and sauerkraut (Nielsen et al., 2018; Raak, Ostermann, Boehm & Molsberger, 2014). Human trials are still needed to understand the in vivo benefits of LA fermented cucumber pickles, juices, carrots, olives, turşu, tomatoes and mixed fermentations. Furthermore, determination of dose-dependent relationships between food and positive health impacts is essential for making recommendations for consumer health.

6.17 Conclusion

Globally, LA fermentation is used to convert sugars in fruits and vegetables to organic acids leading to the preservation of perishable produce. LA fermented products range from individual fruits and vegetables (i.e., sauerkraut, cucumber pickles, cocoa) to mixtures (i.e., kimchi, hardaliye, shalgam) and come in a wide array of formats from whole pieces to slices, shreds, relishes, purees and juices. In addition to protecting fresh fruits and vegetables against spoilage and pathogenic organisms, LA fermentation transforms the aroma, flavor, taste, and

texture of foods to enhance desirability. During fermentation, LAB incur additional compositional changes that lead to enhanced health-promoting properties of fruits and vegetables either through producing bioactive compounds or aiding in their release from or transformation within the food matrix. Some of these bioactive compounds include bioactive peptides, amino acids, GABA, phenolic compounds, β-carotene, glucosinolates, hydroxytyrosol, CLA and EPS. These compounds possess antioxidant, anticancer, antimicrobial, anti-inflammatory and/or antihypertensive activities, among others.

Preparation of fermented food products aids in nutrient release and bioavailability through mechanical size reduction (i.e., shredding, slicing, pureeing) and cellular disruption due to the presence of salt or generation of carbon dioxide by LAB. Fermentation aids in the release of bound, insoluble phenolic compounds and vitamins from food matrices to improve antioxidant potential and bioavailability. While spontaneous fermentation is most often used in the manufacturing of LA fermented fruits and vegetables, selection and use of starter cultures is being explored for enhancing health promoting properties of these products. Starter cultures have been used to optimize phenolic biotransformation and increase production of GABA, hydroxytyrosol, ascorbigen, CLA and EPS in various foods including olives, black carrots, kimchi and cucumber juice. Many studies have evaluated bioactive compound formation during fermentation but neglected to determine the impact of long-term storage or downstream processing on these compounds. In order to fully evaluate bioactive compound changes in fermented foods, studies must incorporate manufacturing processes and storage conditions into their experimental designs.

Areas of future research in LA fermented fruits and vegetables include development of novel mixed fermentations and food formats, reclamation of waste streams containing bioactive compounds, utilization of produce nearing spoilage, fermentation of traditionally acidified fruits and vegetables, and conversion of processing by-products such as peels and pomace to value-added foods. Unique fermentations isolated to geographical regions, such as turşu from Turkey or khalpi from the Eastern Himalayas, should be taken as examples for innovation in global fermented foods, inspiring researchers to develop new mixed fermentations or food formats, such as beverages and purees. Foods with significant potential for enhanced bioactive compounds through LA fermentation include broccoli, cauliflower, prickly pear cactus and sweet potato as well as acidified foods such as giardiniera, chow-chow, relishes, and coleslaw. Furthermore,

several commonly consumed fermented fruits and vegetables, such as capers and cucumbers, lack significant research into their health promoting potential and investigations focused on these foods have the potential for widespread impact on human populations.

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Table 1. Formation of bioactive compounds during lactic acid fermentation of fruits and vegetables

Formation		Bioactivity	Fermented Food Sources	References
Ginsenoside RB1	β-glucosidase → Compound K	• Tumor suppresser	• Ginger (kimchi, sauces)	Quan et al., 2011
Linoleic Acid	Microbial Metabolism → Conjugated Linoleic Acid	• Anti-cancer • Anti-inflammatory • Body Fat Reduction	• *In vitro*	Gong et al., 2019; Liu et al., 2011; Renes et al., 2017
Sugars	Microbial Metabolism → Exopoly-saccharides	• Gut colonization • Immune Modulation	• Various	Caggianiello et al., 2016; Das et al., 2014; Dols, et al., 1997; Ruas-Madiedo et al., 2002;
Bound phenolics	Microbial Metabolism / Plant Cell Disruption → Free phenolics	• Antioxidant	• Capers • Carrots (purees, juices, turşu) • Olives	Benincasa et al., 2015; Hu et al., 2019; Jiménez-López et al., 2018
Glutamate	GAD → GABA	• Antianxiety • Antihypertensive • Immune Support	• Cabbage (kimchi, sauerkraut) • Cucumbers (pickles) • Prickly pear cactus • Cocoa	Cho et al., 2011; Filannino et al., 2016;Lee et al., 2018; Marseglia et al., 2014; Moore et al., 2021
Glucosinolates	Myrosinase → Indoles & Isothiocyanates (i.e. ascorbigen)	• Anticancer	• Cabbage (kimchi, sauerkraut) • Capers	Bonneson et al., 2001; Jiménez-López et al., 2018; Peñas et al., 2011; Wagner et al., 2009
Proteins & Peptides	Proteolytic Enzymes → Peptides & Amino Acids	• Anticancer • Antihypertensive • Antioxidant • Antidiabetic • Anti-obesogenic	• Cocoa • Cucumbers (pickles)	Domínguez-Pérez et al., 2020; Fideler et al., 2019
Carbohydrate	Microbial Metabolism → SCFA	• Antidiabetic	• Carrots (purees, juices, turşu)	Li et al., 2014; Park et al., 2016; Wan et al., 2019
Oleuropein	β-glucosidase → Tyrosol & Hydroxytyrosol	• Antioxidant • Antimicrobial	• Carrots (purees, juices, turşu) • Olives	Aponte et al., 2010; Benincasa et al., 2015; Charoenprasert et al., 2012; Tafesh et al., 2011

CHAPTER 7: Conclusion

Globally, fermented foods are commonly perceived as healthful and have been ranked by dietitians as the number one superfood in 2018 and 2019 (Pollock Communications, 2019). Two-thirds of Americans consume various types of cucumber pickles, making them the most commonly consumed fermented vegetable in the United States. Despite the popularity of pickled vegetables, little research exists surrounding the development of health promoting compounds during lactic acid fermentation of cucumbers. During natural fermentation of cucumbers, lactic acid bacteria (LAB) convert naturally present sugars to lactic acid. Additionally, LAB increase the health promoting potential of fermented foods by metabolizing proteins to bioactive peptides and free amino acids, and converting glutamate to γ-aminobutyric acid (GABA). Bioactive peptides have a variety of demonstrated health properties including antihypertensive, anticholesterolemic, and antioxidant activities. Likewise, GABA has been shown to lower blood pressure, decrease anxiety, and support immune function. In this study, the formation of bioactive peptides and GABA in naturally fermented cucumbers was investigated.

Proteolytic activity of LAB resulted in the formation of peptides in fermented cucumbers, including four angiotensin converting enzyme (ACE) inhibitory peptides. Isoleucine-proline-proline (0.42-0.49 mg/kg), leucine-proline-proline, (0.30-0.33 mg/kg), and valine-proline-proline (0.32-0.35 mg/kg) were formed in both natural and starter culture fermented cucumbers while lysine-proline increased 3 to 5-fold in fermented cucumbers (0.93-1.5 mg/kg) compared to acidified cucumbers. GABA formation, stability, and enhancement were also studied in LA fermented cucumbers. Fresh and acidified cucumbers contained 0.83 ± 0.16 mM and 0.56 ± 0.30 mM GABA, respectively, with additional GABA formed in fermented cucumbers (1.21 ± 0.35 mM). GABA remained stable during pasteurization and after 6 months of storage, demonstrating its likelihood to be present in pickle products that reach consumers. The effect of brine salt content (2%, 3%, 4%, 6%) on GABA formation in naturally fermented cucumbers was studied, with the highest GABA formation observed in cucumbers brined in 2% NaCl (1.38 ± 0.31 mM). GABA content of commercially available pickle products was also analyzed with the highest GABA found in directly packed, fermented products (1.32 ± 0.25 mM), indicating that development of lower salt (2% NaCl) fermented pickles that do not require desalting or repacking is optimal for GABA formation and retention. Addition of 10 mM glutamate, a food additive with Generally Recognized as Safe (GRAS) status in the USA, to natural and starter

culture fermented cucumbers brined in 2% NaCl resulted in significantly higher GABA (10.39 – 14.86 mM) compared to naturally fermented cucumbers (1.12 ± 0.16 mM). Cucumber pickles with added glutamate contained 36.5 mg of GABA in a 1 oz serving compared with 4.7 mg per 1 oz serving in naturally fermented cucumbers.

Our work demonstrated that natural lactic acid fermentation of cucumbers enhanced the bioactive peptide and GABA content of pickled cucumbers. Furthermore, glutamate addition to natural cucumber fermentations increased GABA formation during fermentation to clinically relevant levels. Consumption of bioactive peptides and GABA in pure form or as components of select fermented foods has produced antihypertensive effects in human studies. However, the impact of consuming fermented cucumber pickles containing these bioactive compounds has not yet been studied. Further exploration of the effects of fermented vegetable consumption on human health will shed light on the potential to positively impact human lives and provide guidance for developing GABA-enhanced, fermented vegetable products.

7.1 References

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APPENDICES

APPENDIX A: Total Protein Quantification in Raw and Fermented Cucumbers

A.1 Overview

In order to assess proteolysis and potential bioactive peptide formation in lactic acid fermented cucumbers, methods for quantification of total protein in raw and fermented cucumbers must be identified. Quantification of total protein was performed on raw and fermented cucumbers using the bicinchoninic acid (BCA) method and the Bradford method. Additionally, preparation of protein samples for SDS-PAGE using a phenol extraction method was explored. A comparison between these methods' advantages and disadvantages is presented.

A.2 Materials & Methods

A.2.1 BCA Total Protein Assay

A.2.1.1 Microtiter Plate Procedure

Equipment List:

- Analytical balance (15 g capacity)
- Tekmar Tissumizer with TR-10 power controller and "10 N" flat bottom stator
- Vortexer
- Centrifuge with 15 mL tube capacity
- Microplate Reader
- Pipettes with working range 25 µL-
- Incubator at 37°C

Materials List:

- Pierce BCA Protein Assay Kit (Thermo Scientific)
  - BCA reagent A
  - BCA reagent B
  - BSA stock solution, 2000µg/mL (BSA Standard #1)

- BSA Standard #2 stock solution mixed in house, 2000µg/mL (Sigma Aldrich, item #A7030)
- Phosphate-Buffered Saline (PBS) ~pH 7.4 prepared from tablets (Sigma Aldrich, item #P4417)
- Deionized water
- Mock Brine
    - NaCl (99.7% purity)
    - Lactic Acid (>85% purity)
- 15 mL falcon tubes (polypropylene – centrifuge safe)
- Pop-top microcentrifuge tubes
- Screw-top microcentrifuge tubes
- 96-well microtiter plate

Protocol:

1. Prepare 50mL BSA Standard #2 stock solution by dissolving powdered BSA in deionized water. Store in tightly closed container in fridge (4°C) when not using.
2. Prepare two sets of standards using Standards #1 and #2 per instructions provided in the Pierce BCA Protein Assay Kit. Dilute with PBS. Store in microcentrifuge pop-top tubes until ready to add to microtiter plate.
3. Prepare brine and calculate density.
4. Prepare unknown samples:
    a. Thaw frozen slurry.
    b. Prepare 50ml of brined slurry using a 45:55 ratio of brine to slurry by weight using the densities of the brine and cucumber slurry. Mix on stir plate for one hour.
    c. Weigh PBS, slurry, and brined slurry into 15ml centrifuge safe falcon tubes following the treatment plan.
    d. Add Standard #2 by pipette to all treatments receiving a BSA spike.

e. Vortex and then tissumize sample by immersing the stator into falcon tube and increase power regulator to 60% for six, 10-second periods. Move the falcon tube up and down to ensure even mixing during the "on" cycles.

f. Centrifuge tubes at 9000 RPM x 10 minutes

5. Prepare BCA working reagent (WR):

a. Calculate volume needed for samples and mix 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B, 51 fold dilution).

6. Pipette 25µL of each standard and treatment into randomly selected wells in the microtiter plate.

7. Choose one treatment and pipette 25µL into four more wells to test for variability within the plate.

8. Using a multichannel pipette, add 200µL WR to each well (sample to WR ratio = 1:20)

9. Add 225µL of deionized water to three wells for blanks.

10. Cover plate and incubate for 30 min at 37°C. Remove from incubator and let cool to room temperature (~10 minutes)

11. Turn on microplate reader and computer 30 minutes before reading sample.

12. Measure the absorbance of microtiter plate at 550nm and report data with and without blank adjustments.

13. Prepare a standard curve by plotting the average zeroed, blank-corrected measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

A.2.1.2 Test Tube Procedure

Equipment List:

- Analytical balance (15 g capacity)
- Tekmar Tissumizer with TR-10 power controller and "10 N" flat bottom stator
- Vortexer
- Centrifuge with 15 mL tube capacity
- Pipettes
- Water bath at 37°C

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- Spectrophotometer

Materials List:

- Pierce BCA Protein Assay Kit (Thermo Scientific, item # 23227)
    - BCA reagent A
    - BCA reagent B
    - BSA stock solution, 2000µg/mL (BSA Standard #1) *(optional, prefer to make in-house)*
- BSA Standard stock solution mixed in house, 2000µg/mL (Sigma Aldrich, item #A7030)
- Phosphate-Buffered Saline (PBS) ~pH 7.4 prepared from tablets (Sigma Aldrich, item #P4417)
- 15 mL falcon tubes, polypropylene, centrifuge safe
- Pop-top microcentrifuge tubes

Protocol:

1. Prepare 50mL BSA Standard #2 stock solution by dissolving powdered BSA in deionized water. Store in tightly closed container in fridge (4°C) when not using.
2. Prepare two sets of standards using Standards #1 and #2 per instructions provided in the Pierce BCA Protein Assay Kit. Dilute with PBS. Store in microcentrifuge pop-top tubes until ready to add to microtiter plate.
3. Prepare brine and calculate density.
4. Prepare unknown samples:
    a. Thaw frozen slurry.
    b. Prepare 50ml of brined slurry using a 45:55 ratio of brine to slurry by weight using the densities of the brine and cucumber slurry. Mix on stir plate for one hour.
    c. Weigh PBS, slurry, and brined slurry into 15ml centrifuge safe falcon tubes following the treatment plan.
    d. Add Standard #2 by pipette to all treatments receiving a BSA spike.

e. Vortex and then tissumize sample by immersing stator into falcon tube and increasing power regulator to 60% for six, 10-second periods. Move the falcon tube up and down to ensure even mixing during the "on" cycles.

f. Centrifuge tubes at 9000 RPM x 10 minutes

5. Prepare standard and unknown sample dilutions in microcentrifuge pop-top tubes. Use same diluent for standards and unknowns.

6. Prepare working reagent (WR):

a. Calculate volume needed for samples and mix 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B, 51 fold dilution).

7. Working with 10-12 samples at a time, pipette 0.1mL of each standard and unknown sample replicate into an appropriately labeled centrifuge tube.

8. Add 2.0 mL WR to each tube and mix well.

9. Cover and incubate tubes in a water bath at 37°C for 30 minutes.

10. Cool all tubes to room temperature.

11. With the spectrophotometer set to 562 nm, zero the instrument on a cuvette filled only with water.

12. Measure the absorbance of all the samples within 10 minutes.

13. Subtract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm absorbance measurement of all other individual standard and unknown sample replicates.

14. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample

A.2.2 Bradford Assay

Equipment List

- Tekmar Tissumizer with TR-10 power controller and "10 N" flat bottom stator
- Vortexer
- Centrifuge with 15 mL tube capacity
- Pipettes

- Spectrophotometer

Materials List

- Bradford Reagent (Sigma Aldrich, item #B6916)
- BSA Standard #2 stock solution mixed in house, 2000 µg/mL (Sigma Aldrich, item #A7030)
- Phosphate-Buffered Saline (PBS) ~pH 7.4 prepared from tablets (Sigma Aldrich, item #P4417)
- Mock Brine
    - NaCl (99.7% purity)
    - Lactic Acid (>85% purity)
- 15mL falcon tubes (polypropylene – centrifuge safe)
- Disposable polystyrene cuvettes, 3mL
- Microcentrifuge tubes (1.2mL)
- Future:
    - 0.1N or 1.0 N sodium hydroxide (NaOH)
    - Trichloroacetic Acid (TCA)

Protocol

1. Remove Bradford reagent from refrigerator, gently mix in bottle, and let warm to room temperature.
2. Prepare one set of serial dilutions (100 to 1400 µg/mL BSA) using stock solution and PBS.
3. Prepare unknown samples:
    a. Thaw frozen slurry.
    b. Prepare 50 ml of brined slurry using a 45:55 ratio of brine to slurry by weight using the densities of the brine and cucumber slurry. Mix on stir plate for one hour.
    c. Weigh PBS, slurry, and brined slurry into 15ml centrifuge safe falcon tubes following the treatment plan.
    d. Add standard by pipette to all treatments receiving a BSA spike.

e. Vortex and then tissumize sample by immersing stator into falcon tube and set power regulator to 60% for one, 30-second period. Move the falcon tube up and down to ensure even mixing.

f. Between samples, immerse stator in DI water and run at 60% power for 10 seconds. Rinse stator into receptacle and dab dry with paper towel.

g. Centrifuge tubes at 9000 RPM x 15 minutes.

h. Decant supernatant into microcentrifuge tubes and use for assay.

4. Turn spectrophotometer on at least 30 minutes before using to allow bulb to warm up.

5. Add 100 µL of unknown sample to a 15mL non-centrifuge safe falcon tube. Add 3 mL of Bradford reagent to each tube.

6. Let color develop for 5 to 45minutes at room temperature.

7. Transfer samples to cuvettes and measure absorbance at 595 nm including a water and reagent blank.

8. Measure all samples within one hour of mixing and within 10 minutes of each other.

9. Prepare a standard curve by plotting the average Blank-corrected 595 nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

A.2.2 Phenol Extraction and SDS-PAGE analysis

Equipment List
- Centrifuge
- Freezers: -20C and -80C
- Tekmar Tissumizer with TR-10 power controller and "10 N" flat bottom stator
- Incubator
- SDS-PAGE
- Speed vacuum

Materials List
- Freeze-dried cucumber samples (raw and fermented)
- 50-ml conical tubes (compatible with phenol and acetone)

- Sucrose

- Potassium chloride (KCl)

- Tris-HCl

- Ethylenediaminetetraacetic acid (EDTA)

- Hydrochloric acid (HCl)

- Sodium hydroxide (NaOH)

- β-mercaptoethanol

- Phenylmethylsulfonyl fluoride (PMSF)

- Isopropanol

- Ammonium acetate

- Phenol

- Methanol

- Water

- Sodium dodecyl sulfate (SDS)

- Glycine

- SDS-PAGE standards

Protocol: SDS-PAGE

Resulting pellets from the phenol protein extraction were analyzed using SDS-PAGE in the
Muddiman Lab. Sample preparation was as follows: pellets were added to a speed vac to
evaporate residual acetone. 10 volumes of running buffer were added to the pellets and then
centrifuged. One replicate of each sample was centrifuged with a 10 kDa filter while other
replicates were not. Supernatants from the first centrifuge step and filtrates from the second
centrifuge step diluted 2X, 10X, and 100X then electrophoresed. Running buffer consisted of
250mM Tris, 1.92M glycine and 1% SDS (pH 8.3)

Protocol: phenol extraction

Table A.1. Phenol protein extraction protocol

Category	Step	Action	Reagents	Purpose	Retain	Discard
Cell Disruption	1	Freeze plant tissue in liquid nitrogen	Liquid nitrogen	Cease plant processes and enzymatic action Prepare tissue for disruption	Powdered plant tissue (frozen)	
	2	Grind plant powder in chilled mortar and pestle.	Liquid nitrogen	Further disrupt plant cells	Powdered, pulverized plant tissue (frozen)	
	3	Grind plant powder again with polytron/glass homogenizer	Liquid nitrogen	Further disrupt plant cells. We may not need to do this because ours were freeze dried and then liquid nitrogen macerated; the material had much less water than fresh plant.	Powdered, pulverized plant tissue (frozen)	
Protein Extraction	4	Add plant powder and cold extraction buffer to tube.	Extraction buffer	Disperse and solubilize plant powder. Will serve as aqueous phase in next step.	Powder in buffer	
	5	Add cold Tris-buffered phenol to tube. Shake for 30 min at 4C	Tris-buffered phenol	Mix. Allow components to partition per their solubilities (aqueous vs. phenol).	Powder in buffer and phenol	
	6	Centrifuge and siphon off top layer. Discard bottom layer and pellet.		Partition into phenol phase (top): proteins, pigments, and lipids. Aqueous (bottom): insoluble cell debris, carbohydrates, salts, nucleic acids.	Phenol Layer: proteins + impurities (pigments, lipids)	Extraction buffer and pellet: insoluble cell debris, CHO, salts, nucleic acids.
	7	Add equal volume cold extraction buffer. Shake 30 minutes at 4C.	Extraction buffer	Further partitioning		

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Table A.1. (cont.)

Protein Extraction	8	Centrifuge and siphon off top layer. Discard bottom layer and pellet.		Further refine protein extract by removing more interfering substances.	Phenol Layer: proteins + impurities (pigments, lipids)	Extraction buffer and pellet: insoluble cell debris, CHO, salts, nucleic acids.
	9	Add equal volume cold extraction buffer. Shake 30 minutes at 4C.	Extraction buffer	Further partitioning		
	10	Centrifuge and siphon off top layer. Discard bottom layer and pellet.		Further refine protein extract by removing more interfering substances.	Phenol Layer: proteins + impurities (pigments, lipids)	Extraction buffer and pellet: insoluble cell debris, CHO, salts, nucleic acids.
Protein Precipitation	11	Add ammonium acetate to collected phenol-protein extract. Let sit overnight at -20C.	Ammonium acetate	Precipitate proteins from phenol phase	Precipitate: protein + impurities (lipids, pigments, am. acetate)	Solution: phenol with some protein
	12	Centrifuge. Discard sup. Retain pellet.		Proteins should ppt after 30 minutes but best to wait overnight. Centrifuge to completely separate protein particulates from phenol.	Pellet: protein + impurities (lipids, pigments, ammonium acetate)	Solution: phenol

Table A.1. (cont.)

Protein Purification	13	Rise pellet with methanol.	Methanol	Resuspend pellet. Methanol used to replace remaining phenol in pellet and solubilize ammonium acetate, lipids, and pigments.		
	14	Centrifuge. Discard sup. Retain pellet.		Remove impurities from protein pellet.	Pellet: protein + minor impurities	Retentate: MeOH, lipids, pigments, am. acetate
	15	Rise pellet with methanol.	Methanol	Resuspend pellet and solubilize remaining ammonium acetate, lipids, and pigments.		
	16	Centrifuge. Discard sup. Retain pellet.		Remove impurities from protein pellet.	Pellet: protein + minor impurities	Retentate: MeOH, lipids, pigments, am. acetate
	17	Rise pellet with methanol.	Methanol	Resuspend pellet and solubilize remaining ammonium acetate, lipids, and pigments.		
	18	Centrifuge. Discard sup. Retain pellet.		Remove impurities from protein pellet.	Pellet: protein	Retentate: MeOH, lipids, pigments, am. acetate
	19	Rise pellet with acetone.	Acetone	Resuspend pellet. Replace remaining methanol with acetone which will evaporate quicker.		
	20	Centrifuge. Discard sup. Retain pellet.		Remove methanol from protein pellet.	Pellet: protein	Retentate: methanol and acetone
	21	Rise pellet with acetone.	Acetone	Resuspend pellet.		
	22	Centrifuge. Discard sup. Retain pellet.		Remove methanol from protein pellet.	Pellet: protein	Retentate: methanol and acetone

Table A.1. (cont.)

Protein Purification	23	Rise pellet with acetone.	Acetone	Resuspend pellet.		
	24	Centrifuge. Discard sup. Retain pellet.		Remove methanol from protein pellet.	Pellet: protein	Retentate: methanol and acetone
	25	Dry pellet under gentle stream of nitrogen gas	Nitrogen (g)	Dry pellet and remove acetone.	Dried pellet	
	26	Store pellet at -80C or use immediately				

A.2.4 Experimental Approach

Standard curves using BSA were prepared for each assay according to manufacturer's instructions. Assays were assessed for standard curve quality, standard curve and sample reproducibility, interaction with components of typical cucumber fermentations (brines made with 6% NaCl, 18 mM $CaCl_2$, and 53 mM acetic acid), recovery of spiked BSA and protein extraction and solubility. For recovery studies, cucumber slurries were diluted with either brine or PBS and spiked with known levels of BSA.

A.2.4.1 Experiment 1: Total Protein Quantification by BCA Assay in Raw Cucumbers

Overview

Quantification of total protein in raw and fermented cucumbers using a Thermo Scientific Pierce BCA Protein Assay kit and microtiter plate method is explored.

Objectives

1. Determine if BSA standard prepared in lab is equivalent to kit standard and achieve reproducible absorbance measurements for said standards
2. Determine if raw cucumber matrix interferes with BCA color development (BSA spike)
3. Determine if brine components influence absorbance in raw cucumber samples.
4. Determine if brine components in cucumber matrix interfere with BCA + BSA color reaction

Table A.2. Treatments for total protein quantification by BCA in raw cucumbers

Matrix	Treatment	Dilutions	Technical Replicates	Total Samples
BSA Standard - Pierce kit	Prepare as directed in manual	0-2000ug/ml (9)	1	9
BSA Standard - mixed in-house	Prepare as directed in manual	0-2000ug/ml (9)	1	9
Raw Cucumber	Dilution with PBS	1:8 (1)	3	3
Raw Cucumber	Dilution with PBS + brine	1:8 (1)	3	3
Raw Cucumber	Dilution with PBS + BSA (250µg/ml - post dilution, 2000 µg added)	1:8 (1)	3	3
Raw Cucumber	Dilution with PBS + brine, + BSA (250µg/ml - post dilution, 2000 µg added)	1:8 (1)	3	3
Raw Cucumber	Dilution with PBS,+ BSA (500µg/ml - post dilution, 4000 µg added)	1:8 (1)	3	3
Raw Cucumber	Dilution with PBS + brine, + BSA (500µg/ml - post dilution, 4000 µg added)	1:8 (1)	3	3
Total Treatments:	8		Sum:	36
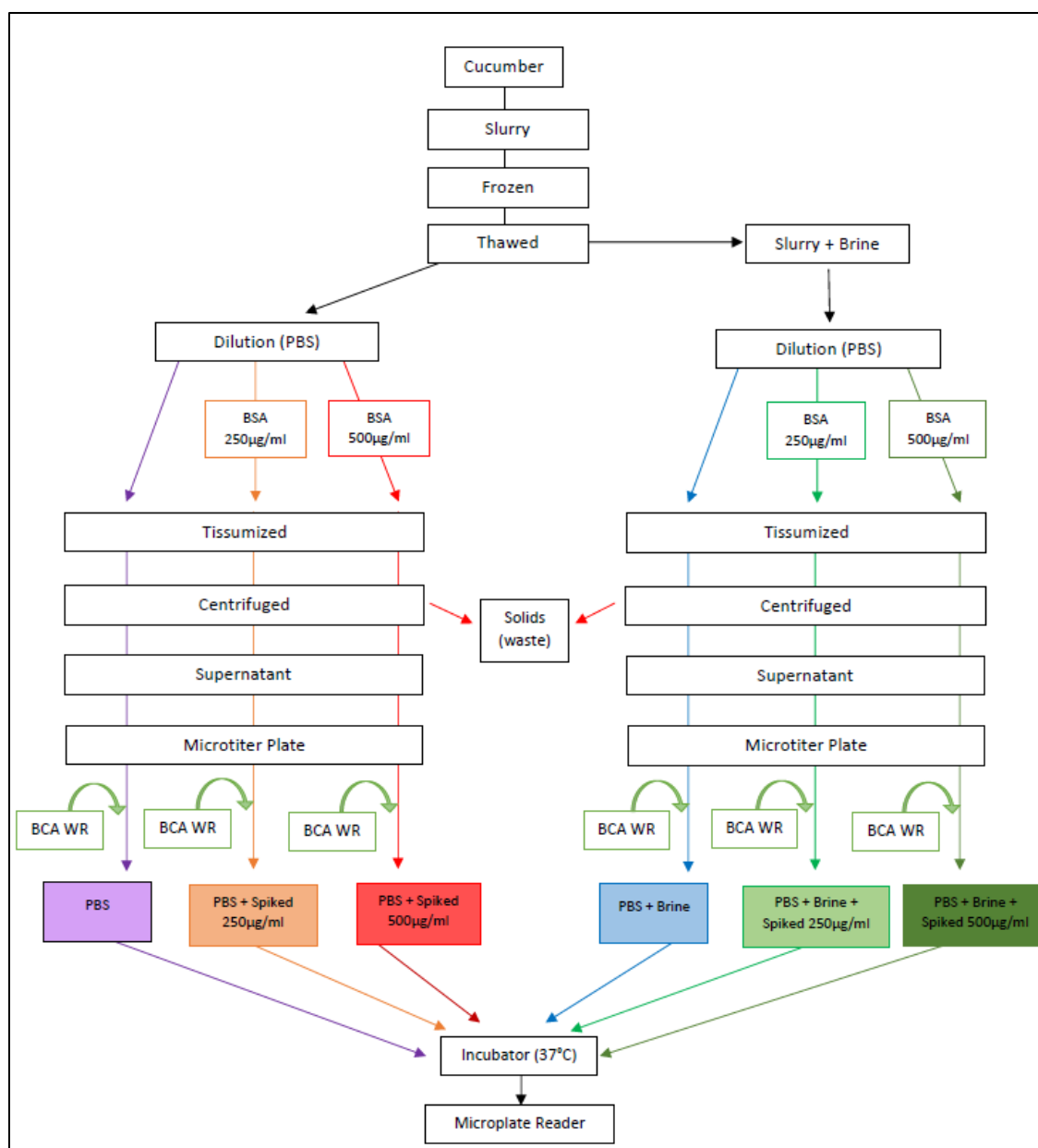
Figure A.1. Experimental flow chart for total protein quantification by BCA in raw cucumbers. Mock brine composition was 1 M NaCl + 100 mM lactic acid.

A.2.4.2 Experiment 2: Total Protein Quantification by Bradford Assay in Raw Cucumbers

Objectives:

1. Obtain reproducible standard curve technical replicates using Bradford reagent.

2. Obtain reproducible standard curve cuvette replicates.

3. Obtain reproducible absorbance measurements for raw cucumber samples.

4. Determine if raw cucumber matrix interferes with color development (BSA spike)

5. Determine if brine components influence absorbance in raw cucumber samples.

Table A.3. Treatments and replicates for total protein quantification by Bradford assay in raw cucumbers

Matrix	Treatment	Dilution Factor	Tech. Reps	Total Samples*	Primary Objectives Explored
BSA Standard	Technical replicate set 2	Various (6)	1	6	1, 2
BSA Standard	Technical replicate set 2, reagent step replicates	Various (6)	1	6	1, 2
Raw Cucumber	Dilution with PBS	1:8 (1)	3	5	3
Raw Cucumber	Dilution with PBS + BSA (250µg/ml - post dilution, 2000 µg added)	1:8 (1)	3	5	4
Raw Cucumber	Dilution with PBS,+ BSA (500µg/ml - post dilution, 4000 µg added)	1:8 (1)	3	5	4
Raw Cucumber	Dilution with PBS + brine	1:8 (1)	3	5	5
Raw Cucumber	Dilution with PBS + brine, + BSA (250µg/ml - post dilution, 2000 µg added)	1:8 (1)	3	5	6
Raw Cucumber	Dilution with PBS + brine, + BSA (500µg/ml - post dilution, 4000 µg added)	1:8 (1)	3	5	66

A.3 Results & Discussion

A.3.1 Experiment 1: Total Protein Quantification by BCA Assay in Raw Cucumbers
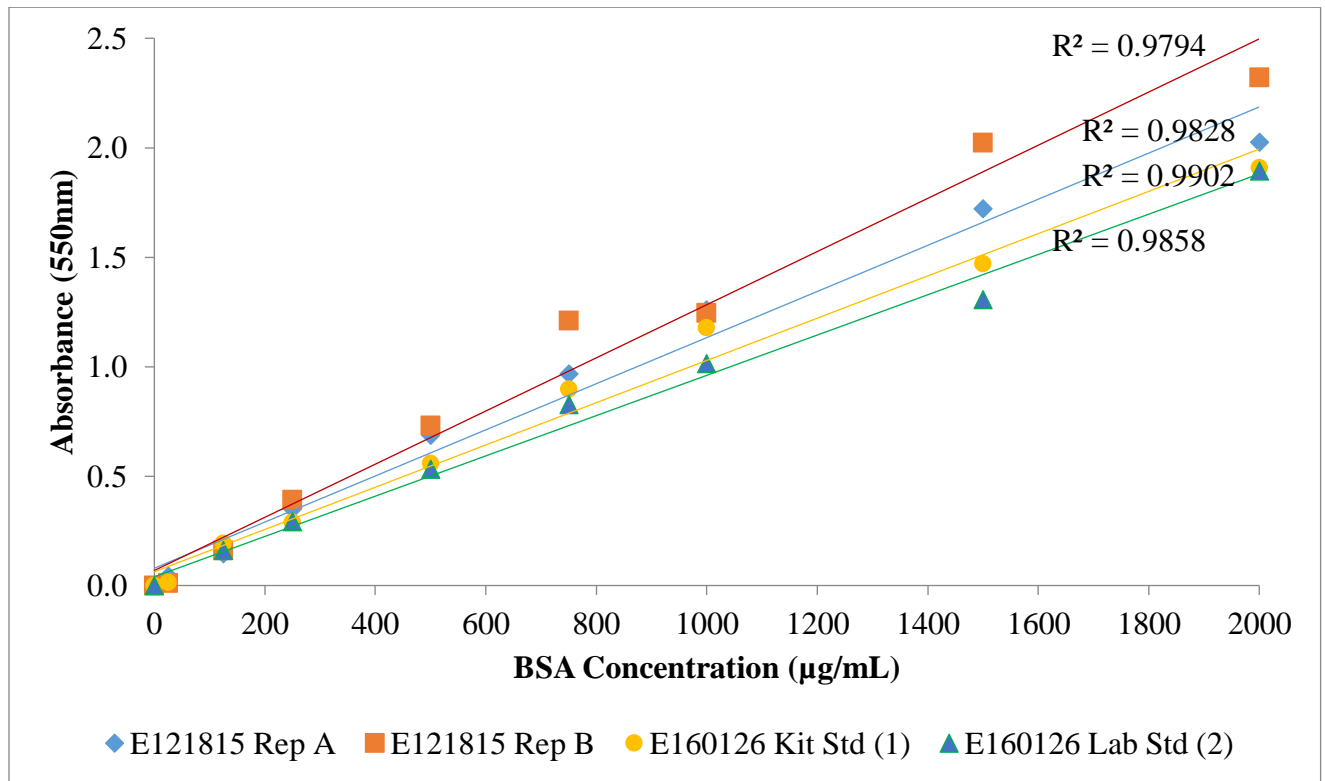
Figure A.2 BSA standard curves prepared for BCA assay. E121815 Rep A and Rep B: BSA standard that came with BCA kit; E160126 Kit Std (1): BSA standard that came with BCA kit; E160126 Lab Std (2): BSA in PBS mixed in-lab

Table A.4. Effect of BSA spike on average protein concentration in BCA assay

Treatment	Avg. protein in control samples (µg/mL BSA)	Avg. protein in spiked samples (µg/mL BSA)	Projected Recovery (µg/mL BSA)	Spiked – Control (µg/mL BSA)	Recovery
Standard E - BSA 500 µg/mL	N/A	483.2	500	483.2	96.6%
Standard F - BSA 250 µg/mL	N/A	249.3	250	249.3	99.7%
PBS (250ug/mL, 2000ug total)	888.9	1223.7	250	334.9	133.9%
PBS + Brine (250ug/mL, 2000ug total)	699.2	805.5	250	106.3	42.5%
PBS (500ug/mL, 4000ug total)	888.9	1325.4	500	436.5	87.3%
PBS + Brine (500ug/mL, 4000ug total)	699.2	840.6	500	141.5	28.3%
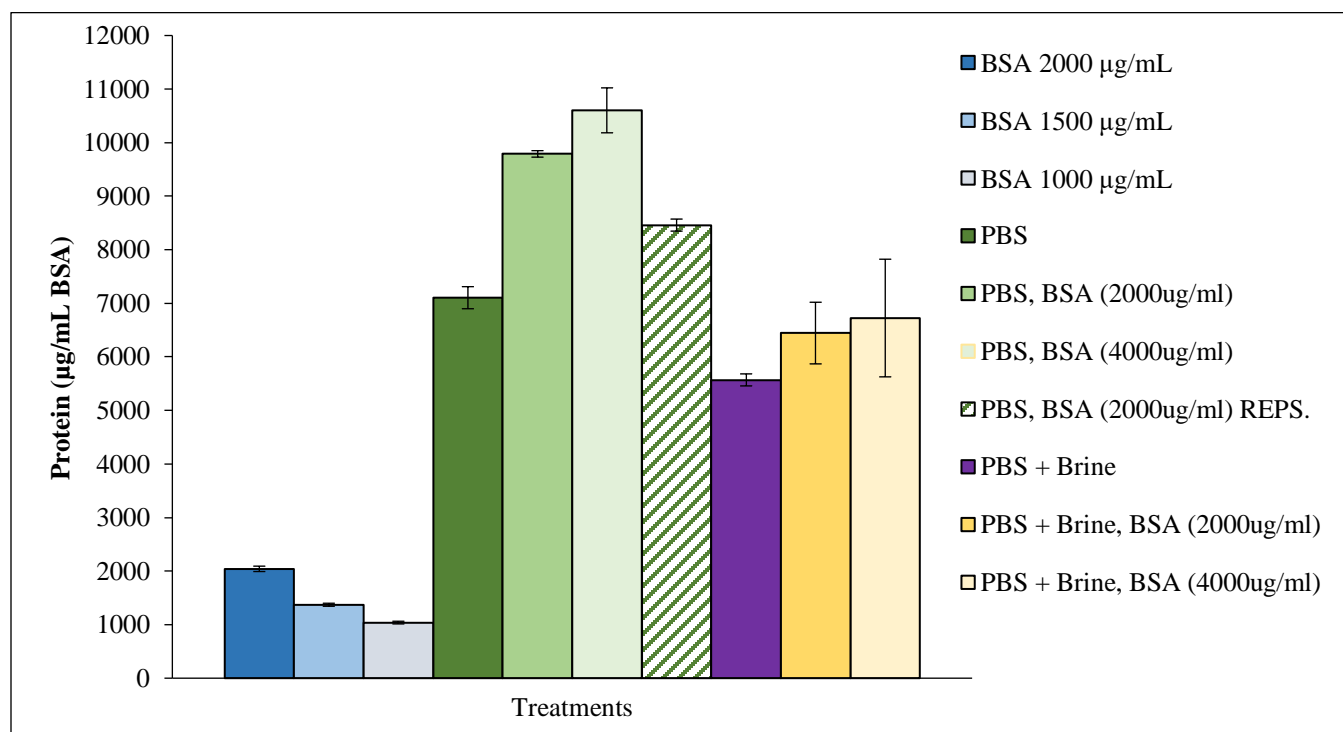
Figure A.3. Average protein content by treatment in BCA assay. Treatment *PBS, BSA (2000µg/mL) REPS.* is an average of four replicate wells in the microplate from one technical replicate of treatment *PBS, BSA (2000µg/mL).*
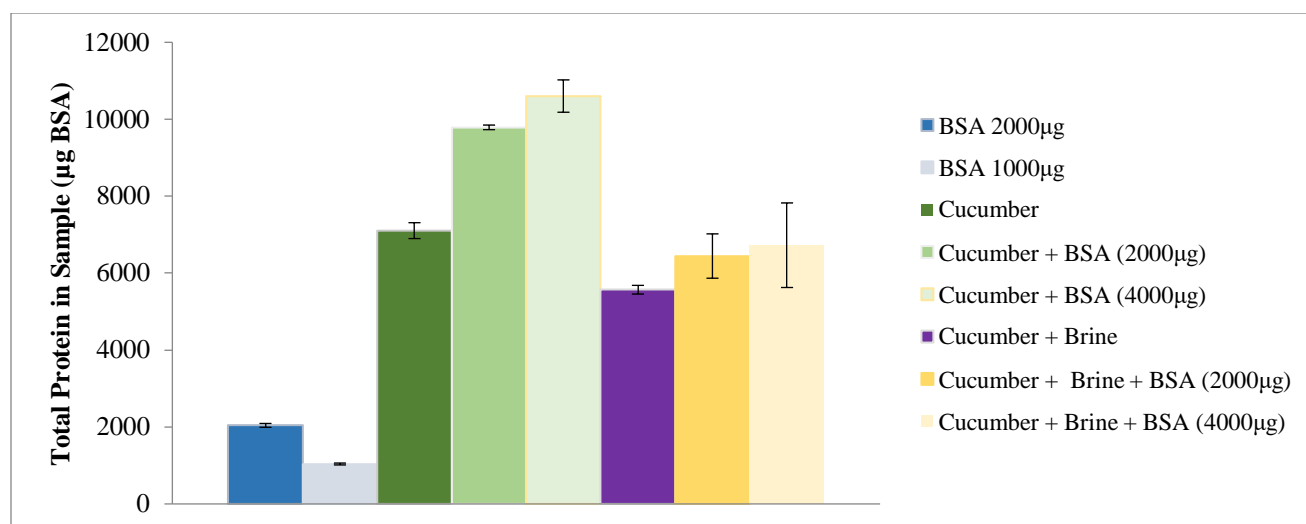
Figure A.4. Average total protein in diluted samples by treatment in BCA assay. Absorbance values of cucumber + brine treatments were corrected for dilution.

- The projected protein recovery for spiked samples was not achieved (Table A.4).
- Projected recovery was below 100% in all but the PBS only sample which had a 134% recovery (Table A.4).
- Samples with acidified brine had the lowest recovery percentages (Table A.4).
- Both PBS and PBS + brine diluted samples that were spiked with BSA had increasingly greater projected protein concentrations as compared with the raw samples (Figure A.3)
- There are significant matrix interactions present in this assay. When cucumber is spiked with BSA it has a greater absorbance than when cucumber is diluted with brine and spiced with BSA (after corrections have been made for dilutions) (Figure A.4)

A.3.2 Experiment 2: Total Protein Quantification by Bradford Assay in Raw Cucumbers
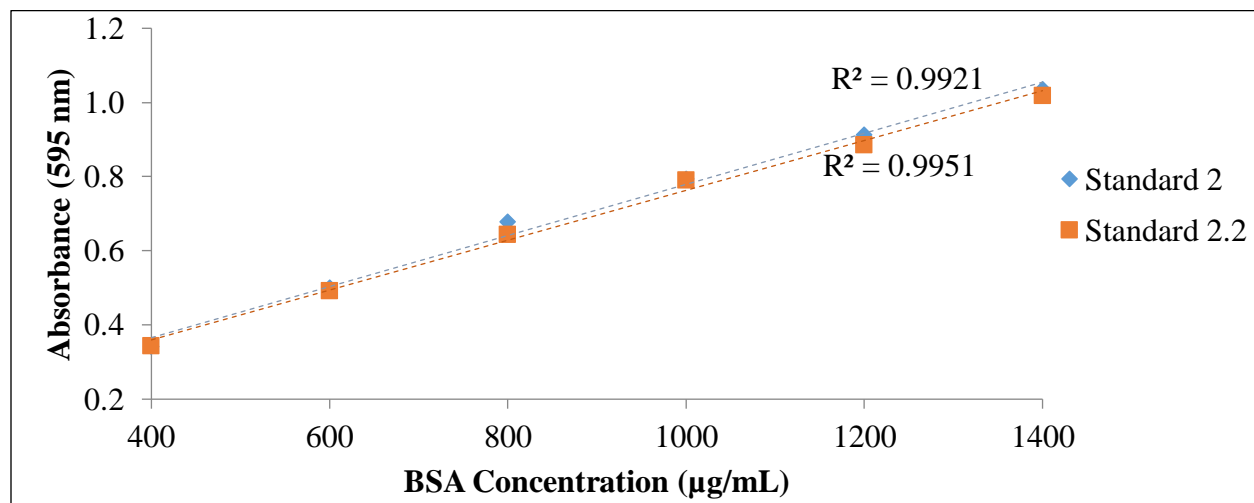
Figure A.5 Standard curves for technical replicate #2 dilution series (400µg/ml – 1400µg/ml) in the Bradford assay. "Standard 2" was run sequentially at the beginning of the experiment. "Standard 2.2" represents cuvette replicates of Standard 2. Their absorbance was read in random order over the course of three measurement groups.

- Standard curves were prepared and fit with polynomial trend lines ($R^2 > 0.998$) (Figure A.5).
- Reproducibility between two replicates was achieved with RSD < 4% for all dilution levels.
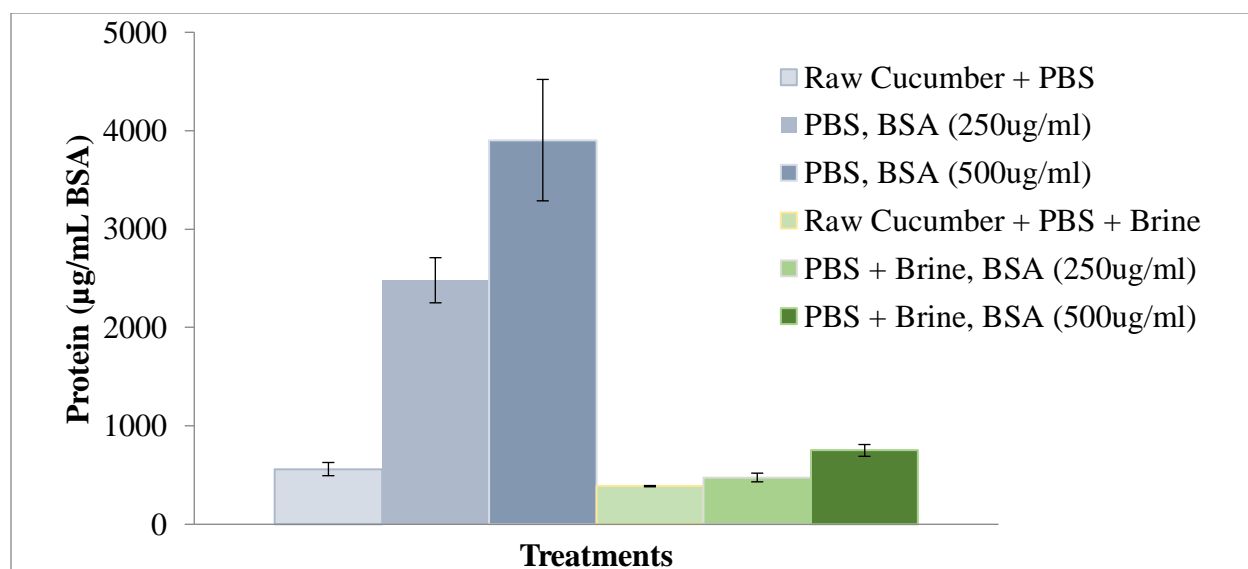
Figure A.6. Estimated protein content of raw cucumber and BSA spiked treatments in the Bradford assay

Table A.5 Percent recovery of BSA spiked samples in the Bradford assay

Treatment	Avg. protein conc. control samples (µg/mL BSA equivalent)	Avg. protein conc. spiked samples (µg/mL)	Projected Recovery (µg/mL)	Spiked - Control	Recovery
PBS (250µg/ml, 2000 µg total)	561.2	2480.1	2000	1919.0	95.9%
PBS + Brine (250µg/ml, 2000 µg total)	387.4	475.6	2000	88.2	4.4%
PBS (500µg/ml, 4000 µg total)	561.2	3904.2	4000	3343.0	83.6%
PBS + Brine (500µg/ml, 4000 µg total)	387.4	750.9	4000	363.4	9.1%

- Recovery of spiked protein varied greatly between the brine and non-brine cucumber slurries. Recovery was very poor (<10%) in the brined samples and somewhat acceptable in the non-brined samples (>80%) (Table A.5, Figure A.6).
- The cucumber matrix diluted with PBS may not interfere with the recovery of BSA from spiked samples, however dilution with brine has an effect on BSA spike recovery (Table A.5, Figure A.6).

- The level of protein estimated in cucumbers was far below the USDA estimate of 6500 µg/mL.
- The cucumber protein and BSA in the brined samples was not measured well with the Bradford method.

A.3.3 Protein extraction using phenol extraction method and SDS-PAGE
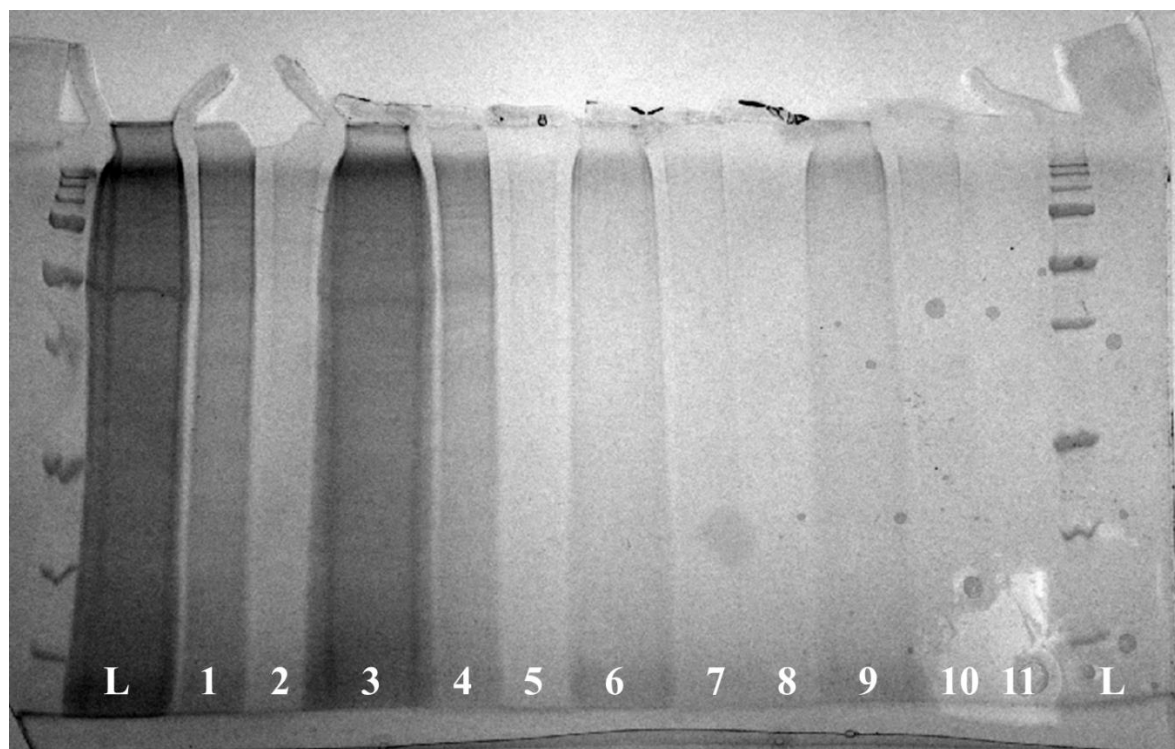
Figure A.7 SDS-PAGE results from cucumber protein extracted with phenol method. L = ladder, 1 = 2x diluted raw cucumber, 2 = 10x diluted raw cucumber, 3 = 100x diluted raw cucumber, 4 = 2x diluted fermented cucumber, 5 = 10x diluted fermented cucumber, 6 = 100x diluted fermented cucumber, 7 = 2x diluted raw cucumber with 10 kDa filtration, 8 = 10x diluted raw cucumber with 10 kDa filtration, 9 = 100 x diluted raw cucumber with 10 kDa filtration, 10 = 2x diluted fermented cucumber with 10 kDa filtration, 11 = 10x diluted fermented cucumber with 10 kDa filtration, 12 = 100 x diluted fermented cucumber with 10 kDa filtration

- Significant streaking occurred when performing SDS-PAGE with phenol-extracted cucumber protein pellets, suggesting that the protein was not adequately separated from interfering compounds. The phenol extraction method was not successful in preparing pure cucumber proteins for analytical methods.

A.3.4 Comparison between BCA and Bradford protein assays used for cucumber protein quantification

While seemingly accurate and repeatable, the BCA assay was lengthy and utilized extensive amounts of reagent and standard. A limited number of samples can be run using the test tube BCA assay and only 12-14 prepared at once because they must be run within a 10 minute window. The BCA microplate method utilized far less materials but could not achieve reproducible results. Reproducibility for cucumber technical replicates was very good ($< 4\%$) but there was unexplained variation between well replicates and between the well replicates and their respective technical replicates (i.e., 10000 µg/ml measured for tech rep A, and when that same sample was pipetted into four more wells their average was 8500ug/ml). The Bradford assay's standard curves were prepared and fit with linear trend lines ($R^2 > 0.998$) and reproducibility between standard replicates was achieved with RSD < 4% for all dilution levels. However, absorbance values for cucumber samples were not reproducible after correction for the reagent's absorbance (RSD > 10%). This variation is likely due to the fact that absorbances were similar to that of the reagent blank and, when corrected, the differences were exacerbated.

The BCA assay was able to quantify protein in some samples near the USDA reported value of 6500 µg/mL (USDA, 2016), but the results are questionable due to the significant matrix interactions observed. The level of protein estimated in cucumbers using the Bradford assay was < 600 µg/mL which is less than 10% of the USDA estimate of 6500 µg/mL. Conversely, the Contrarily, 80% recovery of BSA was achieved in spiked samples which indicates the availability of protein in this matrix to bind with the Coomassie blue dye. Mayer, Schick & Setchell (1986) found that an average of 80% recovery (70-90% range) was achievable in marine sediment samples spiked with casein while Jones, Hare & Compton (1989) observed 98% recovery of RUDP in plant tissues and Crossman, Clements & Cooper (2000) reported very low recovery of BSA from macroalgae. With regards to matrix interactions, both the BCA and Bradford assays had significant interactions. Protein values differed significantly between brined

and non-brined samples and both spiked and non-spiked versions. BSA spiking was unsuccessful (28% to 134% recovery – not reproducible or logical) for the BCA assay. Similar results were observed in brined and non-brined samples indicating that the brine is not the only influential factor. There may be an interaction between the BCA working reagent, BSA, brine and cucumber matrix. Recovery of spiked protein in the Bradford assay varied greatly between the brine and non-brine cucumber slurries. Recovery was very poor ($<10\%$) in the brined samples and acceptable in PBS samples ($>80\%$, consistent with literature) which suggests PBS may not interfere with the recovery of BSA while mock brine does. This interaction may not explain lack of cucumber protein recovery, rather we hypothesized that inadequate protein extraction and solubility may explain it. It is possible that the protein was not extracted from cucumber tissue with our current method, resulting in low protein levels in the Bradford assay. BCA-interacting substances such as amino acids and peptides may have provided the reactive groups necessary for color development in that assay and therefore reported falsely elevated levels of protein. An attempt to optimize protein extraction using the phenol-based method was unsuccessful in preparing a purified protein sample from either raw or fermented cucumbers.

A.4 Conclusion

The BCA and Bradford assays were not successful in quantifying total protein in fermented or fresh cucumber. Significant interactions with cucumber brine components and low reproducibility of technical replicates were evident. These methods are not recommended for use with cucumber slurries or freeze-dried cucumber powders without further optimization. Additionally, phenol extraction of cucumber protein did not achieve purified protein fractions from raw or fermented cucumbers and should not be used for this purpose.

A.5 References

Crossman DJ, Clements KD, Cooper GJS. 2000. Determination of protein for studies of marine herbivory: a comparison of methods. J.Exp.Mar.Biol.Ecol. 244(1):45-65.

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Mayer L, Schick L, Setchell F. 1986. Measurement of protein in nearshore marine sediments. Marine Ecology 30(2-3):159-65.

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Appendix B: Standard operating procedure: Liquid Chromatography Mass Spectrometry

(LC-MS) Method for Peptide Analysis in Fresh, Acidified, and Fermented Cucumbers

B.1. Process Fundamentals

Free amino acid separation is achieved using a reverse phase column. Reverse phase columns have a nonpolar stationary phase and therefore are excellent at separating compounds with nonpolar. Gradient elution beginning with high aqueous phase, allowing for polar compounds to elute first while retaining nonpolar compounds on the column. The latter are eluted as the percentage of organic phase increases.

B.2. Materials
- LC-MS grade methanol
- LC-MS grade formic acid
- LC-MS grade water
- Liquid nitrogen
- Mock brine with components at levels that will equilibrate to the same salt and acid levels of finished fermented and/or acidified cucumbers (only necessary if processing fresh cucumber samples that have not had any brine added to them, "raw" or "fresh" samples).
- Fermented, acidified, and/or raw cucumber slurries frozen at -80°C (3g for each sample)
- Mobile phase A (0.2% Formic Acid in water)
- Mobile phase B (0.2% Formic Acid in methanol)
- Non-labeled standards: mixtures for 5 levels (Table A1)

Table B.1. Peptide standard mixtures

Peptide	Manufacturer	Cat. Number	Concentration in LC-MS vial (nM)				
			Level 1	Level 2	Level 3	Level 4	Level 5
Arginine-tyrosine (RY)	Bachem	G-1535.0001	0.0007	0.0003	0.0007	0.0015	0.0030
Isoleucine-proline-proline (IPP)	Bachem	H-4632.0250	0.0002	0.0003	0.0008	0.0015	0.0031
Leucine-proline-proline (LPP)	Bachem	H-6226.0250	0.0002	0.0003	0.0008	0.0015	0.0031
Lysine-proline (KP)	Bachem	G-4190.0050	0.0010	0.0021	0.0041	0.0103	0.0206
Valine-proline-proline (VPP)	Bachem	H-4634.0250	0.0002	0.0003	0.0008	0.0016	0.0032

B.3. Equipment

- Shimadzu LCMS-8040 triple quadrupole LC-MS/MS
- LC-MS grade volumetric flasks of various sizes
    - "LC-MS grade" indicates glassware that has been cleaned appropriately for use with LC-MS grade solvents. Cleaning for LC-MS grade: wash all glassware 10 x with LC-grade water, 10x with LC-grade methanol, and 10x with LC-grade water again.
- Retsch Mill with 2 x 25 mL capsule, 6 x 12 mm balls. Alternate: 5mL capsules with 2 x 9 mm beads
- Analytical balance to 4 decimal places
- Microcentrifuge
- Liquid nitrogen, dewar, tongs, and cryogenic protective gloves
- DB biphenyl column (100 × 2.1 mm, 1.9 μm) (Restek, Bellefonte, PA, USA)
- Pipettes (10-1000 μL) and pipet tips
- Microcentrifuge tubes, screw-top
- Microcentrifuge tubes, pop-top
- 3 kDa centrifugal filters and tubes (Amicon Ultra, Ultracel-3 regenerated cellulose membrane, 0.5 mL sample volume)

B.4. Safety Information & Hazard Warnings

- Chemical contact
  - Hazards – see individual Safety Data Sheets for each chemical used.
    - Methanol
    - Formic acid
  - Personal Protective Equipment (PPE)
    - Safety glasses
    - Gloves
    - Lab coat
  - Storage – see individual Safety Data Sheets for each chemical used.
  - Spill Measures – see individual Safety Data Sheets for each chemical used.
  - First Aid Measures – see individual Safety Data Sheets for each chemical used.
- Liquid nitrogen
  - Hazards
    - Severe frost bite. Liquid nitrogen is -196°C (-320°F) at atmospheric pressure.
    - Explosion due to use of liquid nitrogen in a sealed container.
    - Explosion due to use of pressurized gas tanks.
    - Displacement of atmospheric oxygen if used in a non-ventilated space.
  - Personal Protective Equipment (PPE)
    - Safety glasses
    - Face shield
    - Cryogenic apron
    - Cryogenic gloves
      - Should be insulated, loose-fitting, and easily removable in case liquid is spilled on them. These gloves are *not* intended for submersion in liquid nitrogen; they provide short-term protection during accidental contact.
    - Lab coat
  - Storage

- Nitrogen tanks should be stored similar to all gas cylinders (i.e., out of direct sunlight)
- Liquid nitrogen should be transferred from the tank into an acceptable dewar and never into a sealed container. Trapping nitrogen in a sealed container will result in pressure buildup due to sublimation.
  - Spill Measures – see individual Safety Data Sheets for each chemical used.
  - First Aid Measures
    - Suffocation
      - Acute symptoms: dizziness, loss of consciousness
      - Action: move person to well-ventilated area immediately. If breathing has stopped, apply artificial respiration. If breathing is difficult, give oxygen. Call a physician. Keep warm and at rest. (https://engineering.dartmouth.edu/microeng/ln2.html)
    - Frost bite
      - Symptoms: cold skin, prickling feeling, numbness. Red, white, bluish-white or grayish-yellow skin; hard or waxy-looking skin. Clumsiness due to joint and muscle stiffness. (https://www.mayoclinic.org/diseases-conditions/frostbite/symptoms-causes/syc-20372656)
      - Action: restore tissue to normal body temperature, 98.6°F (37°C), followed by protection of the injured tissue from further damage and infection. Remove or loosen clothing that may constrict blood circulation to the frozen area. Call a physician. Rapid warming of the affected part is best achieved by using water at 106°F (42°C). Under no circumstances should the water be over 112°F (44°C), nor should the frozen part be rubbed either before or after rewarming. The patient should neither smoke, nor drink alcohol. (https://engineering.dartmouth.edu/microeng/ln2.html)
- High voltage on electrospray ionization housing
  - Hazards
    - Electric shock

- Personal Protective Equipment (PPE) - N/A

- Storage - N/A

- Spill Measures - N/A

- First Aid Measures

    - Acute Symptoms

        - Pain

            - Actions: remove effected person from the area and monitor for side effects or any symptoms not related to pain.

- Compressed gas cylinder (nitrogen and helium)

    - Hazards

        - Explosion

    - Personal Protective Equipment (PPE)

        - Safety glasses

        - Gloves

        - Lab coat

    - Storage

        - All compressed gas cylinders should be chained to the wall individually

        - Regulators should be installed correctly and functioning without leaks

    - Spill Measures - N/A

    - First Aid Measures - N/A

B.5. Training Requirements

- Compressed gas safety training.

- Retsch Mixer Mill training.

- Training by Suzanne Johanningsmeier or Rong Reynolds for specific LC-MS procedures

B.6. Procedure

Mobile Phase Preparation

1. Follow proper safety procedures for each solvent or compound used and use the appropriate PPE (glasses, lab coat, gloves, mask if needed).

2. Prepare mobile phases in the fume hood when appropriate.

3. Use only LC-MS grade glassware (beakers, volumetric flasks, glass bottles) when preparing and storing solutions. This glassware is kept in the acids cabinet under the hood and marked as "LC-MS grade". If you are unsure if your glassware has been cleaned and prepared for LC-MS work, perform proper cleaning on it:

   a. Wash all glassware 10 x with water, 10x with LC-grade methanol, and 10x with LC-grade water again.

   b. Volumetric glassware should only be used to make the solutions labeled on the glassware (i.e., "Mobile Phase A 95% Acetonitrile, 5% water)

   c. Beakers are labeled for either LC-grade water or LC-grade organic (methanol or acetonitrile) based on which method is currently being used on the LC-MS.

4. Prepare solutions in volumetric glassware. It is acceptable to use beakers for measuring into volumetric flasks, but graduated cylinders and beakers should never be used for volumetric measurements.

5. Use only LC-MS grade solvents for LC-MS mobile phases. Solvent bottles will indicate LC-MS grade.

6. Pour solutions from volumetric glassware into LC-grade screw-top glass bottles. Bottles may be reused without rinsing if they contained the same solution prior to emptying (i.e., use the same bottle for Mobile Phase A as yesterday, last week, etc.). If a new solution must be placed in a bottle that contained a different solution it must be rinsed as indicated above with water, methanol, and water again.

7. All solutions must be labeled with the full name of the contents ("methanol", not "MeOH"), health risk stickers, date, and initials of the preparer.

8. After preparation, all mobile phases must be sonicated for at least 20 minutes to remove air bubbles from solution. There is no need to vent the bottles as sonication will not cause pressure buildup.

9. Sonicated solutions are typically used right away, however if they are prepared in advance they may be stored in the appropriate cabinet. Fresh mobile phases should be made every 48 hours.

    a. Organic solutions (containing > 50% methanol or acetonitrile) should be stored in the FLAME CABINET

    b. Aqueous solutions (containing < 50% organic) should be stored in the secondary containment bin in the ACIDS CABINET.

Mobile phases:

Mobile Phase A: 0.2% formic acid in water

Mobile Phase B: 0.2% formic acid in methanol

Materials:

- LC-MS grade water
- LC-MS grade methanol
- LC-MS grade formic acid (liquid)
- (2) 250 mL volumetric flask
- (2) 500 ml glass bottles

1. Prepare Mobile Phase A

    a. Fill a 250 mL volumetric flask half way with water.

    b. Pipette 500 µL formic acid into the flask and swirl to mix.

    c. Add water to volume. You may pour directly from bottle, and when close to the meniscus, use a LC-MS grade beaker and/or transfer pipette to finish.

2. Prepare Mobile Phase B

    a. Fill a 250 mL volumetric flask half way with methanol.

    b. Pipette 500 µL formic acid into the flask and swirl to mix.

    c. Add methanol to volume. You may pour directly from bottle, and when close to the meniscus, use a LC-MS grade beaker and/or transfer pipette to finish.

Table B.2. Sample preparation of cucumber slurries for peptide analysis

Step:	Protocol:
1	Thaw raw and fermented cucumber slurries
2	*For raw cucumber samples:* Mix mock brine; equilibrate with raw cucumber at same ratio as fermented cucumbers for at least 2 hours or overnight at 4C (i.e., 11g raw cucumber + 9g brine = 55:45)
3	Aliquot 2.5 to 3.0 g of sample into each Retsch mill capsule and add 3 x 12mm beads. Ensure that both capsules contain the same weight of sample for each round of grinding.
4	Fill dewar 1/3 to 1/2 way with liquid nitrogen. Using cryogenic gloves and tongs, place both capsules into the dewar and freeze for 30 seconds.
5	Place frozen capsules in Retsch mill and grind at 30 Hz for 4 minutes (240 seconds)
6	Pour ground samples into pop-top microcentrifuge tubes: Open small size (cap) 1st and scrape into tube with scoopula or a gloved finger. Add beads to the small side (may remove them with tweezers) and scrape/pour the material from the larger side into the tube.
7	Centrifuge immediately or store tubes at 4C until centrifugation
8	Centrifuge samples (11,000 rpm for 5 minutes at 4°C)
9	Aliquot 500 µL of supernatant into 3 kDa centrifugal filters
10	Centrifuge filters (12,000 rpm for 15 minutes at 4°C)
11	Run samples on LC-MS and quantify peptides

Table B.3. Sample preparation of brine for peptide analysis

Step:	Protocol:
1	Centrifuge samples (11,000 rpm for 5 minutes at 4°C)
2	Aliquot 500 µL of supernatant into 3 kDa centrifugal filters
3	Centrifuge filters (12,000 rpm for 15 minutes at 4°C)
4	Run samples on LC-MS and quantify peptides

Standard preparation

Five levels of standards are prepared for peptide quantification. See table in Materials section for manufacturer name and catalog number. These mixtures should be made from individual peptide standards made in volumetric flasks in 0.1 % formic acid. Standards should be prepared every 48 hours of analysis. Here is an example of how to prepare standards:

Table B.4. Standard preparation for 1 mL each of five levels of peptide mixtures

	Component	Stock Concentration (nM)	Volume added to mix (µL)	Concentration in LC-MS vial (nM)
Level 1	IPP	0.0025	62.1	0.0002
	VPP	0.0089	18.0	0.0002
	KP	0.0055	185.3	0.0010
	RY	0.0063	112.2	0.0007
	LPP	0.0014	112.2	0.0002
	Formic acid	0.2%	510.2	N/A
Level 2	IPP	0.0025	124.1	0.0003
	VPP	0.0089	36.1	0.0003
	KP	0.0277	74.1	0.0021
	RY	0.0063	46.9	0.0003
	LPP	0.0014	224.4	0.0003
	Formic acid	0.2%	494.3	N/A
Level 3	IPP	0.0025	31.0	0.0003
	VPP	0.0089	90.2	0.0003
	KP	0.0277	148.2	0.0021
	RY	0.0063	117.3	0.0003
	LPP	0.0014	561.0	0.0003
	Formic acid	0.2%	52.3	N/A
Level 4	IPP	0.0025	620.7	0.0015
	VPP	0.0668	24.0	0.0016
	KP	0.5550	18.5	0.0103
	RY	0.0474	31.3	0.0015
	LPP	0.0411	37.4	0.0015
	Formic acid	0.2%	305.4	N/A
Level 5	IPP	0.0025	124.1	0.0015
	VPP	0.0668	48.1	0.0016
	KP	0.5550	37.1	0.0103
	RY	0.0474	62.5	0.0015
	LPP	0.0411	74.8	0.0015
	Formic acid	0.2%	728.2	N/A

LCMS Running Parameters

LC-MS running parameters are as follows per Fideler et al., 2019.

- Column temperature: 45°C

- Flow rate: 0.3 mL/min

- Segmented gradient:

    o 0-25% B (0-3 min)

    o 25-30% B (3-5 min)

    o 30-35% B (5-8 min)

    o 35-0 % B (8-8.1 min)

    o 0% B (8.1-10.1 min)

- The first 1.25 min of flow is diverted to waste to reduce salt accumulation on the ion source.

- Nebulizing and drying gas (nitrogen) flow rates were 3 L/min and 15 L/min, respectively.

- Heat block temperature: 400 °C.

- Loop time: of 1 s

- Q1 resolution: 0.7 Da

- Q3 resolution: 0.7 Da

- Injection volume for all standards and unknowns is 1 µL

Table B.5. Peptide transition and quantification information

Peptide (precursor m/z)	Retention Time (min)	Product Ion	Reference Ions
IPP [M+H]$^+$ 326.15 m/z	7.29	213.10 (-17.0 CE)	116.1 (-26.0 CE) 183.15 (-17.0 CE)
LPP [M+H]$^+$ 326.15 m/z	7.79	213.10 (-17.0 CE)	116.1 (-26.0 CE) 183.15 (-17.0 CE)
KP [M+H]$^+$ 244.20 m/z	1.84	84.05 (-22.0 CE)	116.05 (-26.0 CE) 129.20 (-13.0 CE)
RY [M+H]$^+$ 338.15 m/z	3.05	175.10 (-21.0 CE)	112.05 (-27.0 CE)
VPP [M+H]$^+$ 312.15 m/z	6.24	197.15 (-13.0 CE)	169.10 (-17.0 CE)

B.7. Quality Assurance

- Ensure that standard curves have an r^2 value of at least 0.98 for quantification. If values drop below 0.98 there may be instrumental issues (i.e., clogged desolvation line).

- During data analysis verify that for each target compound peak, the corresponding ISTD peak is accurately chosen and integrated.

B.8. Storage Requirements

- Once prepared, samples should be stored at 4°C and analyzed within 24 hours.

- Mobile phases should be made fresh every 48 hours

- Standards should be prepared fresh every 48 hours.

B.9. Waste Disposal

Waste from the LCMS should be disposed of according to the SDS for each chemical. Almost always, waste will be submitted as Unwanted Materials according to North Carolina State University's (NCSU) Environmental Health and Safety (EHS). Liquids that are purged or diverted away from the MS will collect below the LCMS in a bottle with a cap that allows tubing through. Keep this bottle capped to avoid the release of harmful vapors from organic solvents. Sample vials containing organic solvents should also be submitted through NCSU EHS's Unwanted Materials portal.

B.10. References

1. Fideler J, Johanningsmeier SD, Ekelöf M, Muddiman DC. 2019. Discovery and quantification of bioactive peptides in fermented cucumber by direct analysis IR-MALDESI mass spectrometry and LC-QQQ-MS. Food Chem. 271715-23.

Appendix C: Liquid Chromatography Mass Spectrometry (LC-MS) Method for Free Amino Acid Analysis in Fresh, Acidified, and Fermented Cucumbers.

C.1. Process Fundamentals

Free amino acid separation is achieved using a normal phase hydrophilic interaction liquid chromatography (HILIC) column. HILIC columns have a polar stationary phase and therefore are excellent at separating compounds that are too polar to be retained on traditional reversed-phase approaches. Gradient elution beginning with high organic mobile phase (>80% acetonitrile) allows non-polar compounds to elute first while retaining polar compounds on the column. The latter are eluted as the percentage of aqueous phase increases.

C.2. Materials

- LC-MS grade acetonitrile
- LC-MS grade formic acid
- Ammonium formate
- LC-MS grade water
- Liquid nitrogen
- Mock brine with components at levels that will equilibrate to the same salt and acid levels of finished fermented and/or acidified cucumbers (only necessary if processing fresh cucumber samples that have not had any brine added to them, "raw" or "fresh" samples).
- Fermented, acidified, and/or raw cucumber slurries frozen at -80°C (3g for each sample)
- Mobile phase A (0.15% Formic Acid, 10mM Ammonium Formate, 85% Acetonitrile)
- Mobile phase B (0.15% Formic Acid, 10mM Ammonium Formate in water)
- Stable Isotope Labeled standards (SIL) – see table below
- Non-labeled standards: mixtures for 8 levels – see table below

Table C.1 Amino acid standard manufacturing information

Amino Acid	Abbreviation	Manufacturer	Catalog Number
α-ketoglutarate	2KG	Aldrich	75890
L-α-Aminoadipic Acid	AAA	Sigma	A0637
Alanine	Ala	MP Biomedicals	100423
Arginine	Arg	MP Biomedicals	100743
Asparagine	Asn	MP Biomedicals	100794
Aspartate	Asp (ASX)	MP Biomedicals	100803
L-Citrulline	Cit	Sigma	C7629
γ-Amino-n-butyric Acid	GABA	Sigma	a5835
Glutamine	Gln	Sigma	G3202
Glutamate	Glu (GLX)	Sigma	49449
Glycine	Gly	MP Biomedicals	100570
Histidine	His	MP Biomedicals	101957
Isoleucine	Ile	MP Biomedicals	102082
Leucine	Leu	MP Biomedicals	102154
Lysine	Lys	MP Biomedicals	102214
Methionine	Met	MP Biomedicals	102281
L-Ornithine	Orn	Sigma	W419001
Pyroglutamic acid	pGlu	Aldrich	83160
Phenylalanine	Phe	MP Biomedicals	194723
Proline	Pro	MP Biomedicals	102730
Serine	Ser	MP Biomedicals	102868
Threonine	Thr	MP Biomedicals	103050
Tryptophan	Trp	Sigma	93659
Tyrosine	Tyr	Acros	AC140641000
Valine	Val	MP Biomedicals	103229
β-Alanine	β-Ala	Sigma	146064

Table C.2 Amino acid internal standard manufacturing information

Amino Acid	Three letter code	Manufacturer	Catalog Number	μM std. in vial
Alanine (IS)	Ala SIL	Sigma	767964	28.26
Arginine (IS)	Arg SIL	Sigma	767965	9.29
Asparagine (IS)	Asn SIL	Sigma	767966	7.35
Aspartate (IS)	Asp SIL	Sigma	767967	22.45
Cysteine (IS)	Cys SIL	Sigma	767968	7.74
Glutamate (IS)	Glu SIL	Sigma	767969	18.19
Glutamine (IS)	Gln SIL	Sigma	767970	7.74
Glycine (IS)	Gly SIL	Sigma	767971	20.90
Histidine (IS)	His SIL	Sigma	767972	1.94
Isoleucine (IS)	Ile SIL	Sigma	767973	9.29
Leucine (IS)	Leu SIL	Sigma	767974	19.74
Lysine (IS)	Lys SIL	Sigma	767975	8.13
Methionine (IS)	Met SIL	Sigma	767976	3.87
Phenylalanine (IS)	Phe SIL	Sigma	767977	7.35
Proline (IS)	Pro SIL	Sigma	767978	8.13
Serine (IS)	Ser SIL	Sigma	767979	12.00
Threonine (IS)	Thr SIL	Sigma	767980	12.39
Tryptophan (IS)	Trp SIL	Sigma	767981	0.77
Tyrosine (IS)	Tyr SIL	Sigma	767982	5.81
Valine (IS)	Val SIL	Sigma	767983	12.39
2-ketoglutaric acid, d_6	2KG SIL	Aldrich	615390	32.49
4-aminobutyric acid-2,2,3,3,4,4-d_6	GABA SIL	Sigma	615587	23.35
L-Citrulline-4,4,5,5-d_4	Cit SIL	Sigma	578886	15.72
L-Ornithine-$^{13}C_5$ hydrochloride	Orn SIL	Sigma	736147	22.88

Note: all standards marked "(IS)" are universally labeled on C and N

C.3. Equipment

- Shimadzu LCMS-8040 triple quadrupole LC-MS/MS
- LC-MS grade volumetric flasks of various sizes
  - "LC-MS grade" indicates glassware that has been cleaned appropriately for use with LC-MS grade solvents. Cleaning for LC-MS grade: wash all glassware 10 x with LC-grade water, 10x with LC-grade methanol, and 10x with LC-grade water again.
- Retsch Mill with 2 x 25 mL capsule, 6 x 12 mm balls. Alternate: 5mL capsules with 2 x 9 mm beads
- Analytical balance to 4 decimal places
- Microcentrifuge

- Liquid nitrogen, dewar, tongs, and cryogenic protective gloves

- Atlantis HILIC Silica Column, 3 µm 4.6 x 100mm, P/N 186002029

- Pipettes (10-1000 µL) and pipet tips

- Microcentrifuge tubes, screw-top

- Microcentrifuge tubes, pop-top

- 10 kDa centrifugal filters and tubes (Amicon Ultra, Ultracel-10 regenerated cellulose membrane, 0.5 mL sample volume)

C.4. Safety Information & Hazard Warnings

- Chemical contact
    - Hazards – see individual Safety Data Sheets for each chemical used.
        - Acetonitrile
        - Ammonium formate
        - Formic acid
    - Personal Protective Equipment (PPE)
        - Safety glasses
        - Gloves
        - Lab coat
    - Storage – see individual Safety Data Sheets for each chemical used.
    - Spill Measures – see individual Safety Data Sheets for each chemical used.
    - First Aid Measures – see individual Safety Data Sheets for each chemical used.
- Liquid nitrogen
    - Hazards
        - Severe frost bite. Liquid nitrogen is -196°C (-320°F) at atmospheric pressure.
        - Explosion due to use of liquid nitrogen in a sealed container.
        - Explosion due to use of pressurized gas tanks.
        - Displacement of atmospheric oxygen if used in a non-ventilated space.
    - Personal Protective Equipment (PPE)
        - Safety glasses

- Face shield
- Cryogenic apron
- Cryogenic gloves
  - Should be insulated, loose-fitting, and easily removable in case liquid is spilled on them. These gloves are *not* intended for submersion in liquid nitrogen; they provide short-term protection during accidental contact.
- Lab coat
- Storage
  - Nitrogen tanks should be stored similar to all gas cylinders (i.e., out of direct sunlight)
  - Liquid nitrogen should be transferred from the tank into an acceptable dewar and never into a sealed container. Trapping nitrogen in a sealed container will result in pressure buildup due to sublimation.
- Spill Measures – see individual Safety Data Sheets for each chemical used.
- First Aid Measures
  - Suffocation
    - Acute symptoms: dizziness, loss of consciousness
    - Action: move person to well-ventilated area immediately. If breathing has stopped, apply artificial respiration. If breathing is difficult, give oxygen. Call a physician. Keep warm and at rest. (https://engineering.dartmouth.edu/microeng/ln2.html)
  - Frost bite
    - Symptoms: cold skin, prickling feeling, numbness. Red, white, bluish-white or grayish-yellow skin; hard or waxy-looking skin. Clumsiness due to joint and muscle stiffness. (https://www.mayoclinic.org/diseases-conditions/frostbite/symptoms-causes/syc-20372656)
    - Action: restore tissue to normal body temperature, 98.6°F (37°C), followed by protection of the injured tissue from further damage and infection. Remove or loosen clothing that may constrict blood

circulation to the frozen area. Call a physician. Rapid warming of the affected part is best achieved by using water at 106°F (42°C). Under no circumstances should the water be over 112°F (44°C), nor should the frozen part be rubbed either before or after rewarming. The patient should neither smoke, nor drink alcohol. (https://engineering.dartmouth.edu/microeng/ln2.html)

- High voltage on electrospray ionization housing
  - Hazards
    - Electric shock
  - Personal Protective Equipment (PPE) - N/A
  - Storage - N/A
  - Spill Measures - N/A
  - First Aid Measures
    - Acute Symptoms
      - Pain
        - Actions: remove effected person from the area and monitor for side effects or any symptoms not related to pain.
- Compressed gas cylinder (nitrogen and helium)
  - Hazards
    - Explosion
  - Personal Protective Equipment (PPE)
    - Safety glasses
    - Gloves
    - Lab coat
  - Storage
    - All compressed gas cylinders should be chained to the wall individually
    - Regulators should be installed correctly and functioning without leaks
  - Spill Measures - N/A
  - First Aid Measures - N/A

C.5. Training Requirements

- Compressed gas safety training.
- Retsch Mixer Mill training.
- Training by Suzanne Johanningsmeier or Rong Reynolds for specific LC-MS procedures

C.6. Procedure

Mobile Phase Preparation

10. Follow proper safety procedures for each solvent or compound used and use the appropriate PPE (glasses, lab coat, gloves, mask if needed).
11. Prepare mobile phases in the fume hood when appropriate.
12. Use only LC-MS grade glassware (beakers, volumetric flasks, glass bottles) when preparing and storing solutions. This glassware is kept in the acids cabinet under the hood and marked as "LC-MS grade". If you are unsure if your glassware has been cleaned and prepared for LC-MS work, perform proper cleaning on it:
    a. Wash all glassware 10 x with water, 10x with LC-grade methanol, and 10x with LC-grade water again.
    b. Volumetric glassware should only be used to make the solutions labeled on the glassware (i.e., "Mobile Phase A 95% Acetonitrile, 5% water)
    c. Beakers are labeled for either LC-grade water or LC-grade organic (methanol or acetonitrile) based on which method is currently being used on the LC-MS.
13. Prepare solutions in volumetric glassware. It is acceptable to use beakers for measuring into volumetric flasks, but graduated cylinders and beakers should never be used for volumetric measurements.
14. Use only LC-MS grade solvents for LC-MS mobile phases. Solvent bottles will indicate LC-MS grade.
15. Pour solutions from volumetric glassware into LC-grade screw-top glass bottles. Bottles may be reused without rinsing if they contained the same solution prior to emptying (i.e., use the same bottle for Mobile Phase A as yesterday, last week, etc.). If a new solution

must be placed in a bottle that contained a different solution it must be rinsed as indicated above with water, methanol, and water again.

16. All solutions must be labeled with the full name of the contents ("acetonitrile", not "ACN"), health risk stickers, date, and initials of the preparer.

17. After preparation, all mobile phases must be sonicated for at least 20 minutes to remove air bubbles from solution. There is no need to vent the bottles as sonication will not cause pressure buildup.

18. Sonicated solutions are typically used right away, however if they are prepared in advance they may be stored in the appropriate cabinet. Fresh mobile phases should be made every 48 hours.

    a. Organic solutions (containing > 50% methanol or acetonitrile) should be stored in the FLAME CABINET

    b. Aqueous solutions (containing < 50% organic) should be stored in the secondary containment bin in the ACIDS CABINET.

Mobile phases for HILIC column (amino acid analysis method):

Mobile Phase A: 85% acetonitrile with 10 mM ammonium formate and 0.15% formic acid

Mobile Phase B: 10 mM ammonium formate and 0.15% formic acid

Materials:

- LC-MS grade water
- LC-MS grade acetonitrile
- LC-MS grade formic acid (liquid)
- Ammonium formate (solid)
- (3) 250 mL volumetric flask
- (1) 250 mL glass bottle
- (2) 500 ml glass bottles

3. Prepare 67 mM ammonium formate in water. When mixed into these solutions it will be diluted down to 10 mM.

a. Add 1.056 g ammonium formate to a 250 mL volumetric flask and fill to volume with water.

b. Cap flask and invert to mix until crystals are no longer visible.

c. Pour into 250 mL glass bottle and screw on cap.

4. Prepare Mobile Phase A

a. Add 37.5 mL of 67 mM ammonium formate to a 250 mL volumetric flask. Use a 5 mL pipette to do so (7 aliquots of 5 mL + 1 aliquot of 2.5 mL)

b. Pipette 375 µL of formic acid into the volumetric flask and swirl to mix.

c. Add acetonitrile to volume. You may pour directly from bottle, and when close to the meniscus, use a LC-MS grade beaker and/or transfer pipette to finish it.

d. For 500 mL flask:

    i. 75 mL ammonium formate (50 mL flask + 5 aliquots of 5 mL)

    ii. 750 µL formic acid

    iii. To volume with acetonitrile

5. Prepare Mobile Phase B

a. Add 37.5 mL of 67 mM ammonium formate to a 250 mL volumetric flask. Use a 5 mL pipette to do so (7 aliquots of 5 mL + 1 aliquot of 2.5 mL)

b. Add water to volume. You may pour directly from bottle, and when close to the meniscus, use a LC-MS grade beaker and/or transfer pipette to finish it.

c. For 100 mL flask:

    i. 15 mL ammonium formate (3 aliquots of 5 mL)

    ii. 150 µL formic acid

    iii. To volume with water

Table C.3 Sample preparation of cucumber slurries for amino acid analysis

Step:	Protocol:
1	Thaw raw and fermented cucumber slurries
2	*For raw cucumber samples:* Mix mock brine; equilibrate with raw cucumber at same ratio as fermented cucumbers for at least 2 hours or overnight at 4C (i.e., 11g raw cucumber + 9g brine = 55:45)
3	Aliquot 2.5 to 3.0 g of sample into each Retsch mill capsule and add 3 x 12mm beads. Ensure that both capsules contain the same weight of sample for each round of grinding.
4	Fill dewar 1/3 to 1/2 way with liquid nitrogen. Using cryogenic gloves and tongs, place both capsules into the dewar and freeze for 30 seconds.
5	Place frozen capsules in Retsch mill and grind at 30 Hz for 4 minutes (240 seconds)
6	Pour ground samples into pop-top microcentrifuge tubes: Open small size (cap) 1st and scrape into tube with scoopula or a gloved finger. Add beads to the small side (may remove them with tweezers) and scrape/pour the material from the larger side into the tube.
7	Store tubes at 4C until all samples have been ground
8	Centrifuge all samples (11,000 rpm for 5 minutes at 4°C)
9	Aliquot into a screw-top tube: 90 µL supernatant 565 µL Mobile Phase A (sample dilution factor = 7.2778)
10	Store tubes at 4C for 1 hour to crash out particulates.
11	Centrifuge all samples (11,000 rpm for 5 minutes at 4°C)
12	Aliquot 500 µL of supernatant into 10 kDa centrifugal filters
13	Centrifuge all filters (12,000 rpm for 10 minutes at 4°C) and retain all permeate (total needed: 368 µL)
14	Aliquot into an LC-MS vial: 10 µL internal standard 323 µL permeate (sample dilution factor = 1.0310)
15	Prepare each standard (levels 1-8) by adding predetermined volumes of standard mix, Asn, Gln, SIL mix, and Mobile Phase A.
16	Run samples on LC-MS and quantify all amino acids
17	FOR DILUTED SAMPLES (*if needed*): Aliquot into an LC-MS vial: 10 µL internal standard 45 µL of the permeate from Step 13 345 µL mock brine (added to account for salt and acid dilution) 233 µL Mobile Phase A (sample dilution factor = 7.400)
18	Run samples on LC-MS and quantify all amino acids
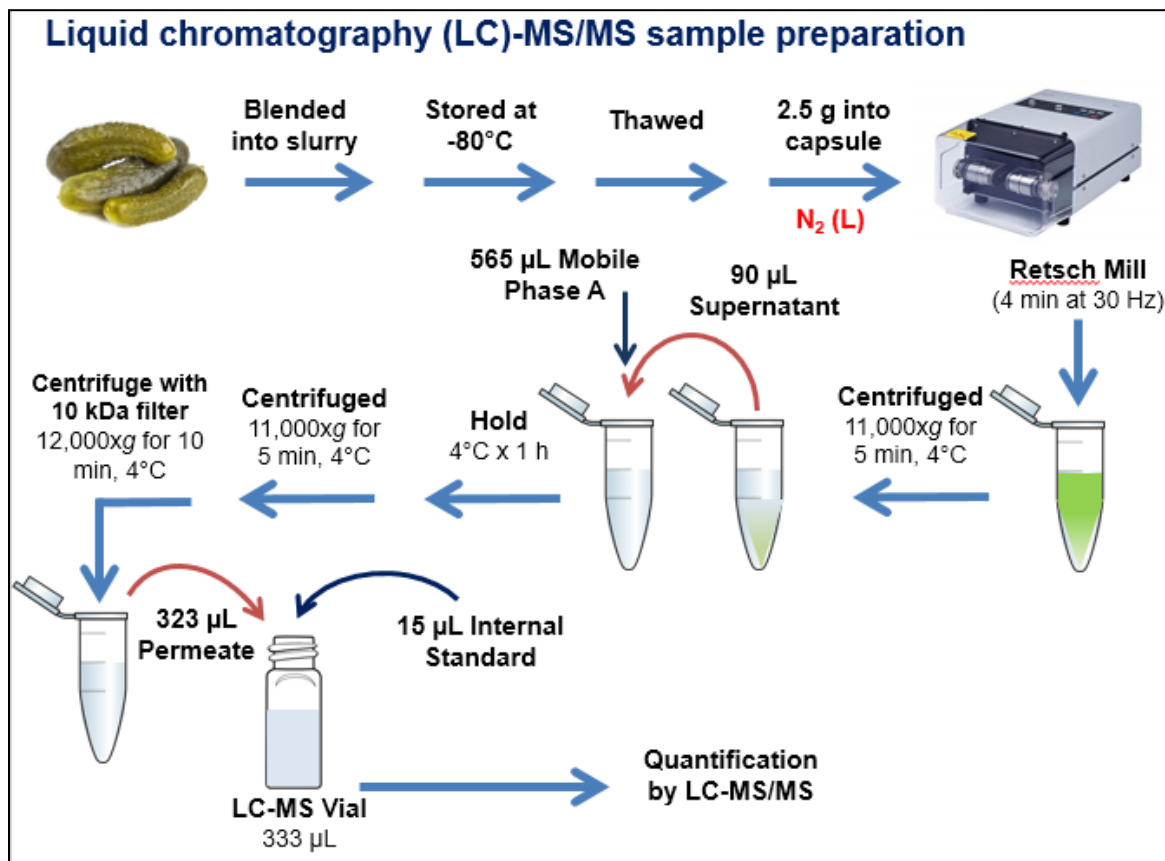
Figure C.1 Visual representation of cucumber slurry sample preparation for amino acid analysis

Table C.4 Sample preparation of brines for amino acid analysis

Step:	Protocol:
1	Thaw brines and vortex to mix.
2	Aliquot into a screw-top tube: 90 µL brine 565 µL Mobile Phase A (sample dilution factor = 7.2778)
3	Store tubes at 4C for 1 hour to crash out particulates.
4	Centrifuge all samples (11,000 rpm for 5 minutes at 4°C)
5	Aliquot 500 µL of supernatant into 10 kDa centrifugal filters
6	Centrifuge all filters (11,000 rpm for 10 minutes at 4°C) and retain all permeate (total needed: 368 µL)
7	Aliquot into an LC-MS vial: 10 µL internal standard 323 µL permeate (sample dilution factor = 1.0310)
8	Prepare each standard (levels 1-8) by adding predetermined volumes of standard mix, Asn, Gln, SIL mix, and Mobile Phase A (See "Standards 052019 v2" tab)
9	Run samples on LC-MS and quantify all amino acids
10	FOR DILUTED SAMPLES (*if needed*): Aliquot into an LC-MS vial: 10 µL internal standard 45 µL of the permeate from Step 13 345 µL mock brine (added to account for salt and acid dilution) 233 µL Mobile Phase A (sample DF = 7.400)
11	Run samples on LC-MS and quantify all amino acids

Standard preparation

Eight levels of standard are prepared for amino acid quantification. See table in Materials section for manufacturer name and catalog number. These mixtures should be made from individual amino acid standards made in volumetric flasks either in LC-MS grade water (asparagine and glutamine) or 0.1 N HCl (all other amino acids) and diluted to volume when necessary with 0.1 N HCl. Standard mixtures should be stored in amber glass bottles at < 0°C and thawed at refrigeration temperature when needed. Standards should be prepared every 48 hours of analysis.

Table C.5 Example standard preparation for amino acid analysis

Standard Level	Injection Volume (µL)	HPLC Vial Volume. (µL)	Mobile Phase A (µL)	Asparagine (µL)	Glutamine (µL)	Internal Standard Mix (µL)	Standard Mix Volume (µL)
1	2	1000	781.97	17.15	75.00	30.00	95.88
2	2	1000	755.53	68.59	50.00	30.00	95.88
3	2	1000	728.55	20.57	125.00	30.00	95.88
4	2	1000	786.41	37.72	50.00	30.00	95.88
5	2	1000	744.26	54.86	75.00	30.00	95.88
6	2	1000	702.12	72.00	100.00	30.00	95.88
7	2	1000	770.56	89.15	14.42	30.00	95.88
8	2.0	1000.0	751.10	106.29	16.73	30.00	95.88

- Each level of standard has a different amino acid standard mix added to it.
- Note that asparagine and glutamine (made in water) are not included in the amino acid standard mixtures as they will degrade in the presence of 0.1 N HCl.

LCMS Running Parameters

Separation is achieved with the following LC parameters per Qiu and others (2020) with slight adaptation:

- Column temperature: 37°C
- Mobile phase A: 85% acetonitrile with 10 mM ammonium formate and 0.15% formic acid.
- Mobile phase B: 10 mM ammonium formate and with 0.15% formic acid in water.
- Flow rate: 0.6 mL/min
- Segmented gradient:
  - 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)
- The first 2 min of flow is diverted to waste to reduce salt accumulation on the ion source.

- Nebulizing and drying gas (nitrogen) flow rates were 3 L/min and 15 L/min, respectively.

- Heat block temperature: 400 °C.

- Loop time: of 1 s

- Q1 resolution: 1.0 Da or 0.7 Da (glu and gln)

- Q3 resolution: 1.0 Da

- Injection volume for all standards and unknowns is 2 μL

Table C.6 Amino acid transitions and retention times

Amino Acid	Type	ISTD Group	Transition	Reference Ions	Ret. Time (min)
2KG	Target	23	144.95>101.10	145.05>57.10	4.812
2KG SIL	ISTD	23	148.95>105.00	149.15>61.15	0.001
AAA	Target	13	162.00>98.00	161.90>115.95	8.602
Ala	Target	22	90.05>44.05		8.566
Ala SIL	ISTD	1	95.10>46.85		0.001
Arg	Target	2	175.00>70.00	175.00>60.00	10.4
Arg SIL	ISTD	2	185.10>75.05	185.00>64.05	10.795
Asn	Target	3	133.10>74.15	133.00>87.00	8.539
Asn SIL	ISTD	3	139.10>77.10		8.767
Asp	Target	4	134.05>88.05	133.95>74.00	0.001
Asp SIL	ISTD	4	139.10>76.90		8.756
B-ala	Target	13	89.95>90.00	89.95>72.05	9.157
Cit	Target	21	176.00>70.05	176.00>113.00	9.37
Cit SIL	ISTD	21	180.10>74.10		8.773
Cys SIL	ISTD	5	127.15>79.05	125.95>61.00	0.001
GABA	Target	20	104.00>87.10	103.90>69.15	8.535
GABA SIL	ISTD	20	110.00>93.10		8.531
Gln	Target	6	147.20>84.05	147.30>56.10 147.30>42.05	8.875
Gln SIL	ISTD	6	154.10>89.00	153.90>107.00 153.90>60.10	8.902
Glu	Target	7	148.05>84.05	148.25>102.00 148.25>130.15	8.871
Glu SIL	ISTD	7	154.10>89.00		8.902
Gly	Target	22	76.05		8.803
His	Target	8	156.00>110.10	155.90>83.05	11.393
His SIL	ISTD	8	165.15>118.00	164.95>89.00	11.026
Table C.6 (cont.)					
Ile	Target	9	133.10>87.10	132.00>69.15 132.80>44.00	7.171

Ile SIL	ISTD	9	138.35>92.05	138.95>74.00	0.001
Leu	Target	10	132.00>86.15	131.90>44.00	7.466
Leu SIL	ISTD	10	139.15>92.05		7.514
Lys	Target	11	147.10>84.10	146.90>56.05	10.634
Lys SIL	ISTD	11	155.15>90.15	155.25>59.95	11.262
Met	Target	10	149.95>56.05	150.35>60.80	6.971
Met SIL	ISTD	12	155.00>63.25	155.30>76.70	0.001
Orn	Target	22	132.95>70.00		11.063
Orn SIL	ISTD	22	138.10>74.10		11.061
pGlu	Target	13	130.10>84.10	130.10>56.10	8.894
Phe	Target	13	166.05>120.10	165.95>102.95	6.801
Phe SIL	ISTD	13	176.15>129.15		6.801
Pro	Target	14	116.00>70.05	115.90>42.95	8.87
Pro SIL	ISTD	14	122.10>75.10		8.868
Ser	Target	13	106.00>60.00	105.90>88.00	0.001
Ser SIL	ISTD	15	110.00>63.20		0.001
Thr	Target	14	119.30>73.55	119.90>56.05	0.001
Thr SIL	ISTD	16	124.00>59.95	124.90>78.15	0.001
Trp	Target	17	205.10>146.05	204.90>118.00	5.874
Trp SIL	ISTD	17	218.10>156.05	217.90>127.00	6.038
Tyr	Target	18	182.00>136.05	182.00>90.90	7.084
Tyr SIL	ISTD	18	192.15>127.25		0.001
Val	Target	20	118.00>72.10	118.10>55.10 118.1000>57.05	7.899
Val SIL	ISTD	19	124.15>77.10	123.95>58.95	7.503

SIL = standard isotope labelled, ISTD = internal standard.

Table C.7 Amino acid quantification information for 2 µL injections.

Amino Acid	Concentration (µM)							
	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7	Level 8
2KG	0.1	100	500	1500	3000	4500	6000	7000
2KG SIL	16.2	16.2	16.2	16.2	16.2	16.2	16.2	16.2
AAA	0.2	0.7	3	10	15	20	30	45
Ala	15	45	90	150	200	250	300	350
Ala SIL	14.1	14.1	14.1	14.1	14.1	14.1	14.1	14.1
Arg	0.5	10	50	100	200	300	400	500
Arg SIL	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6
Asn	18.9	75.7	227.2	416.5	605.8	795.1	984.4	1173.8
Asn SIL	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7
Asp	0.4	1	2.5	5	10	15	20	25
Asp SIL	11.2	11.2	11.2	11.2	11.2	11.2	11.2	11.2

Table C.7 (cont.)

B-ala	2.5	25	50	75	100	125	150	200
Cit	0.5	10	50	100	200	300	400	500
Cit SIL	7.9	7.9	7.9	7.9	7.9	7.9	7.9	7.9
Cys SIL	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9
GABA	0.4	4	40	60	120	180	250	350
GABA SIL	13.8	13.8	13.8	13.8	13.8	13.8	13.8	13.8
Gln	1.5	100	250	500	750	1000	1250	1450
Gln SIL	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9
Glu	0.5	1	5	10	20	30	45	60
Glu SIL	9.1	9.1	9.1	9.1	9.1	9.1	9.1	9.1
Gly	0.4	4	40	60	120	180	250	350
His	0.6	2	5	10	35	50	70	95
His SIL	1	1	1	1	1	1	1	1
Ile	1.5	7	25	50	75	100	150	200
Ile SIL	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6
Leu	2	7	25	50	80	110	175	230
Leu SIL	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9
Lys	1.5	7	25	50	75	100	150	200
Lys SIL	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1
Met	0.6	2	5	10	35	50	70	95
Met SIL	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
Orn	0.5	10	50	100	200	300	400	500
Orn SIL	11.4	11.4	11.4	11.4	11.4	11.4	11.4	11.4
pGlu	1000	2000	3000	4000	5000	6000	7000	8000
Phe	0.6	2	5	10	35	50	75	110
Phe SIL	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7
Pro	0.2	1	5	10	40	80	110	140
Pro SIL	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1
Ser	0.9	3	10	35	50	75	110	130
Ser SIL	6	6	6	6	6	6	6	6
Thr	5	10	20	40	60	80	100	125
Thr SIL	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2
Trp	30	40	60	80	100	120	140	160
Trp SIL	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Tyr	0.8	2	5	15	25	35	50	70
Tyr SIL	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9
Val	10	100	500	1000	2500	4000	5000	6000
Val SIL	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2

SIL = standard isotope labelled, ISTD = internal standard.

C.7 Quality Assurance

- Internal standards (ISTD) are used for as many transitions as possible.

- Ensure that standard curves have an r^2 value of at least 0.98 for quantification. If values drop below 0.98 there may be instrumental issues (i.e., clogged desolvation line).
- During data analysis verify that for each target compound peak, the corresponding ISTD peak is accurately chosen and integrated.

C.8 Storage Requirements

- Once prepared, samples should be stored at 4°C and analyzed within 24 hours. Glutamine and asparagine begin to degrade in acidic conditions and quantification may be compromised with extended storage.
- Mobile phases should be made fresh every 48 hours
- Standards should be prepared fresh every 48 hours.
- When not using the HILIC column for more than 5 days, the column should be flushed with 80 volumes of 95% acetonitrile/5% water. Prior to beginning analysis, flush with 80 volumes mobile phase A.

C.9 Waste Disposal

Waste from the LCMS should be disposed of according to the SDS for each chemical. Almost always, waste will be submitted as Unwanted Materials according to North Carolina State University's (NCSU) Environmental Health and Safety (EHS). Liquids that are purged or diverted away from the MS will collect below the LCMS in a bottle with a cap that allows tubing through. Keep this bottle capped to avoid the release of harmful vapors from organic solvents. Sample vials containing organic solvents should also be submitted through NCSU EHS's Unwanted Materials portal.

C.10. References

1. Qiu, X., Reynolds, R., Johanningsmeier, S., & Truong, V. (2020). Determination of free amino acids in five commercial sweetpotato cultivars by hydrophilic interaction liquid chromatography-mass spectrometry. Journal of Food Composition and Analysis, 103522.

APPENDIX D: Appendix D: Development of Colorimetric Assays for Glutaminase and Glutamate Decarboxylase Activity in Lactic Acid Bacteria

D.1 Overview

Conversion of glutamate to γ-aminobutyric acid (GABA) by glutamate decarboxylase (GAD) during lactic acid fermentation of cucumbers has been demonstrated, with 1.21 ± 0.35 mM GABA produced in a typical natural fermentation with 4% NaCl brine (Moore, DuVivier & Johanningsmeier, 2021). Furthermore, the glutamate to GABA conversion rate during cucumber fermentation is approximately 100%. GABA production is limited by the amount of free glutamate available to lactic acid bacteria (LAB). Cucumbers are a significant source of glutamine (~10 mM, Moore, DuVivier & Johanningsmeier, *in preparation*) which can be converted to glutamate by glutaminase (GlsA or GlsB, represented by "GlsX"), however GlsX expression by LAB is strain specific. Inclusion of GlsX expressing LAB in cucumber fermentations may increase overall GABA content by providing additional glutamate for conversion to GABA.

Three culture collections are available within the North Carolina State University Department of Food, Bioprocessing and Nutrition Sciences for screening for GlsX expressing LAB. The first collection belongs to the United States Department of Agriculture Food Science Research Unit housed at NCSU and it contains LAB isolated from cucumbers, cucumber fermentations, and other non-cucumber associated sources. A second USDA culture collection is maintained by Dr. Ilenys Pérez-Díaz and contains both LAB and enteric organisms isolated from cucumbers and cucumber fermentations. The third collection is maintained by the Barrangou Lab at NCSU and contains LAB isolated from various sources. Metagenomic analysis of the species contained in these collections using the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) database, GenBank, for GAD or GlsX activity revealed 125 isolates from vegetable fermentations, 95 isolates from non-vegetable or unknown fermentations, and 18 enteric organisms with potential GAD and/or GlsX activity (Table D.1). Quantification of glutamate, glutamine and GABA produced in pure culture fermentations would require significant time and resources. Alternatively, screening this collection for GlsX and GAD expressing bacteria using qualitative, colorimetric techniques provides the potential to rapidly

evaluate hundreds of isolates and identify suitable GlsX and/or GAD expressing candidates for cucumber fermentations.

Qualitative, colorimetric assays have been published for GAD (Rice, Johnson, Dunnigan & Reason, 1993) and GlsX (Pennacchietti, D'Alonzo, Freddi, Occhialini, and De Biase, 2018) screening of microorganisms, however these assays were both developed with enteric microorganisms and their application to LAB has not been verified. Both assays utilize bromocresol green as a color indicator, with the Rice assay reagent containing glutamate as the amino acid substrate and the GlsX assay reagent containing glutamine. In the Rice assay, as LAB express GAD, glutamate is decarboxylated to GABA, increasing the pH of the reagent and changing the color from blue to green or yellow. In the GlsX assay, when GlsX is expressed glutamine is deamidated to form glutamate, resulting in a pH increase that turns the blue reagent green. A subsequent decarboxylation of glutamate to GABA results in further pH increase and the reagent transitions to a yellow color (Figure D.1)
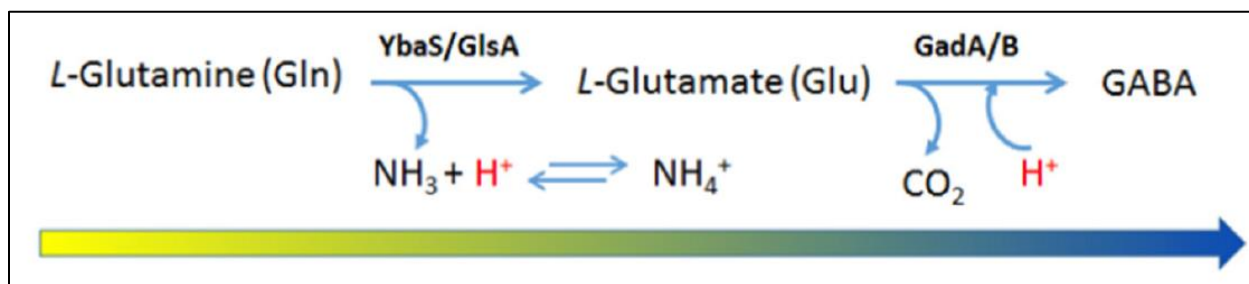
Figure D.1 Assay color change schematic (Pennacchietti et al., 2018). YbaS/GlsA (glutaminase), GadA/B (glutamate decarboxylase)

A third colorimetric method developed by Daeschel, McFeeters, Fleming, Klaenhammer & Sanozky (1983) to screen LAB for malate decarboxylase (MD) activity has potential to be adapted for screening of GlsX and GAD activity. Similar to the Rice and GlsX assay solutions, bromocresol green is utilized as a pH color indicator in the MD assay medium. The medium contains glucose as an energy source for lactic acid production and malic acid as a substrate for decarboxylation by MD. The starting pH of the medium is 7.0 and its color is deep blue. Decarboxylation of malic acid results in the uptake of a proton, therefore LAB performing this reaction will neutralize the lactic acid produced during fermentation, maintaining the deep blue color. Media fermented with bacteria lacking MD will transition to a green or yellow color

corresponding with pH decline. Adaptation of this assay by replacing malic acid with glutamate or glutamine has potential to serve as a rapid, colorimetric screening method for GAD and GlsX activity in LAB.

- MDG assay solution begins at pH 7 with blue color.
- Fermentation of glucose by LAB produces lactic acid, decreasing pH and resulting in yellow color.
- Deamidation of glutamine or decarboxylation of glutamate counteracts pH decline by lactic acid production, resulting in a green or blue color.
- Inability to produce lactic acid (i.e. by enteric organisms) may also result in maintenance of a high pH and green or blue color.

Figure D.2 Color and pH indicated by malate decarboxylase assay adapted for glutamate decarboxylase and glutaminase screening in LAB

The objectives of this study were to (1) determine if the Rice and GlsX assays were applicable to LAB and (2) adapt the malate decarboxylase assay to test for GAD and GlsX (known hereafter as the MDG assay) in LAB. The organisms selected for screening were divided into five phases indicating priority of testing (Table D.2). Phase I includes the control organisms outlined in table D.3. Phase II microorganisms were isolated from cucumber or vegetable fermentations, are homofermenters or facultative heterofermenters and have the highest likelihood of surviving and proliferating in a cucumber fermentation matrix. These organisms have the lowest potential for CO_2 production which can contribute to the formation of hollow cavities inside fermenting cucumbers (bloater defects). If suitable Phase II organisms are not identified, CO_2 producing heterofermentative isolates from vegetable fermentations, specifically *Lb. brevis*, will be screened in Phase III. If successful in increasing GABA content, alternative procedures for controlling bloating can be implemented for these value-added fermentations. Phase IV microorganisms were isolated from other food sources such as dairy, grain, fruit, and human subjects and have a lower likelihood of surviving in and completing fermentation in a

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cucumber fermentation matrix. Phase V microorganisms are enteric bacteria isolated from cucumber fermentations.

Table D.1 Microorganisms selected for GlsX and GAD assay screening from culture collections housed at NCSU.

Phase	Collection	Internal ID Number	Other ID Number(s)	Genus	Species	Metabolism*	Notes
1	N/A	N/A	ATCC 700926	*Escherichia*	*coli*	Enteric	Type strain
1	N/A	N/A	ATCC 367	*Lactobacillus*	*brevis*	Het.	
1	FSRU	LA0284	NRRL B-1922, ATCC 393	*Lactobacillus*	*casei*	Fac. Het.	ATCC 393 Type strain Isolated from cheese
1	FSRU	LA0272A	B-4562, ATCC 25601	*Lactobacillus*	*curvatus*	Fac. Het.	Type strain
1	FSRU	LA0272B	B-4562, ATCC 25601	*Lactobacillus*	*curvatus*	Fac. Het.	Type strain
1	FSRU	LA0257	B-14168, ATCC 33323	*Lactobacillus*	*gasseri*	Hom.	From Lauer and Kandler (1980) re: *Lb. gasseri* "No growth below 22 C; growth generally at 45 C; optimum at 35-38 c. Growth occurs at initial pH values of 5-7; optimum 5.5-6.0."
1	FSRU	LA0285	NRRL B-531, ATCC 8014	*Lactobacillus*	*plantarum*	Fac. Het.	ATCC 8014 - website does not give origin of culture
1	FSRU	LA1196	ATCC BAA-793; NCIMB 8826; WCFS1	*Lactobacillus*	*plantarum*	Fac. Het.	Caldwell Notebook 7, pp. 131-133. Wegkamp et al. 2009 Letters in Appl. Microbiol. Minimal growth media for LP.

Table D.1 (cont.)

2	FSRU	LA0602	BI0231, JM-8	*Lactobacillus*	*casei*	Fac. Het.	Isolated from low salt spoiled cucumber fermentation brine by Dr. Jae-Ho Kim, 2002.
2	FSRU	LA0602	BI0231, JM-8	*Lactobacillus*	*casei*	Fac. Het.	Isolated from low salt spoiled cucumber fermentation brine by Dr. Jae-Ho Kim, 2002.
2	FSRU	LA1133		*Lactobacillus*	*casei*	Fac. Het.	Isolate from cucumber pickles, Sue Hale 2005, Identified by partial 16S sequence, Ilenys Diaz-Muniz 6/2006.
2	FSRU	LA1141	33	*Lactobacillus*	*casei*	Fac. Het.	From MOP spoilage tank 30-12, from Wendy Franco's culture plates 11/24/09 See Caldwell Notebook 4, p. 146
2	FSRU	LA0471	BI0074, LY31	*Lactobacillus*	*paracasei*	Fac. Het.	F. Breidt; isolated from "red" pickle. Identified by partial 16S sequence, Ilenys Diaz-Muniz, 6/2006.
2	FSRU	LA0520	BI0149, DM8	*Lactobacillus*	*paracasei*	Fac. Het.	Pickle isolate (EX-35-00, Jar 1C) identified by 16S sequence and isolated by Laura Reina

Table D.1 (cont.)

2	FSRU	LA1049	DQ682983, 14F3	*Lactobacillus*	*paraplantarum*	Fac. Het.	DNA fingerprinting of LAB in sauerkraut; V. Plengvidhya, 2007
2	FSRU	LA1074	DQ682984, 14H4	*Lactobacillus*	*paraplantarum*	Fac. Het.	DNA fingerprinting of LAB in sauerkraut; V. Plengvidhya, 2007
2	IPD	1.2.7	7	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	1.8.18	68	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	14.2.16	466	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	14.2.1RB - B		*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	14.2.3	340	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	14.8.42	429	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	3.2.36	329	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	3.8.39RB - B		*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019

Table D.1 (cont.)

2	IPD	3.8.45	284	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.2.11	130	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.2.15	134	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.2.20	139	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.2.23	142	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.2.25FB - G		*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.2.27FB - E		*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.2.30RB - G		*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.2.33	152	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.8.2	352	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019

Table D.1 (cont.)

2	IPD	7.8.46 RB - K		*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.8.48	217	*Lactobacillus*	*pentosus*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	FSRU	LA0233	ATCC 8041	*Lactobacillus*	*pentosus*	Fac. Het.	Type strain - Fred EB, et al. The characteristics of certain pentose destroying bacteria, especially as concerns their action on arabinose. Suggests this organism may originate from sauerkraut. Same as LA0136
2	FSRU	LA0514	BI0143, DM2	*Lactobacillus*	*pentosus*	Fac. Het.	Pickle isolate (EX-35-00, Jar 13C) identified by 16S sequence and isolated by Laura Reina
2	IPD	3.2.21	314	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	3.2.8	301	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	3.8.3	203	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019

Table D.1 (cont.)

2	IPD	3.8.35	274	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	3.8.41	280	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.2.26	326	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.2.4	304	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.2.7	307	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.2.8	308	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.8.1	170	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.8.3	172	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.8.33 FB - K	383	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.8.4 (MN - Cluster 1, best fermenters)	354	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019

Table D.1 (cont.)

2	IPD	7.8.4 (NC - cluster 7)	173	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.8.4 FB - K	354 or 173	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.8.42	392	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.8.5	355	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	IPD	7.8.50	400	*Lactobacillus*	*plantarum*	Fac. Het.	Cucumber fermentation Dr. Perez-Diaz 2019
2	FSRU	LA0070	ATCC 14917	*Lactobacillus*	*plantarum*	Fac. Het.	ATCC received 10/83; isolated from pickled cabbage (ATCC)
2	FSRU	LA0098	ATCC 14917, 1752	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England). ATCC says isolated from pickled cabbage
2	FSRU	LA0308	14-C8	*Lactobacillus*	*plantarum*	Fac. Het.	Isolated from commercial sauerkraut by Jhongjing Lu in 2001. Identified by 16S sequence. 2/2003.

Table D.1 (cont.)

2	FSRU	LA0309	22-D10	*Lactobacillus*	*plantarum*	Fac. Het.	Isolated from commercial sauerkraut by Jhongjing Lu in 2001. Identified by 16S sequence. 2/2003.
2	FSRU	LA0436		*Lactobacillus*	*plantarum*	Fac. Het.	Isolate from spoiled MOP brine (butyric acid) received 9/3/04. Large mucoid white colonies. Identified by partial 16S sequence, Sue Hale 2005.
2	FSRU	LA0437		*Lactobacillus*	*plantarum*	Fac. Het.	Isolate from spoiled MOP brine (propionic acid) received 9/22/04. Medium dome shaped yellow colonies. Identified by partial 16S sequence, Sue Hale 2005.
2	FSRU	LA0438		*Lactobacillus*	*plantarum*	Fac. Het.	Isolate from spoiled MOP brine (propionic acid) received 9/22/04. Medium dome shaped white colonies. Identified by partial 16S sequence, Sue Hale 2005.

Table D.1 (cont.)

2	FSRU	LA0439		*Lactobacillus*	*plantarum*	Fac. Het.	Isolate from spoiled MOP brine (propionic acid) received 9/22/04. Large white translucent colonies. Identified by partial 16S sequence, Sue Hale 2005.
2	FSRU	LA0442		*Lactobacillus*	*plantarum*	Fac. Het.	Isolate from spoiled DeGraffenreid pickle brine, white, dome shaped colonies, received 10/11/04. Identified by partial 16S sequence, Sue Hale 2005.
2	FSRU	LA0599	BI0228, JM-2	*Lactobacillus*	*plantarum*	Fac. Het.	Isolated from low salt spoiled cucumber fermentation brine by Dr. Jae-Ho Kim, 2002.
2	FSRU	LA0599	BI0228, JM-2	*Lactobacillus*	*plantarum*	Fac. Het.	Isolated from low salt spoiled cucumber fermentation brine by Dr. Jae-Ho Kim, 2002.
2	FSRU	LA0822	DQ628955, 7C4	*Lactobacillus*	*plantarum*	Fac. Het.	DNA fingerprinting of LAB in sauerkraut; V. Plengvidhya, 2007
2	FSRU	LA0834	DQ682957, 7D4	*Lactobacillus*	*plantarum*	Fac. Het.	DNA fingerprinting of LAB in sauerkraut; V. Plengvidhya, 2007

Table D.1 (cont.)

2	FSRU	LA0990	DQ682977, 14A4	*Lactobacillus*	*plantarum*	Fac. Het.	DNA fingerprinting of LAB in sauerkraut; V. Plengvidhya, 2007
2	FSRU	LA1018	DQ682981, 14C8	*Lactobacillus*	*plantarum*	Fac. Het.	DNA fingerprinting of LAB in sauerkraut; V. Plengvidhya, 2007
2	FSRU	LA1044	DQ682982, 14E10	*Lactobacillus*	*plantarum*	Fac. Het.	DNA fingerprinting of LAB in sauerkraut; V. Plengvidhya, 2007
2	FSRU	LA1142	37	*Lactobacillus*	*plantarum*	Fac. Het.	From MOP spoilage tank 30-12, from Wendy Franco's culture plates 11/24/09 See Caldwell Notebook 4, p. 146
2	FSRU	LA1204	9-B4	*Lactobacillus*	*plantarum*	Fac. Het.	Bacteriophage host isolate, Lu et al., 2003. Bacteriophage Ecology in Commercial Sauerkraut Fermentations.
2	FSRU	LA1205	??	*Lactobacillus*	*plantarum*	Fac. Het.	Bacteriophage host isolate, Lu et al., 2003. Bacteriophage Ecology in Commercial Sauerkraut Fermentations.
2	FSRU	LA0219		*Lactobacillus*	*plantarum*	Fac. Het.	Cm(r), L. R.

Table D.1 (cont.)

2	FSRU	LA0516	BI0145, DM4	*Lactobacillus*	*plantarum*	Fac. Het.	Pickle isolate (EX-35-00, Jar 13B) identified by 16S sequence and isolated by Laura Reina
2	FSRU	LA0003	FFL#48 ATCC 43201	*Pediococcus*	*pentosaceus*	Hom.	syn=61 FBB61 (plasmid bac+imm+)-TSA only. According to ATCC, isolated from fermenting cucumbers in North Carolina
2	FSRU	LA0061	ATCC 43201, L-7230	*Pediococcus*	*pentosaceus*	Hom.	ATCC 43201, FFL-NCSU. bac+ (plasmid bac+ imm) missing 5.0? Isolated from fermenting cucumbers in North Carolina (ATCC)
2	FSRU	LA0187	ATCC 10791	*Pediococcus*	*pentosaceus*	Hom.	ATCC, 1-25-93. Isolation: Fermented food (ATCC); "cucumber pickle fermentation"
2	FSRU	LA0805	DQ682952, 7A11	*Pediococcus*	*pentosaceus*	Hom.	DNA fingerprinting of LAB in sauerkraut; V. Plengvidhya, 2007
3	FSRU	LA0743	DQ682939, 3D9	*Lactobacillus*	*brevis*	Het.	DNA fingerprinting of LAB in sauerkraut; V. Plengvidhya, 2007

Table D.1 (cont.)

3	FSRU	LA0825	DQ682956, 7C7	*Lactobacillus*	*brevis*	Het.	DNA fingerprinting of LAB in sauerkraut; V. Plengvidhya, 2007
3	FSRU	LA0028	MD#30	*Lactobacillus*	*acidophilus*	Hom.	Mundt-TN.
3	FSRU	LA0029	MD#50	*Lactobacillus*	*acidophilus*	Hom.	Syn=NC7M, Dr. Klaenhammer NCSU.
3	FSRU	LA0025	MD#42	*Lactobacillus*	*brevis*	Het.	Syn=50=FBB50, FFL.
3	FSRU	LA0026	MD#43	*Lactobacillus*	*brevis*	Het.	Syn=70, FFL.
3	FSRU	LA0036	ATCC 14869, NRRL B-4527	*Lactobacillus*	*brevis*	Het.	Nakamura NRRL.
3	FSRU	LA0188	ATCC 4006	*Lactobacillus*	*brevis*	Het.	ATCC 1-25-93.
3	FSRU	LA0200	ATCC 8287	*Lactobacillus*	*brevis*	Het.	ATCC, 8-16-93.
3	FSRU	LA0227	ATCC 14869	*Lactobacillus*	*brevis*	Het.	Type strain, small colony.
3	FSRU	LA0228	ATCC 14869	*Lactobacillus*	*brevis*	Het.	Type strain, big colony.
3	FSRU	LA0311	7-E1	*Lactobacillus*	*brevis*	Het.	Isolated from commercial sauerkraut by Jhongjing Lu in 2001.Identified by 16S sequence. 2/2003.
3	FSRU	LA0443		*Lactobacillus*	*brevis*	Het.	Isolate from spoiled DeGraffenreid pickle brine, white, tiny, flat colonies, received 10/11/04. Identified by partial 16S sequence, Sue Hale 2005.

Table D.1 (cont.)

3	FSRU	LA0515	BI0144, DM3	*Lactobacillus*	*brevis*	Het.	Pickle isolate (EX-35-00, Jar 13A) identified by 16S sequence and isolated by Laura Reina
3	FSRU	LA0517	BI0146, DM5	*Lactobacillus*	*brevis*	Het.	Pickle isolate (EX-35-00, Jar 1A) identified by 16S sequence and isolated by Laura Reina
3	FSRU	LA0518	BI0147, DM6	*Lactobacillus*	*brevis*	Het.	Pickle isolate (EX-35-00, Jar 1A) identified by 16S sequence and isolated by Laura Reina
3	FSRU	LA0519	BI0148, DM7	*Lactobacillus*	*brevis*	Het.	Pickle isolate (EX-35-00, Jar 13A) identified by 16S sequence and isolated by Laura Reina
3	FSRU	LA0521	BI0150, DM9	*Lactobacillus*	*brevis*	Het.	Pickle isolate (EX-29-00, Jar 4B) identified by 16S sequence and isolated by Laura Reina
3	FSRU	LA0600	BI0229, JM-4	*Lactobacillus*	*brevis*	Het.	Isolated from low salt spoiled cucumber fermentation brine by Dr. Jae-Ho Kim, 2002.

Table D.1 (cont.)

3	FSRU	LA0600	BI0229, JM-4	*Lactobacillus*	*brevis*	Het.	Isolated from low salt spoiled cucumber fermentation brine by Dr. Jae-Ho Kim, 2002.
3	FSRU	LA1145	47	*Lactobacillus*	*brevis*	Het.	From MOP spoilage tank 30-12, from Wendy Franco's culture plates 11/24/09 See Caldwell Notebook 4, p. 146
3	FSRU	LA0230		*Lactobacillus*	*delbrueckii*	Hom.	Probiotic if Subsp. *bulgaricus.* It grows in anaerobic conditions. It takes 48 hours to grow and colonies seem to be different (star and round). This was star form.
3	FSRU	LA0231		*Lactobacillus*	*delbrueckii*	Hom.	It grows under anaerobic conditions. It takes 48 hours to grow. Colony form; round.
3	FSRU	LA0114	481	*Lactobacillus*	*helveticus*	Hom.	BAC(+) Helveticin J (from Klaenhammer 6/87).
3	FSRU	LA1170	incorrectly labeled as LA819	*Lactobacillus*	*paracasei*	Fac. Het.	Transferred from ECT-labelled box. Was not entered in Culture Collection JMC 11/15/11

Table D.1 (cont.)

3	FSRU	LA0274	NRRL #B-23115 ATCC 700211	*Lactobacillus*	*paraplantarum*	Fac. Het.	Type strain, isolated from fermented beverages, Strasbourg France
3	FSRU	LA0136	ATCC 8041	*Lactobacillus*	*pentosaceus*	Hom.	Type strain - Fred EB, et al. The characteristics of certain pentose destroying bacteria, especially as concerns their action on arabinose. J. Biol. Chem. 48: 385-412, 1921 ... suggests this organism may originate from sauerkraut. Same as LA0233
3	FSRU	LA1171	incorrectly labeled as LA820	*Lactobacillus*	*pentosus*	Fac. Het.	Transferred from ECT-labelled box. Was not entered in Culture Collection JMC 11/15/11
3	FSRU	LA0027	MD#20	*Lactobacillus*	*brevis*	Het.	Mundt-TN.
3	FSRU	LA0513	BI0142, DM1	*Lactobacillus*	*brevis*	Het.	Pickle isolate (EX-35-00, Jar 1C) identified by 16S sequence and isolated by Laura Reina

Table D.1 (cont.)

3	FSRU	LA0232		*Lactobacillus*	*delbrueckii*	Hom.	It grows under anaerobic conditions. Mix colonies star and round.
3	FSRU	LA0054	135	*Pediococcus*	*acidilactici*	Hom.	Syn=PC 135. Dr. J. B. Evans Dept. Microbiology NCSU.
3	FSRU	LA0056	SP4	*Pediococcus*	*acidilactici*	Hom.	Syn=PC SP4 Dr. J. B. Evans Dept. Microbiology NCSU.
3	FSRU	LA0057	SP5	*Pediococcus*	*acidilactici*	Hom.	Dr. J. B. Evans Dept. Microbiology NCSU.
3	FSRU	LA0074	ATCC 33314	*Pediococcus*	*acidilactici*	Hom.	Gonzalez study;1983 Appl. Environ. 46:81-84 (1/17/83) ATCC type strain.
3	FSRU	LA0082		*Pediococcus*	*acidilactici*	Hom.	PAC 1.0 Ref. Gonzalez; Hoover.
3	FSRU	LA0001	MD23	*Pediococcus*	*pentosaceus*	Hom.	Mundt-TN.
3	FSRU	LA0002	FFL#47	*Pediococcus*	*pentosaceus*	Hom.	Syn=39
3	FSRU	LA0046	B15	*Pediococcus*	*pentosaceus*	Hom.	Syn=B15 Dr. F. Radler Institute foe Mikrobiologie und Weinforschung.

Table D.1 (cont.)

3	FSRU	LA0047	B56	*Pediococcus*	*pentosaceus*	Hom.	Syn=B56 Dr. F. Radler Institute foe Mikrobiologie und Weinforschung Johannes Gutenbreg Universitat 6500 Mainz Postfach 3980 West. Germany. Terminal pH 3.86.
3	FSRU	LA0053	B1325	*Pediococcus*	*pentosaceus*	Hom.	Syn=PC 1325 NRRL.
3	FSRU	LA0055	P-20	*Pediococcus*	*pentosaceus*	Hom.	Syn=PC 20 Dr. J. B. Evans Dept. Microbiology NCSU.
3	FSRU	LA0058	183-1W	*Pediococcus*	*pentosaceus*	Hom.	Dr. J. B. Evans Dept. Microbiology NCSU.
3	FSRU	LA0060	E66	*Pediococcus*	*pentosaceus*	Hom.	J. R. Stamer, Cornell Univ.
3	FSRU	LA0072	NRRL B-11465	*Pediococcus*	*pentosaceus*	Hom.	Gonzalez study; 1983 Appl. Environ. 46:81-84 (1/17/83). Plasmid transfer in Pediococcus spp.: intergeneric and intrageneric transfer of pIP501.

Table D.1 (cont.)

3	FSRU	LA0073	ATCC 25745	*Pediococcus*	*pentosaceus*	Hom.	Gonzalez study; 1983 Appl. Environ. 46:81-84 (1/17/83). Plasmid transfer in Pediococcus spp.: intergeneric and intrageneric transfer of pIP501.
3	FSRU	LA0076	ATCC 33316	*Pediococcus*	*pentosaceus*	Hom.	Gonzalez study; 1983 Appl. Environ. 46:81-84 (1/17/83). Plasmid transfer in Pediococcus spp.: intergeneric and intrageneric transfer of pIP501.
3	FSRU	LA0198	B-1325	*Pediococcus*	*pentosaceus*	Hom.	LA53 Nip(r) 200 ug/mL nisin broth, K. Crowley, 8/17/93.
3	FSRU	LA0199	183-1W	*Pediococcus*	*pentosaceus*	Hom.	LA58, Nip(r) 200 ug/mL nisin broth, K. Crowley, 8/17/93.
3	FSRU	LA0358	LR47	*Pediococcus*	*pentosaceus*	Hom.	Isolated from FRESCU by Laura Reina. Identified by 16S sequence. 4/2003.
3	FSRU	MU0089	FBB-63	*Pediococcus*	*pentosaceus*	Hom.	Syn=DG3; see AEM 50: 532-534, 1985; from Donald Graham 1/26/86.
3	FSRU	LA0052	L-728	*Pediococcus*	*pentosaceus*	Hom.	Syn=PC 72-8 NRRL.

Table D.1 (cont.)

4	FSRU	LA0115	88	*Lactobacillus*	*acidophilus*	Hom.	BAC(+) Lactocin F (from Klaenhammer 6/87).
4	FSRU	LA0116	117592E	*Lactobacillus*	*acidophilus*	Hom.	BAC(+) Lactocin M (from Klaenhammer 6/87).
4	FSRU	LA0117	12B	*Lactobacillus*	*acidophilus*	Hom.	BAC(+) from Klaenhammer 6/87.
4	FSRU	LA0118	C7-C1	*Lactobacillus*	*acidophilus*	Hom.	BAC(+) from Klaenhammer 6/87.
4	FSRU	LA1135	NCK 56, NCFM#2	*Lactobacillus*	*acidophilus*	Hom.	One of strains used commercially to make culture concentrates for Sweet Acidophilus milk. Obtained from Todd Klaenhammer Lab, NCSU, 6/2006.
4	FSRU	LA1136		*Lactobacillus*	*acidophilus*	Hom.	Sweet Acidophilus from Rhodia Inc., Dairy Business, Madison, WI, 7/2006.
4	FSRU	LA0038	NRRL B-4560	*Lactobacillus*	*casei*	Fac. Het.	Nakamura NRRL. Source: milking machine
4	FSRU	LA0037	ATCC 7469, NRRL B-1445, NCTC 6372, NCDO243	*Lactobacillus*	*casei (rhamnosus)*	Fac. Het.	Nakamura NRRL. 37 C. Source according to NRRL is Flynn, L. M. (U Missouri, Columbia, MO) <- ATCC
4	FSRU	LA0226	ATCC 9649	*Lactobacillus*	*delbrueckii*	Hom.	Type strain - isolated from sour grain mash

Table D.1 (cont.)

4	Barrangou	NCSU_RB330	2634	*Lactobacillus*	*fermentum*	Het.	TBD
4	Barrangou	NCSU_RB201	1341	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB197	334	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB215	2376	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB216	2377	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB251	2253	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB319	2378	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB195	99	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB196	102	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB203	1343	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB193	1084	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB194	1085	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB326	2630	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB327	2631	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB328	2632	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB331	1338	*Lactobacillus*	*gasseri*	Hom. (?)	TBD
4	Barrangou	NCSU_RB271	1370	*Lactobacillus*	*johnsonii*	Hom. (?)	TBD
4	Barrangou	NCSU_RB263	948	*Lactobacillus*	*johnsonii*	Hom. (?)	TBD
4	Barrangou	NCSU_RB265	957	*Lactobacillus*	*johnsonii*	Hom. (?)	TBD
4	Barrangou	NCSU_RB267	964	*Lactobacillus*	*johnsonii*	Hom. (?)	TBD
4	Barrangou	NCSU_RB269	979	*Lactobacillus*	*johnsonii*	Hom. (?)	TBD
4	FSRU	LA0278	NRRL B-4560, ATCC 22598	*Lactobacillus*	*paracasei*	Fac. Het.	ATCC 25598 Type strain Isolated from: milking machine
4	FSRU	LA0286	NRRL B-4564, ATCC 27216	*Lactobacillus*	*paracasei*	Fac. Het.	ATCC 27216 Type strain Isolated from saliva, child

Table D.1 (cont.)

4	FSRU	LA0021	MD#15	*Lactobacillus*	*plantarum*	Fac. Het.	Mundt-TN.
4	FSRU	LA0022	MD#16	*Lactobacillus*	*plantarum*	Fac. Het.	Mundt-TN.
4	FSRU	LA0023	MD#40	*Lactobacillus*	*plantarum*	Fac. Het.	Syn=WSO, FFL.
4	FSRU	LA0084	NC-8	*Lactobacillus*	*plantarum*	Fac. Het.	Dobrogoz 12/12/85 transconjugant (pAMB1).
4	FSRU	LA0086	NC-8	*Lactobacillus*	*plantarum*	Fac. Het.	Plasmid free from Dobrogoz 1/9/86.
4	FSRU	LA0087	15	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England).
4	FSRU	LA0088	16	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England).
4	FSRU	LA0089	ATCC8014	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England). Source: corn silage (NCIMB)
4	FSRU	LA0091	NCDO341 NCTC5914 NCIMB 5914	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England).
4	FSRU	LA0092	ATCC10241	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England).

Table D.1 (cont.)

4	FSRU	LA0093	ATCC10012	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England).
4	FSRU	LA0094	NCDO354 NCTC6461 NCIMB 6461	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained from NCDO (Reading, England).
4	FSRU	LA0095	ATCC8041	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England). NCIMB says its Lb. pentosus
4	FSRU	LA0096	NCDO1193 NCIMB 8299	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England). Source: ensiled vegetable matter (NCIMB)
4	FSRU	LA0097	NCDO1194 NRC716 NCIMB 8531	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England). Source: Lactic acid fermentation of waste sulphite liquor. NCIMB calls this strain Lb. pentosus.

Table D.1 (cont.)

4	FSRU	LA0100	NCDO1988 NCIMB 701988	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England).
4	FSRU	LA0101	68	*Lactobacillus*	*plantarum*	Fac. Het.	T. R. Klaenhammer who obtained them from YAKULT Institute for Microbiological Research (Tokyo, Japan).
4	FSRU	LA0135		*Lactobacillus*	*plantarum*	Fac. Het.	LA86.
4	FSRU	LA0146	C2R	*Lactobacillus*	*plantarum*	Fac. Het.	
4	FSRU	LA0153	FBNC3	*Lactobacillus*	*plantarum*	Fac. Het.	LA86, Cm(r), lux(+), F. Breidt.
4	FSRU	LA0154	FBNC4	*Lactobacillus*	*plantarum*	Fac. Het.	LA86, Cm(r), LUX(+), F. Breidt.
4	FSRU	LA0155	FBNC5	*Lactobacillus*	*plantarum*	Fac. Het.	LA86, Cm (r), lux(+), F. Breidt.
4	FSRU	LA0156	FBNC6	*Lactobacillus*	*plantarum*	Fac. Het.	LA86, Cm(r), lux(+), F. Breidt.
4	FSRU	LA0201		*Lactobacillus*	*plantarum*	Fac. Het.	LA90, Nis(r) 20,000 I.U./mL nisin, 8-23-93, KC.
4	FSRU	LA0202		*Lactobacillus*	*plantarum*	Fac. Het.	LA96, Nis(r) 20,000 I.U./mL nisin, 8-23-93, KC.
4	FSRU	LA0204		*Lactobacillus*	*plantarum*	Fac. Het.	LA90, Nis(r) 20,000 I.U./mL nisin, CM(r), Em(r), 9-22-93, KC.

Table D.1 (cont.)

4	FSRU	LA0205		*Lactobacillus*	*plantarum*	Fac. Het.	Nis(r) 20,000 I.U./mL, Cm(r), EM(r), 9-22-93, KC.
4	FSRU	LA0206		*Lactobacillus*	*plantarum*	Fac. Het.	LA90, Nis(r) 20,000 I.U./mL, Cm(r), Em(r), 9-22-93, KC.
4	FSRU	LA0207		*Lactobacillus*	*plantarum*	Fac. Het.	LA96 Nis(r) 20,000 I.U/mL, Cm(r), Em(r), 9-22-93, KC.
4	FSRU	LA0208		*Lactobacillus*	*plantarum*	Fac. Het.	LA96, Nis(r) 20,000 I.U./mL nisin, Cm(r), Em(r), 9-22-93, KC.
4	FSRU	LA0209		*Lactobacillus*	*plantarum*	Fac. Het.	LA96, Nis(r) 20,000 I.U./mL nisin, Cm(r), Em(r), 9--22-93, KC.
4	FSRU	LA0211		*Lactobacillus*	*plantarum*	Fac. Het.	LA96, Nis(r) 20,000 I.U./mL nisin, 8-23-93, KC,Cm(r).
4	FSRU	LA0212		*Lactobacillus*	*plantarum*	Fac. Het.	LA96, Nis(r) 20,000 I.U./mL nisin, 8-23-93, KC, Cm(r).
4	FSRU	LA0215		*Lactobacillus*	*plantarum*	Fac. Het.	LA90, Nis(r) 20,000 I.U/mL nisin, 8-23-93, KC, Cm(r).
4	FSRU	LA0216		*Lactobacillus*	*plantarum*	Fac. Het.	LA90, Nis(r) 20,000 I.U./mL nisin, 8-23-93, KC, Cm(r).
4	FSRU	LA0220		*Lactobacillus*	*plantarum*	Fac. Het.	Cm(r), F. Breidt.
4	FSRU	LA0235	ATCC 43199, NCDO1869	*Lactobacillus*	*plantarum*	Fac. Het.	Type strain, isolated from frozen peas (ATCC)

Table D.1 (cont.)

4	FSRU	LA0402	LR91	*Lactobacillus*	*plantarum*	Fac. Het.	Isolated from FRESCU by Laura Reina. Identified by 16S sequence. Additionally, identified by ITS PCR product as L. plantarum. 4/2003.
4	FSRU	LA0403	LR92	*Lactobacillus*	*plantarum*	Fac. Het.	Isolated from FRESCU by Laura Reina. Identified by ITS PCR product. 4/2003.
4	FSRU	LA0404	LR93	*Lactobacillus*	*plantarum*	Fac. Het.	Isolated from FRESCU by Laura Reina. Identified by ITS PCR product. 4/2003.
4	FSRU	LA0405	LR94	*Lactobacillus*	*plantarum*	Fac. Het.	Isolated from FRESCU by Laura Reina. Identified by ITS PCR product. Additionally, identified by API 50 CHL as L. plantarum. 4/2003.
4	FSRU	LA0406	LR95	*Lactobacillus*	*plantarum*	Fac. Het.	Isolated from FRESCU by Laura Reina. Identified by ITS PCR product. 4/2003.

Table D.1 (cont.)

4	FSRU	LA0445	BI0007, MOP3	*Lactobacillus*	*plantarum*	Fac. Het.	L. plantarum isolate from expt. 15-83; 7/83, MOP 3, originally BI0007
4	FSRU	LA1148		*Lactobacillus*	*plantarum*	Fac. Het.	Sent by Bonjoon Kim of CJ Cheiljedang 8/6/10 JMC. See CRADA notebook 58-3K95-0-1429-MTA
4	FSRU	MU0045	M6	*Lactobacillus*	*plantarum*	Fac. Het.	Mop3-1; assay 8C. MU45, MOP3-M6, MDC- strain
4	FSRU	MU0079		*Lactobacillus*	*plantarum*	Fac. Het.	Str(R) rif(R), 304B+ 3 mg/mL strep., 0.25 mg/mL rif., Ann Schirago? 12/15/86. Isolated from exp6-85; Tank B-2VI-B, Bloch+Guggenhiemer.
4	FSRU	MU0080		*Lactobacillus*	*plantarum*	Fac. Het.	304 A(-), spontaneous non-polymer producing mutants.
4	FSRU	MU0081		*Lactobacillus*	*plantarum*	Fac. Het.	304 B(_), spontaneous non- polymer producer mutants.
4	FSRU	MU0082		*Lactobacillus*	*plantarum*	Fac. Het.	NC8-VI, str(R), rif(R), 3mg/mL str\.05mg/mL rif SV=rif.

Table D.1 (cont.)

4	FSRU	MU0083		*Lactobacillus*	*plantarum*	Fac. Het.	NC5-V31, str(R), rif(R), 3mg/mL str/.25 mg rifamycin Ann Schirago? 12/15/86.
4	FSRU	MU0084		*Lactobacillus*	*plantarum*	Fac. Het.	NC8-V1, str(R), rif(R), 3mg/mL. str/.25 mg rif Ann Schirago? 12/15/86.
4	FSRU	MU0085		*Lactobacillus*	*plantarum*	Fac. Het.	NC5-V31, str(R), rif(R), 3 mg/mL str/.1 mg rif Ann Schirago? 12/15/86.
4	FSRU	MU0086		*Lactobacillus*	*plantarum*	Fac. Het.	NC1-V3, str(R) rif(R), 3mg/mL str/.1 mg rif Ann Schirago? 12/15/86.
4	FSRU	MU0087		*Lactobacillus*	*plantarum*	Fac. Het.	Str(R) rif(R), 3mg/mL str/0.1mg/mL rif Ann Schirago? 12/15/86.
4	FSRU	MU0109	MU45, Mop 3-M6	*Lactobacillus*	*plantarum*	Fac. Het.	Transformed by F. Breidt.
4	FSRU	MU0065		*Lactobacillus*	*plantarum*	Fac. Het.	Origin: Bac(-), imm(+) that reverted to Bac(+) due to repeated culture 3/19/85.
4	FSRU	MU0066		*Lactobacillus*	*plantarum*	Fac. Het.	Origin: "Original culture Bac(+) immun(+)". No culture present 2014

Table D.1 (cont.)

4	FSRU	MU0078		Lactobacillus	plantarum	Fac. Het.	Str(R) rif(R), 304 A(+), 3mg/mL strep, 0.5mg, rif., 5V, Ann Schirago? 12/15/86. Isolated from exp6-85; Tank B-2-VI-B. Block + Guggenhiemer.
4	FSRU	LA0024	MD#41	Lactobacillus	plantarum	Fac. Het.	Syn=442, FFL.
4	FSRU	LA0090	NCDO340 NCTC6105 NCIMB 6105	Lactobacillus	plantarum	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England). NCIMB source says silage
4	FSRU	LA0099	ATCC15578 IAM10077 NCDO1939	Lactobacillus	plantarum	Fac. Het.	T. R. Klaenhammer who obtained them from NCDO (Reading, England). Source: Moto yeast mash of sake, Japan (ATCC)
4	FSRU	LA0280	KCCM 11322	Lactobacillus	plantarum	Fac. Het.	From Sing-sik Yoon, Korean culture collection
4	FSRU	MU0064		Lactobacillus	plantarum	Fac. Het.	Origin; Bac(-), immun(+) 3/19/85.
5	IPD	1.2.3E		Citrobacter	freundii	Enteric	Or Citrobacter freundii - Tank A
5	IPD	3.2.3E		Citrobacter	freundii	Enteric	Or Citrobacter freundii - Tank A

Table D.1 (cont.)

5	IPD	3.2.13E		*Enterobacter*	*cancerogenus*	Enteric	Or Enterobacter cancerogenus - Tank B
5	IPD	1.2.10E		*Enterobacter*	*cloacae*	Enteric	Or Pantoea spp. - Tank A
5	IPD	3.2.8E		*Enterobacter*	*cloacae*	Enteric	Or Enterobacter cloacae - Tank A
5	IPD	3.8.2E		*Enterobacter*	*cloacae*	Enteric	Or Enterobacter cloacae - Tank A
5	IPD	1.2.4E		*Enterobacter*	*kobei*	Enteric	Or Enterobacter cloacae - Tank A
5	IPD	3.2.9E		*Enterobacter*	*kobei*	Enteric	Or Enterobacter cloacae - Tank A
5	IPD	7.2.2E		*Enterobacter*	*sp.*	Enteric	Or Enterobacter cloacae - Tank A
5	IPD	1.8.5E		*Kluyvera*	*cryocrescens*	Enteric	Or Kluyvera spp. - Tank A
5	IPD	3.2.17E		*Kluyvera*	*intermedia*	Enteric	Or Acinetobacter baumannii - Tank B
5	IPD	3.2.1E		*Leclercia*	*adecarboxylata*	Enteric	Or Leclercia adecarboxylata - Tank A
5	IPD	7.8.20E		*Leclercia*	*adecarboxylata*	Enteric	Or Leclercia adecarboxylata - Tank B
5	IPD	1.2.1E		*Ochrobactrum*	*pseudogrignonense*	Enteric	Or Ochrobactrum anthropi - Tank A
5	IPD	1.2.21E		*Ochrobactrum*	*pseudogrignonense*	Enteric	Or Ochrobactrum anthropi - Tank B
5	IPD	1.2.4E		*Pantoea*	*agglomerans*	Enteric	Or Pantoea spp. - Tank B

Table D.1 (cont.)

5	IPD	1.2.16E		*Pantoea*	*ananatis*	Enteric	Or Pantoea spp. - Tank B
5	IPD	1.8.2E		*Providencia*	*rettgeri*	Enteric	Or Providencia rettgeri - Tank A
5	IPD	1.8.3E		*Serratia*	*marcescens*	Enteric	Or Serratia marcescens - Tank B

*Fac. Het. (facultative heterofermenters), Het. (heterofermenter), Hom. (homofermenter). FSRU = Food Science Research Unit, IPD =

Ilenys Pérez-Díaz Lab.

Table D.2 Phases of organism screening for GAD and GlsX activity

Metabolism*	Organism	Phase I (Control)	Phase II	Phase III	Phase IV	Phase V
Hom.	*Lactobacillus gasseri*	1			15	
Hom.	*Lactobacillus johnsonii*				5	
Hom.	*Lactobacillus delbrueckii*			3	1	
Hom.	*Lactobacillus acidophilus*			2	6	
Hom.	*Lactobacillus helveticus*			1		
Hom.	*Lactobacillus pentosus*		22	1		
Hom.	*Pediococcus acidilactici*			5		
Hom.	*Pediococcus pentosaceus*		4	17		
Hom.	*Lactobacillus brevis*	1		21		
Hom.	*Lactobacillus fermentum*				1	
Fac. Het.	*Lactobacillus casei*	1	4		2	
Fac. Het.	*Lactobacillus curvatus*	1				
Fac. Het.	*Lactobacillus paracasei*		2	1	2	
Fac. Het.	*Lactobacillus paraplantarum*		2	1		
Fac. Het.	*Lactobacillus plantarum*	2	39		63	
Enteric	*Citrobacter freundii*					2
Enteric	*Enterobacter cancerogenus*					1
Enteric	*Enterobacter cloacae*					3
Enteric	*Enterobacter kobei*					2
Enteric	*Enterobacter sp.*					1
Enteric	*Escherichia coli*	1				
Enteric	*Kluyvera cryocrescens*					1
Enteric	*Kluyvera intermedia*					1
Enteric	*Leclercia adecarboxylata*					2
Enteric	*Ochrobactrum pseudogrignonense*					2
Enteric	*Pantoea agglomerans*					1
Enteric	*Pantoea ananatis*					1
Enteric	*Serratia marcescens*					1
	Total # Strains:	7	73	52	95	18

*Fac. Het. (facultative heterofermenters), Het. (heterofermenter), Hom. (homofermenter)

Table D.3 Organisms selected for development of GAD and GlsX screening protocols

Culture Number	Internal ID Number	Other ID Number(s)	Genus	Species	Propagation	Metabolism*	Enzymes	Isolation Source
1	LA0272A	B-4562, ATCC 25601	*Lactobacillus*	*curvatus*	MRS at 37 C	Fac. Het.	-	Unknown
2	LA0272B	B-4562, ATCC 25601	*Lactobacillus*	*curvatus*	MRS at 37 C	Fac. Het.	-	Unknown
3	LA0285	NRRL B-531, ATCC 8014	*Lactobacillus*	*plantarum*	MRS at 37 C	Fac. Het.	gadB, gdh	Unknown
4	LA1196	ATCC BAA-793; NCIMB 8826; WCFS1	*Lactobacillus*	*plantarum*	MRS at 37 C, 5% CO2	Fac. Het.	gadB, gdh	Saliva - human oral cavity
5	LA0257	B-14168, ATCC 33323	*Lactobacillus*	*gasseri*	MRS at 37 C	Hom.	glsA	Unknown
6	LA0284	NRRL B-1922, ATCC 393	*Lactobacillus*	*casei*	MRS at 37 C	Fac. Het.	gdh	Cheese
7	3.2.13E		*Enterobacter*	*cancerogenus*	BHI at 30C	Enteric	Unknown	Cucumber Fermentation
8	1.2.7E		*Enterobacter*	*nimipressuralis*	BHI at 30C	Enteric	Unknown	Cucumber Fermentation
9	1.2.4E		*Pantoea*	*agglomerans*	BHI at 30C	Enteric	Unknown	Cucumber Fermentation

*Fac. Het. (facultative heterofermenters), Het. (heterofermenter), Hom. (homofermenter)

Table D.4 Organisms selected for development of MDG screening protocols

Culture Number	Internal ID Number	Other ID Number(s)	Genus	Species	Propagation	Metabolism*	Enzymes	Source
1	LA0257	B-14168, ATCC 33323	*Lactobacillus*	*gasseri*	MRS at 37 C	Hom.	glsA	Unknown
2	LA0272A	B-4562, ATCC 25601	*Lactobacillus*	*curvatus*	MRS at 37 C	Fac. Het.	-	Unknown
6	LA1196	ATCC BAA-793; NCIMB 8826; WCFS1	*Lactobacillus*	*plantarum*	MRS at 37 C, 5% CO2	Fac. Het.	gadB, gdh	Saliva - human oral cavity
7	LA1129	ATCC 367	*Lactobacillus*	*brevis*	LAB MRS at 30C	Het.	gadB	Unknown
9	B0545	ATCC 700926	*Escherichia*	*coli*	ATCC Medium 2511: M9 Minimal Agar/Broth at 37C	Enteric	gadA, gadB, gdh, glsA, glsB	Unknown

*Fac. Het. (facultative heterofermenters), Het. (heterofermenter), Hom. (homofermenter)

D.2 Materials & Methods

D.2.1 Rice and GlsX Assays

The Rice assay was performed following **Rice et al., (1993)** and **Pennacchiette et al., (2018)** with adaptations (see **Table D.6)**

Materials:

- Glutamate
- Glutamine
- Bromocresol green
- NaCl
- 0.85% saline
- Triton X-100
- Distilled water
- MRS (de Man, Rogosa & Sharpe) broth
- BHI (brain-heart infusion) broth
- Ethanol (70%)

Rice Assay Solution (1 liter): Combine 1 g glutamate, 0.05 g bromocresol green, 90 g NaCl, 3 mL Triton X-100 and fill to volume with distilled water. Adjust pH to 3.4 and sterilize with 0.2 µm filter prior to use. Reagent is stable for 2 months at 5°C.

GlsX Assay Solution (1 liter): Combine 1 g glutamine, 0.05 g bromocresol green, 90 g NaCl, 3 mL Triton X-100 and fill to volume with distilled water. Adjust pH to 3.1 and sterilize with 0.2 µm filter prior to use. Reagent is stable for 2 months at 5°C.

Equipment:

- 15 ml conical tubes
- Pop-top microcentrifuge tubes
- Centrifuge
- Vortexer

- Filter paper

- Incubator

- Sterile loop

- Pipettes

Table D.5 Original Rice assay protocol per Rice et al., 1993

Step	Instruction	Equipment & Materials	Time
1	Grow cultures in LTB or PA broth for 20-24 hr.	LTB (lauryl tryptose broth) or PA (presence-absence) broth, centrifuge tubes, incubator.	24 hours
2	Transfer 5 mL of culture to 15 mL conical tubes	15 mL centrifuge tubes	10 min
3	Centrifuge at 500 x g for 10 min	Centrifuge	10 min
4	Decant media and ensure all is removed by using a pipette and/or thin strip of filter paper.	Pipette, filter paper	5 min
5	Resuspend pellet in 5 mL phosphate buffer and centrifuge at 500 x g for 10 min at 22°C	Phosphate buffer, centrifuge	15 min
6	Decant phosphate buffer and ensure all is removed by using a pipette and/or thin strip of filter paper.	Pipette, filter paper	5 min
7	Pellet concentration: resuspend pellet in 1 mL Rice assay solution [5x concentration of cells]	Rice assay solution	10 min
8	Incubate at 35°C for 4 hours. Observe hourly for color change.	Incubator	4 hours

Table D.6 Original glutaminase assay protocol per Pennacchiette et al., 2018

Step	Instruction	Equipment & Materials	Time
1	Remove culture isolate from -80°C storage		5 min
2	Use a sterile loop to inoculate 1.5 ml of media contained in a microcentrifuge tube (pop-top).	Loop, microcentrifuge tube (pop-top), MRS (LAB) or BHI (*Enterobacteriaceae*)	10 min
3	Incubate 24 hours at 37°C	Incubator	24 hours
4	Centrifuge at 4,000 rpm for 20 min	Centrifuge	20 min
5	Resuspended in an isovolume (1.5mL) of physiological saline solution	Saline solution, pipette	10 min
6	Repeat steps 4-5 two times for a total of three pellet washings.	Centrifuge, saline solution, pipette	40 min
7	Measure cell density at OD_{600} and adjust volume with saline so that $OD_{600} = 2.0$	Cuvette, saline solution, pipette.	60 min
8	Aliquot 500 µL into two microcentrifuge tubes (~ $0.2–1.0 \times 10^9$ cells/ml), one for each assay	2 microcentrifuge tubes (pop-top), pipette	10 min
9	Centrifuge each tube at 14,000 rpm for 5 min	Centrifuge	5 min
10	Remove supernatants; resuspend pellets by vigorous vortexing or pipetting in 150 µl of GlsX assay solution	Pipette, GlsX assay solution, vortexer	10 min
11	Incubate tubes at 37°C for 30 min - 2 hours	Incubator	120 min
12	Assess color change by eye and record results		10 min

Table D.7 Rice and GlsX assay protocols adapted for LAB

Step	Instruction	Equipment & Materials	Time
1	Remove culture isolate from -80°C storage		5 min
2	Use a sterile loop to inoculate 20 ml of media contained in a 50 mL tube.	Loop, 50 mL falcon tube, MRS (for LAB) or BHI (for E*nterobacteriaceae*)	10 min
3	Incubate 24 hours at 37°C (*Enterobacteriaceae*) or 30°C (LAB)	Incubator	24 hours
4	Centrifuge at 12,000 rpm for 5 min at 22°C.	Centrifuge	5 min
5	Decant media and ensure all is removed by using a pipette and/or thin strip of filter paper. Resuspend pellet in an isovolume (20mL) media.	Pipette, MRS (for LAB) or BHI (for *Enterobacteriaceae*)	5 min
6	Incubate 3-4 hours at 37°C (*Enterobacteriaceae*) or 30°C (LAB)	Incubator	4 hours
7	Centrifuge at 12,000 rpm for 5 min at 22°C.	Centrifuge	5 min
8	**Ethanol wash #1:** Decant media and ensure all is removed. Resuspend pellet in an isovolume (20mL) ethanol and centrifuge at 12,000 rpm for 5 min at 22°C.	Ethanol (70%), pipette, filter paper, centrifuge	10 min
9	**Ethanol wash #2:** Repeat step 8.	Ethanol (70%), pipette, filter paper, centrifuge	10 min
10	**Saline wash #1:** Decant ethanol and ensure all is removed. Resuspend pellet in an isovolume (20mL) physiological saline and centrifuge at 12,000 rpm for 5 min at 22°C.	Saline solution (0.85% NaCl), pipette, filter paper, centrifuge	10 min
11	**Saline wash #2:** repeat step 10.	Saline solution (0.85% NaCl), pipette, filter paper, centrifuge	10 min
12	**Pellet concentration:** Decant saline and ensure all is removed. Resuspend pellet in 4 mL physiological saline. [5x concentration of cells]	Saline solution (0.85% NaCl), pipette, filter paper	10 min
13	Measure cell density using 1 mL of the cell suspension at OD_{600}. Adjust volume with saline so that $OD_{600} = 2.0$.	1.5 mL cuvette, saline solution (0.85% NaCl), pipette.	10 min
14	Aliquot 500 µL into two microcentrifuge tubes (~ $0.2–1.0 \times 10^9$ cells/ml), one for each assay	2 microcentrifuge tubes (pop-top), pipette	10 min
15	Centrifuge each tube at 14,000 rpm for 5 min at 22°C to obtain a pellet.	Centrifuge	5 min

Table D.7 (cont.)

16	Remove supernatants carefully using a pipette and/or thin strip of filter paper. Resuspend pellets by vigorous vortexing or pipetting in 150 µl of Rice or GlsX assay solution.	Pipette, GlsX assay solution, Rice assay solution, vortexer	20 min
17	Incubate tubes at 37°C for 30 min - 2 hours	Incubator	120 min
18	Assess color change by eye and record results		10 min

D.2.2 MDG Assay

The MDG assay reagent was prepared with two levels of glucose. The original assay contained 0.5% glucose and 2% malic acid which allowed for sufficient malic acid decarboxylation and pH increase to maintain a blue color. Addition of 2% glutamine or glutamate is excessive and could interfere with LAB viability, therefore only 0.4% of these amino acids were added (27.19 mM glutamate or 27.37 mM glutamine). Trials of assay solutions made with 0.25% (13.88 mM) and 0.125% (6.94 mM) glucose were conducted to determine which level allowed for noticeable color changes.

Materials:

- Trypticase
- Glucose
- Casamino acids
- Phytone
- Yeast Extract
- Tween 80
- Bromocresol green
- NaOH
- KOH
- Glutamine
- Glutamate

Table D.8 Original MD assay reagent formulation (Daeschel et al. 1983)

Component	%
L-Malic acid	2.00%
Trypticase	1.00%
D-Glucose	0.50%
Casamino Acids	0.30%
Phytone	0.15%
Yeast Extract	0.10%
Tween 80	2.00%
Bromocresol green (0.1g per 30 mL 0.01 N NaOH)	2.00%
Agar (when desired)	2.00%

Adjust pH to 7.0 with 10 N KOH. Autoclave at 15lb pressure for 15 min. Can be stored at room temperature.

Table D.9 Adapted MD assay (MDG) reagent formulation

	Assay Reagent					
	0.25% Glucose			0.125% Glucose		
Component	Control ("A")	Glutamate Added ("AE")	Glutamine Added ("AN")	Control ("B")	Glutamate Added ("BE")	Glutamine Added ("BN")
Glutamate		0.40%			0.40%	
Glutamine			0.40%			0.40%
Trypticase	1.00%	1.00%	1.00%	1.00%	1.00%	1.00%
D-Glucose	0.250%	0.250%	0.250%	0.125%	0.125%	0.125%
Casamino Acids	0.30%	0.30%	0.30%	0.30%	0.30%	0.30%
Phytone	0.15%	0.15%	0.15%	0.15%	0.15%	0.15%
Yeast Extract	0.10%	0.10%	0.10%	0.10%	0.10%	0.10%
Tween 80	2.00%	2.00%	2.00%	2.00%	2.00%	2.00%
Bromocresol green solution*	2.00%	2.00%	2.00%	2.00%	2.00%	2.00%

*Bromocresol green (0.025g per 10 mL 0.01 N NaOH). Adjust pH to 7.0 with 1 N KOH (6.234 g 90% purity KOH into 100 mL water). Autoclave at 15lb pressure for 15 min or sterile filter. Can be stored at room temperature.

Equipment:

- 15 ml conical tubes

- Pop-top microcentrifuge tubes

- Centrifuge

- Vortexer

- Filter paper

- Incubator

- Sterile loop

- Pipettes

Table D.10 Protocol for MDG Assay

Prepare Reagents	
1	Make Assay Reagents in volumetric flasks.
2	Make Assay Reagents Glx Added: Add glutamate or glutamine to volumetric flasks and fill to volume with Assay Reagent (Control). Sonicate for 30 min.
3	Adjust pH of all reagents to 7.0 using 1.0 N KOH and 0.1 N HCl
4	Sterile filter (0.2 um) or autoclave reagents.
Liquid Culture Tests	
1	Prepare LAB liquid culture in MRS, incubate at 30°C for 16-20 hours.
2	Aliquot 1 mL of each culture into microfuge tube.
3	Centrifuge tubes for 5 min at 3000 rpm at room temperature.
4	Decant supernatant and resuspend in isovolume of saline (0.85% NaCl).
5	Aliquot 100 µL culture into a microfuge tube with 900 µL assay reagent.
6	Incubate at 30°C for 24-48 hours and observe color change.
7	Measure pH of cultures at beginning and end of assay.

D.2.3 GABA Quantification by LC-MS

Quantification of GABA by liquid-chromatography mass spectrometry (LC-MS) was performed according to Moore et al., (2021).

D.3 Results & Discussion

D.3.1 Rice and GlsX Assays

 Upon addition of enteric microorganism cultures (*E. cancerogenus*, *E. nimipressuralis*, and *P. agglomerans*) to the Rice (pH 3.14) and GlsX (pH 3.45) assay solutions the color of the solutions immediately turned from yellow to blue. Two ethanol rinse steps were incorporated into the protocol (Table D.7) to remove potentially interfering exopolysaccharides, but were unsuccessful in mitigating this immediate color change (Figure D.3). Addition of LAB to assay solutions initially remained yellow and took at least 15 minutes to begin causing color changes. Metagenomic analysis showed that the enteric organisms did not possess GAD, but did possess GlsX, therefore conversion of glutamate to GABA was not responsible for the Rice assay solution's immediate color change. Likewise, conversion of glutamine to glutamate was likely not responsible for the GlsX assay's blue color given the rapidity of the color change. According to Rice et al., (1994), color changes produced by a variety of enteric microorganisms took up to 4 hours. Likewise, Pennacchiette et al. (2018) reported that color changes in the GlsX assay were observed after 30 minutes to 2 hours, therefore, the immediate color change we observed was abnormal. Negative controls (tubes 1 & 2, *Lb. curvatus*) lacking genes for GAD and GlsX should have had no effect on the assay color, however we observed that these organisms caused a slight green/blue hue to develop in in the GlsX assay (Figure D.3), indicating a false positive. Similarly, one of the GAD-producing organisms (tube 4, *Lb. plantarum*) also caused a significant color change, indicating it possessed glutaminase (Figure D.3) This organism, *Lb. plantarum* WCFS1 has been well-studied and its fully annotated genome published by Kleerebezem et al. (2003). This organism does not possess genes encoding for glutaminase and the color change observed must be due to another metabolic reaction or a matrix effect of the prepared culture on the assay reagent. Organisms were transferred to fresh saline prior to inoculating into the assay reagents to reduce potential matrix effects. Glutaminase producing organisms (tubes 5 & 6) caused the assay reagent to lightly turn green, as expected (Figure D.3).

 Results of the Rice assay after 2 hours of incubation are presented in Figure D.4. The negative controls are slightly green hued (tubes 1 & 2) and the GAD-producing organisms (tubes 3 & 4) are slightly green as well, with greater color change in tube 4 (*Lb. plantarum* WCFS1). The GAD-negative, glutaminase-producing *Lb. casei* (tube 6) also caused a green hue to appear,

however this organism should not have caused any color changes. Both the Rice and GlsX assays produced false positive results and were unable to be used on enteric organisms isolated from cucumber fermentations. The authors of the GlsX assay were contacted by e-mail to inquire about the function of their assay on LAB and they responded explaining that they had not tried it on LAB. Development of functioning Rice and GlsX assays for LAB may take considerable time and resources, therefore we chose to proceed with adapting the MD assay which was already known to function with LAB.
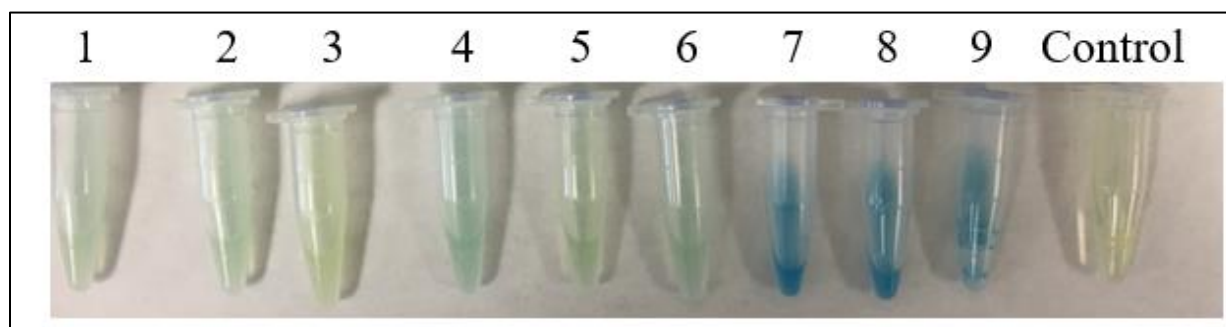
Figure D.3 Photograph of GlsX assay results for LAB and enteric organisms. Negative controls (1 & 2, *Lb. curvatus*), GAD producers (3, 4, *Lb. plantarum*), GlsX producers (5, *Lb. gasseri*; 6, *Lb. casei*), Enterics (7, *E. cancerogenus*; 8, *E. nimipressuralis*; 9, *P. agglomerans*), Control (GlsX assay reagent + saline)
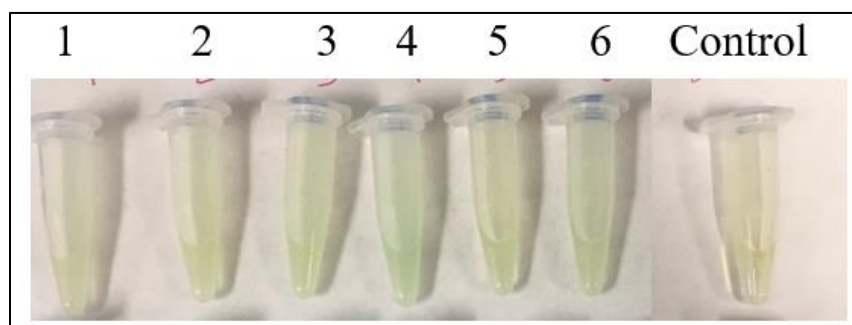
Figure D.4 Photograph of Rice assay results for LAB. Negative controls (1 & 2, *Lb. curvatus*), GAD producers (3, 4, *Lb. plantarum*), GlsX producers (5, *Lb. gasseri*; 6, *Lb. casei*), Control (Rice assay reagent + saline)

D.3.2 MDG Assay

Initial testing with MDG assay reagent revealed that the assay did not function with *E. coli* due to the fact that *E. coli* does not produce an acidic environment during growth. The MDG assay reagent begins with a pH of 7.0 and even if the *E. coli* is expressing GAD and GlsX the color will not change because it is already neutral (results not shown). Conversely, because the Rice and GlsX assays begin with a low pH (3.1 – 3.4) expression of GAD and GlsX by *E. coli* do cause an increase in pH and result in a color change to green or blue. Due to the number of reagents included in the adaptation of this assay (Table D.9), the number of organisms tested was reduced to four (Table D.4), two with GAD-producing capabilities and two with GlsX-producing capabilities. Two technical replicates (Rep A, Rep B) were performed for each MDG assay reagent beginning with propagation from frozen stock cultures. One set of controls was prepared using saline instead of starter cultures for the six MDG assay reagents. Results of this study after 24 hours of incubation are shown in Figure D.5. Controls prepared with saline remained blue and presented no color change, indicating the assay had no contamination. GAD-producing organisms (6, 7) caused significant color changes in the 0.25% glucose reagents (A, AE, AN) indicating their fermentation of glucose to lactic acid. *Lb. brevis* caused the glutamate-containing reagent (AE) to turn yellow-green, indicating production of GABA from glutamate to maintain higher pH. *Lb. plantarum* caused all six reagents to turn yellow, demonstrating its efficient fermentation of glucose to lactic acid. A slight green hue was observed for the BE treatment, indicating that for *Lb. plantarum*, lower levels of glucose were optimal for observing color change in this assay by limiting the amount of lactic acid produced. This organism may produce GABA but not at high enough levels to counteract the pH changes incurred by its lactic acid production. For *Lb. brevis* the 0.125% glucose reagents (B, BE, BN) remained blue-green and nearly indistinguishable from each other. These results indicate that for *Lb. brevis,* 0.125% glucose was not sufficient to produce enough lactic acid to turn the assay reagent yellow and that the 0.25% glucose reagent was optimal to visualize color changes. The assay functioned as expected with the *Lb. curvatus* negative control (tube 2) for both glucose levels, however replicates A and B were inconsistent and replicate B produced a false positive for the 0.125% glucose solutions. Likewise, results from replicates A and B were inconsistent for *Lb. gasseri* fermentations. Metagenomic analysis indicated that this strain of *Lb. gasseri* contains genes encoding for GlsX and not GAD, therefore treatment AN should have been more green or blue

than treatments A or AE, but it they were all blue green in Rep A. In Rep B, all tubes were yellow. The cause for inconsistency of these results could not be determined. Since GlsX activity was not directly measured in the assay we cannot be sure that *Lb. gasseri* produced GlsX under these conditions.
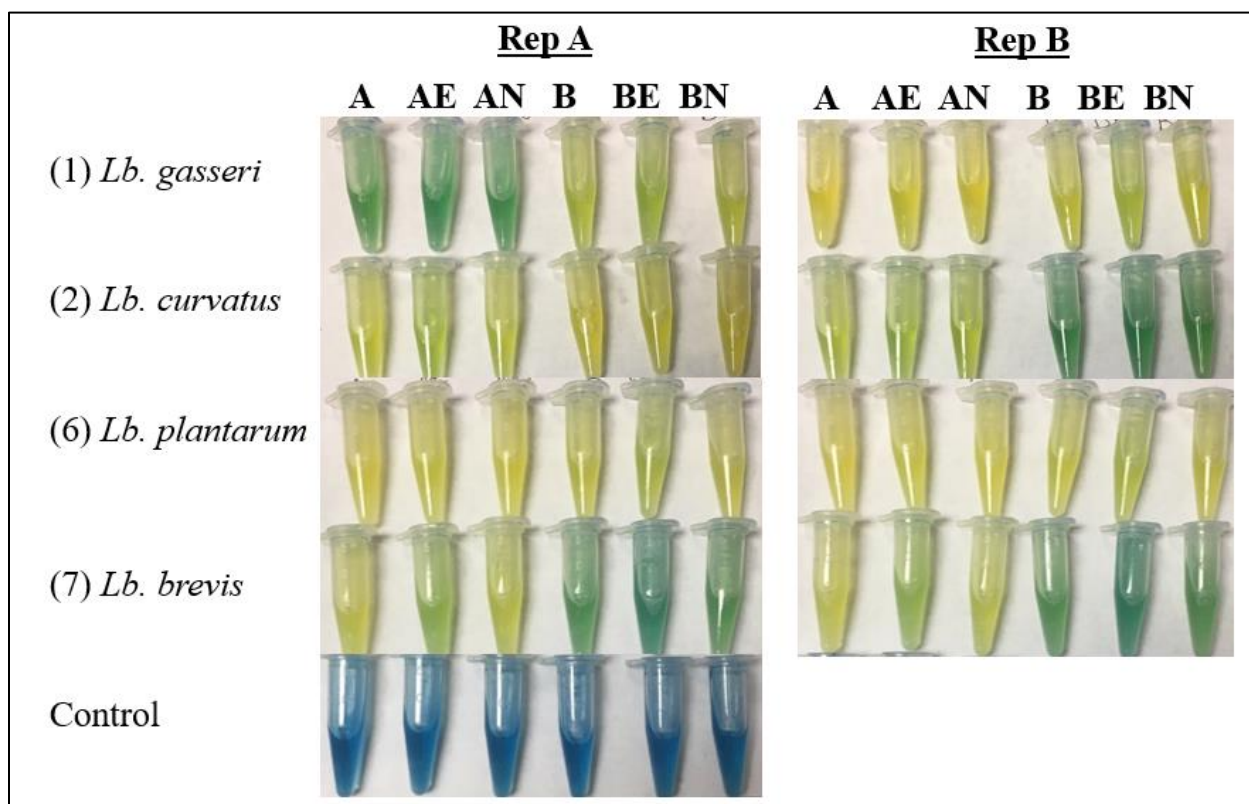
Figure D.5 Photograph of MDG assay results for LAB after 24 hours. A = 0.25% glucose control, AE = 0.25% glucose + glutamate, AN = 0.25% glucose + glutamine, B = 0.125% glucose control, BE = 0.125% glucose + glutamate, BN = 0.125% glucose + glutamine. Glutaminase producer (1, *Lb. gasseri*), negative control (*Lb. curvatus*), GAD producers (6, *Lb. plantarum*; 7, *Lb. brevis*), Control (MDG assay reagent + saline)

Quantification of GABA was performed for control MDG assay reagents and those containing glutamate (AE and BE) to determine if the color indicators aligned with quantitative measurements (Table D.11). False positive results were detected for *Lb. gasseri* as the color of the 0.25% glucose + glutamate reagent turned green but did not contain any GABA. Conversely, *Lb. plantarum* produced a significant amount of GABA in the 0.125% glucose + glutamate reagent (5.96 mM) but did not cause a color change. *Lb. curvatus* colors correctly aligned with a

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lack of GABA production. *Lb. brevis* produced significantly greater GABA than *Lb. plantarum* and had a correspondingly blue/green color. The MDG assay may be applicable to organisms that produce high amounts of GABA, as was the case with *Lb. brevis*, but it is not suitable to determine if lower amounts of GABA are produced, as was the case with *Lb. plantarum*. This assay may be suitable of identifying these "super" producers; however, it still produces false positives as with *Lb. gasseri* and therefore is not a reliable assay for organism screening.

Table D.11 GABA quantification (µM) of MDG assay reagents after 24 hours of incubation

ID No.	Organism	Enzymes	0.25% Glucose		0.125% Glucose		Does the color assay tell us if GABA is produced?
			"A"	"AE"	"B"	"BE"	
LA0257	*Lb. gasseri*	GlsX	25	0	24	0	NO - false positive
LA0272 A	*Lb. curvatus*	-	22	0	24	0	YES
LA1196	*Lb. plantarum*	GAD	925	492	1089	5958	NO - false negative
LA1129	*Lb. brevis*	GAD	700	42324	22	54821	YES

D.4 Conclusion

The Rice and GlsX assays as described in the literature did not produce the predicted results with LAB, but rather produced false positives for GAD and glutaminase negative organisms. Similarly, they produced false positives for the cucumber-associated enteric organisms tested, immediately turning the assay reagents blue. These assays were not optimized to function with LAB or enteric organisms isolated from cucumber fermentations. The MDG assay had similar flaws, including producing false positive and false negative results as confirmed by LC-MS quantification of GABA. None of the assays tested or adapted in this study are suitable for rapid screening of LAB for GAD and GlsX activity. Screening of organisms for GAD and GlsX activity during fermentation will need to be performed using a quantitative method such as LC-MS.

D.5 References

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Appendix E: Table S1. Food-derived Bioactive Peptides and Adducts Used in MSiReader Analysis of Fermented and Acidified Cucumbers

Table E.1 Food-derived Bioactive Peptides and Adducts Used in MSiReader Analysis of Fermented and Acidified Cucumbers

Sequence	m/z	Adduct	Activity	Food source	Reference	IC$_{50}$ value*
AF	219.113	[M+H-H$_2$O]$^+$	ACE inhibitory	Wheat	Matsui et al. (1999)	15.2 μM
AF	237.123	[M+H]$^+$	ACE inhibitory	Wheat	Matsui et al. (1999)	15.2 μM
AF	259.105	[M+Na]$^+$	ACE inhibitory	Wheat	Matsui et al. (1999)	15.2 μM
AH	209.103	[M+H-H$_2$O]$^+$	Antioxidant	Muscle tissue	Boldyrev et al. (1988); Chan and Decker (1994)	NA
AH	227.114	[M+H]$^+$	Antioxidant	Muscle tissue	Boldyrev et al. (1988); Chan and Decker (1994)	NA
AH	249.096	[M+Na]$^+$	Antioxidant	Muscle tissue	Boldyrev et al. (1988); Chan and Decker (1994)	NA
AKK	328.234	[M+H-H$_2$O]$^+$	ACE inhibitory	Sardine hydrolyzate	Matsufuji et al. (1994)	3.13 μM
AKK	346.245	[M+H]$^+$	ACE inhibitory	Sardine hydrolyzate	Matsufuji et al. (1994)	3.13 μM
AKK	368.227	[M+Na]$^+$	ACE inhibitory	Sardine hydrolyzate	Matsufuji et al. (1994)	3.13 μM
AP	169.097	[M+H-H$_2$O]$^+$	ACE inhibitory	Anchovy, sardine, bonito	Ichimura et al. (2003)	29 μM
AP	187.108	[M+H]$^+$	ACE inhibitory	Anchovy, sardine, bonito	Ichimura et al. (2003)	29 μM
AP	209.090	[M+Na]$^+$	ACE inhibitory	Anchovy, sardine, bonito	Ichimura et al. (2003)	29 μM
AVP	268.166	[M+H-H$_2$O]$^+$	ACE inhibitory	Casein hydrolyzate	Meisel (1993)	340 μM
AVP	286.176	[M+H]$^+$	ACE inhibitory	Casein hydrolyzate	Meisel (1993)	340 μM
AVP	308.158	[M+Na]$^+$	ACE inhibitory	Casein hydrolyzate	Meisel (1993)	340 μM
AW	258.124	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk	Rutella et al. (2016)	5 μM
AW	276.134	[M+H]$^+$	ACE inhibitory	Milk	Rutella et al. (2016)	5 μM
AW	298.116	[M+Na]$^+$	ACE inhibitory	Milk	Rutella et al. (2016)	5 μM
AY	235.108	[M+H-H$_2$O]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	100 μM

Table E.1 (cont.)

AY	253.118	[M+H]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	100 μM
AY	275.100	[M+Na]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	100 μM
DG	173.056	[M+H-H$_2$O]$^+$	ACE inhibitory	Soy protein hydrolyzate	Wu and Ding (2002)	12.3 μM
DG	191.066	[M+H]$^+$	ACE inhibitory	Soy protein hydrolyzate	Wu and Ding (2002)	12.3 μM
DG	213.048	[M+Na]$^+$	ACE inhibitory	Soy protein hydrolyzate	Wu and Ding (2002)	12.3 μM
DLP	326.171	[M+H-H$_2$O]$^+$	ACE inhibitory	Soy protein hydrolyzate	Wu and Ding (2002)	4.8 μM
DLP	344.182	[M+H]$^+$	ACE inhibitory	Soy protein hydrolyzate	Wu and Ding (2002)	4.8 μM
DLP	366.164	[M+Na]$^+$	ACE inhibitory	Soy protein hydrolyzate	Wu and Ding (2002)	4.8 μM
EF	277.118	[M+H-H$_2$O]$^+$	ACE inhibitory	Pea	Li and Aluko (2010)	2980 μM
EF	295.129	[M+H]$^+$	ACE inhibitory	Pea	Li and Aluko (2010)	2980 μM
EF	317.111	[M+Na]$^+$	ACE inhibitory	Pea	Li and Aluko (2010)	2980 μM
FAL	332.197	[M+H-H$_2$O]$^+$	ACE inhibitory	Micro algae hydrolyzate	Suetsuna and Chen (2001)	26.3 μM
FAL	350.207	[M+H]$^+$	ACE inhibitory	Micro algae hydrolyzate	Suetsuna and Chen (2001)	26.3 μM
FAL	372.189	[M+Na]$^+$	ACE inhibitory	Micro algae hydrolyzate	Suetsuna and Chen (2001)	26.3 μM
FGK	333.192	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk	Maruyama et al. (1987a)	160 μM
FGK	351.203	[M+H]$^+$	ACE inhibitory	Milk	Maruyama et al. (1987a)	160 μM
FGK	373.185	[M+Na]$^+$	ACE inhibitory	Milk	Maruyama et al. (1987a)	160 μM
FK	276.171	[M+H-H$_2$O]$^+$	ACE inhibitory	Sweet potato	Huang et al. (2011)	265 μM
FK	294.181	[M+H]$^+$	ACE inhibitory	Sweet potato	Huang et al. (2011)	265 μM
FK	316.163	[M+Na]$^+$	ACE inhibitory	Sweet potato	Huang et al. (2011)	265 μM
FP	245.129	[M+H-H$_2$O]$^+$	ACE inhibitory	Whey protein	Abubakar et al. (1998)	315 μM
FP	263.139	[M+H]$^+$	ACE inhibitory	Whey protein	Abubakar et al. (1998)	315 μM
FP	285.121	[M+Na]$^+$	ACE inhibitory	Whey protein	Abubakar et al. (1998)	315 μM
FQ	276.134	[M+H-H$_2$O]$^+$	ACE inhibitory	Buckwheat sprouts	Koyama et al. (2013)	NR

Table E.1 (cont.)

FQ	294.1449	[M+H]$^+$	ACE inhibitory	Buckwheat sprouts	Koyama et al. (2013)	NR
FQ	316.127	[M+Na]$^+$	ACE inhibitory	Buckwheat sprouts	Koyama et al. (2013)	NR
FY	311.139	[M+H-H$_2$O]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	25 μM
FY	329.150	[M+H]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	25 μM
FY	351.132	[M+Na]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	25 μM
GEP	284.124	[M+H-H$_2$O]$^+$	ACE inhibitory	Mushroom	Lee et al. (2004)	3200 μM
GEP	302.1347	[M+H]$^+$	ACE inhibitory	Mushroom	Lee et al. (2004)	3200 μM
GEP	324.117	[M+Na]$^+$	ACE inhibitory	Mushroom	Lee et al. (2004)	3200 μM
GF	205.097	[M+H-H$_2$O]$^+$	ACE inhibitory	Garlic	Suetsuna (1998)	278 μM
GF	223.108	[M+H]$^+$	ACE inhibitory	Garlic	Suetsuna (1998)	278 μM
GF	245.090	[M+Na]$^+$	ACE inhibitory	Garlic	Suetsuna (1998)	278 μM
GFR	361.198	[M+H-H$_2$O]$^+$	ACE inhibitory	Sweet potato	Huang et al. (2011)	94 μM
GFR	379.209	[M+H]$^+$	ACE inhibitory	Sweet potato	Huang et al. (2011)	94 μM
GGY	278.114	[M+H]$^+$	ACE inhibitory	Sake and sake lees	Saito et al. (1994)	1.3 μM
GGY	296.124	[M+Na]$^+$	ACE inhibitory	Sake and sake lees	Saito et al. (1994)	1.3 μM
GGY	318.106	[M+H-H$_2$O]$^+$	ACE inhibitory	Sake and sake lees	Saito et al. (1994)	1.3 μM
GHS	282.120	[M+H]$^+$	ACE inhibitory	Rapeseed	He et al. (2013)	1740 μM
GHS	300.130	[M+Na]$^+$	ACE inhibitory	Rapeseed	He et al. (2013)	1740 μM
GHS	322.112	[M+H-H$_2$O]$^+$	ACE inhibitory	Rapeseed	He et al. (2013)	1740 μM
GKP	283.1765	[M+H]$^+$	ACE inhibitory	Whey protein	Abubakar et al. (1998)	352 μM
GKP	301.187	[M+Na]$^+$	ACE inhibitory	Whey protein	Abubakar et al. (1998)	352 μM
GKP	323.169	[M+H-H$_2$O]$^+$	ACE inhibitory	Whey protein	Abubakar et al. (1998)	352 μM
GPP	252.134	[M+H]$^+$	ACE inhibitory	Buckwheat	Ma et al. (2006)	23 μM
GPP	270.145	[M+Na]$^+$	ACE inhibitory	Buckwheat	Ma et al. (2006)	23 μM
GPP	292.127	[M+H-H$_2$O]$^+$	ACE inhibitory	Buckwheat	Ma et al. (2006)	23 μM
GPV	254.150	[M+H]$^+$	ACE inhibitory	Bovine skin hydrolyzate	Kim et al. (2001)	4.67 μM

Table E.1 (cont.)

GPV	272.161	[M+Na]+	ACE inhibitory	Bovine skin hydrolyzate	Kim et al. (2001)	4.67 µM
GPV	294.142	[M+H-H2O]+	ACE inhibitory	Bovine skin hydrolyzate	Kim et al. (2001)	4.67 µM
GQY	349.151	[M+H]+	ACE inhibitory	Sweet potato	Ishiguro et al. (2012)	52.3 µM
GQY	367.161	[M+Na]+	ACE inhibitory	Sweet potato	Ishiguro et al. (2012)	52.3 µM
GQY	389.143	[M+H-H2O]+	ACE inhibitory	Sweet potato	Ishiguro et al. (2012)	52.3 µM
GY	221.092	[M+H-H2O]+	ACE inhibitory	Garlic	Suetsuna (1998)	72.1 µM
GY	239.103	M+H (calc)	ACE inhibitory	Garlic	Suetsuna (1998)	72.1 µM
GY	261.085	[M+Na]+	ACE inhibitory	Garlic	Suetsuna (1998)	72.1 µM
HHL	388.209	[M+H-H2O]+	ACE inhibitory	Fermented soybean paste	Shin et al. (2001)	5 µM
IA	185.129	[M+H-H2O]+	ACE inhibitory	Soy protein hydrolysate	Chen et al. (2002)	153 µM
IA	203.139	[M+H]+	ACE inhibitory	Soy protein hydrolysate	Chen et al. (2002)	153 µM
IA	225.121	[M+Na]+	ACE inhibitory	Soy protein hydrolysate	Chen et al. (2002)	153 µM
IAE	314.171	[M+H-H2O]+	ACE inhibitory	Micro algae hydrolyzate	Suetsuna and Chen (2001)	34.7 µM
IAE	332.182	[M+H]+	ACE inhibitory	Micro algae hydrolyzate	Suetsuna and Chen (2001)	34.7 µM
IAE	354.164	[M+Na]+	ACE inhibitory	Micro algae hydrolyzate	Suetsuna and Chen (2001)	34.7 µM
IAP	282.181	[M+H-H2O]+	ACE inhibitory	Wheat	Motoi and Kodama (2003)	2.7 µM
IAP	300.192	[M+H]+	ACE inhibitory	Wheat	Motoi and Kodama (2003)	2.7 µM
IAP	322.174	[M+Na]+	ACE inhibitory	Wheat	Motoi and Kodama (2003)	2.7 µM
IAPG	339.203	[M+H-H2O]+	ACE inhibitory	Micro algae hydrolyzate	Suetsuna and Chen (2001)	11.4 µM
IAPG	357.213	[M+H]+	ACE inhibitory	Micro algae hydrolyzate	Suetsuna and Chen (2001)	11.4 µM

Table E.1 (cont.)

IAPG	379.195	[M+Na]$^+$	ACE inhibitory	Micro algae hydrolyzate	Suetsuna and Chen (2001)	11.4 μM
II	227.175	[M+H-H$_2$O]$^+$	Cellular glucose uptake stimulator	Whey protein hydrolyzate	Morifuji et al. (2009)	NA
II	245.186	[M+H]$^+$	Cellular glucose uptake stimulator	Whey protein hydrolyzate	Morifuji et al. (2009)	NA
II	267.168	[M+Na]$^+$	Cellular glucose uptake stimulator	Whey protein hydrolyzate	Morifuji et al. (2009)	NA
IIP	324.228	[M+H-H$_2$O]$^+$	ACE inhibitory	Sweet potato	Ishiguro et al. (2012)	80.8 μM
IIP	342.239	[M+H]$^+$	ACE inhibitory	Sweet potato	Ishiguro et al. (2012)	80.8 μM
IIP	364.221	[M+Na]$^+$	ACE inhibitory	Sweet potato	Ishiguro et al. (2012)	80.8 μM
IKP	339.239	[M+H-H$_2$O]$^+$	ACE inhibitory	Bonito	Yokoyama, Chiba and Yoshikawa (1992)	1.7 μM
IKP	357.250	[M+H]$^+$	ACE inhibitory	Bonito	Yokoyama, Chiba and Yoshikawa (1992)	1.7 μM
IKP	379.232	[M+Na]$^+$	ACE inhibitory	Bonito	Yokoyama, Chiba and Yoshikawa (1992)	1.7 μM
IPP	308.197	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk, (*Lb. helveticus*)	Nakamura et al. (1995)	5 μM
IPP	326.207	[M+H]$^+$	ACE inhibitory	Milk, (*Lb. helveticus*)	Nakamura et al. (1995)	5 μM
IPP	348.189	[M+Na]$^+$	ACE inhibitory	Milk, (*Lb. helveticus*)	Nakamura et al. (1995)	5 μM
IR	270.1925	[M+H-H$_2$O]$^+$	ACE inhibitory	Pea	Li and Aluko (2010)	2250 μM
IR	288.203	M+H (calc)	ACE inhibitory	Pea	Li and Aluko (2010)	2250 μM
IR	310.185	[M+Na]$^+$	ACE inhibitory	Pea	Li and Aluko (2010)	2250 μM
ITF	362.207	[M+H-H$_2$O]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	49 μM
ITF	380.218	[M+H]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	49 μM
ITP	312.192	[M+H-H$_2$O]$^+$	ACE inhibitory	Sweet potato	Ishiguro et al. (2012)	9.5 μM
ITP	330.202	[M+H]$^+$	ACE inhibitory	Sweet potato	Ishiguro et al. (2012)	9.5 μM
ITP	352.184	[M+Na]$^+$	ACE inhibitory	Sweet potato	Ishiguro et al. (2012)	9.5 μM

Table E.1 (cont.)

IV	213.160	[M+H-H$_2$O]$^+$	Cellular glucose uptake stimulator	Whey protein hydrolyzate	Morifuji et al. (2009)	NA
IV	231.170	[M+H]$^+$	Cellular glucose uptake stimulator	Whey protein hydrolyzate	Morifuji et al. (2009)	NA
IV	253.152	[M+Na]$^+$	Cellular glucose uptake stimulator	Whey protein hydrolyzate	Morifuji et al. (2009)	NA
IVY	376.223	[M+H-H$_2$O]$^+$	ACE inhibitory	Wheat, Sesame	Matsui et al. (1999), Nakano et al. (2006)	0.48 µM, 14.74 µM
IVY	394.234	[M+H]$^+$	ACE inhibitory	Wheat, Sesame	Matsui et al. (1999), Nakano et al. (2006)	0.48 µM, 14.74 µM
IY	277.155	[M+H-H$_2$O]$^+$	ACE inhibitory	Sake and sake lees	Saito et al. (1994)	2.4 µM
IY	295.165	[M+H]$^+$	ACE inhibitory	Sake and sake lees	Saito et al. (1994)	2.4 µM
IY	317.147	[M+Na]$^+$	ACE inhibitory	Sake and sake lees	Saito et al. (1994)	2.4 µM
KA	200.1394	[M+H-H$_2$O]$^+$	Hypotriglyceridemic	Soy	Inoue et al. (2011)	NA
KA	218.150	[M+H]$^+$	Hypotriglyceridemic	Soy	Inoue et al. (2011)	NA
KA	240.1319	[M+Na]$^+$	Hypotriglyceridemic	Soy	Inoue et al. (2011)	NA
KP	226.155	[M+H-H$_2$O]$^+$	ACE inhibitory	Anchovy	Ichimura et al. (2003)	22 µM
KP	244.166	[M+H]$^+$	ACE inhibitory	Anchovy	Ichimura et al. (2003)	22 µM
KP	266.148	[M+Na]$^+$	ACE inhibitory	Anchovy	Ichimura et al. (2003)	22 µM
KW	315.182	[M+H-H$_2$O]$^+$	ACE inhibitory	Sardine hydrolyzate	Matsufuji et al. (1994)	1.63 µM
KW	333.192	[M+H]$^+$	ACE inhibitory	Sardine hydrolyzate	Matsufuji et al. (1994)	1.63 µM
KW	355.174	[M+Na]$^+$	ACE inhibitory	Sardine hydrolyzate	Matsufuji et al. (1994)	1.63 µM
LAN	299.171	[M+H-H$_2$O]$^+$	Zinc chelating	Sesame	Wang et al. (2012)	NA
LAN	317.182	[M+H]$^+$	Zinc chelating	Sesame	Wang et al. (2012)	NA
LAN	339.164	[M+Na]$^+$	Zinc chelating	Sesame	Wang et al. (2012)	NA
LGI	284.197	[M+H-H$_2$O]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	29 µM
LGI	302.207	[M+H]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	29 µM
LGI	324.189	[M+Na]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	29 µM

Table E.1 (cont.)

LLF	374.244	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk	Hernández-Ledesma et al. (2002)	79.8 μM
LLF	392.254	[M+H]$^+$	ACE inhibitory	Milk	Hernández-Ledesma et al. (2002)	79.8 μM
LN	228.134	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk	van Platerink et al. (2008)	NR
LN	246.145	[M+H]$^+$	ACE inhibitory	Milk	van Platerink et al. (2008)	NR
LN	268.127	[M+Na]$^+$	ACE inhibitory	Milk	van Platerink et al. (2008)	NR
LPP	308.197	[M+H-H$_2$O]$^+$	ACE inhibitory	Maiz	Maruyama et al. (1989b)	9.6 μM
LPP	326.207	[M+H]$^+$	ACE inhibitory	Maiz	Maruyama et al. (1989b)	9.6 μM
LPP	348.189	[M+Na]$^+$	ACE inhibitory	Maiz	Maruyama et al. (1989b)	9.6 μM
LRP	367.245	[M+H-H$_2$O]$^+$	ACE inhibitory	Wheat, Maiz	Nogata et al. (2009), Miyoshi et al. (1991)	0.21 μM, 0.27 μM
LRP	385.256	[M+H]$^+$	ACE inhibitory	Wheat, Maiz	Nogata et al. (2009), Miyoshi et al. (1991)	0.21 μM, 0.27 μM
LSA	272.161	[M+H-H$_2$O]$^+$	ACE inhibitory	Sesame	Nakano et al. (2006)	7.81μM
LSA	290.171	[M+H]$^+$	ACE inhibitory	Sesame	Nakano et al. (2006)	7.81μM
LSA	312.153	[M+Na]$^+$	ACE inhibitory	Sesame	Nakano et al. (2006)	7.81μM
LSP	298.176	[M+H-H$_2$O]$^+$	ACE inhibitory	Maiz	Miyoshi et al. (1991)	1.7 μM
LSP	316.187	[M+H]$^+$	ACE inhibitory	Maiz	Miyoshi et al. (1991)	1.7 μM
LSP	338.1687	[M+Na]$^+$	ACE inhibitory	Maiz	Miyoshi et al. (1991)	1.7 μM
LVR	369.261	[M+H-H$_2$O]$^+$	ACE inhibitory	Fig tree latex	Maruyama et al. (1989a)	14 μM
LVR	387.271	[M+H]$^+$	ACE inhibitory	Fig tree latex	Maruyama et al. (1989a)	14 μM
MNP	343.144	[M+H-H$_2$O]$^+$	ACE inhibitory	Porcine skeletal muscle	Arihara et al. (2001)	66.6 μM
MNP	361.154	[M+H]$^+$	ACE inhibitory	Porcine skeletal muscle	Arihara et al. (2001)	66.6 μM
MNP	383.136	[M+Na]$^+$	ACE inhibitory	Porcine skeletal muscle	Arihara et al. (2001)	66.6 μM
NCS	305.091	[M+H-H$_2$O]$^+$	Zinc chelating	Sesame	Wang et al. (2012)	NA

Table E.1 (cont.)

NCS	323.102	[M+H]$^+$	Zinc chelating	Sesame	Wang et al. (2012)	NA
NCS	345.084	[M+Na]$^+$	Zinc chelating	Sesame	Wang et al. (2012)	NA
NF	262.119	[M+H-H$_2$O]$^+$	ACE inhibitory	Garlic	Suetsuna (1998)	46.3 μM
NF	280.1292	[M+H]$^+$	ACE inhibitory	Garlic	Suetsuna (1998)	46.3 μM
NF	302.111	[M+Na]$^+$	ACE inhibitory	Garlic	Suetsuna (1998)	46.3 μM
NPP	309.156	[M+H-H$_2$O]$^+$	ACE inhibitory	Porcine skeletal muscle	Arihara et al. (2001)	290.5 μM
NPP	327.166	[M+H]$^+$	ACE inhibitory	Porcine skeletal muscle	Arihara et al. (2001)	290.5 μM
NPP	349.148	[M+Na]$^+$	ACE inhibitory	Porcine skeletal muscle	Arihara et al. (2001)	290.5 μM
PHH	372.178	[M+H-H$_2$O]$^+$	Antioxidant	Soy	Chen et al. (1996)	NA
PHH	390.188	[M+H]$^+$	Antioxidant	Soy	Chen et al. (1996)	NA
PLW	397.223	[M+H-H$_2$O]$^+$	Antihypertensive	Milk	Maruyama et al. (1987b)	18 μM
PQR	382.220	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk	Maruyama et al. (1987a)	400 μM
PR	254.1612	[M+H-H$_2$O]$^+$	ACE inhibitory	Sake and sake lees	Saito et al. (1994)	4.1 μM
PR	272.172	[M+H]$^+$	ACE inhibitory	Sake and sake lees	Saito et al. (1994)	4.1 μM
PR	294.154	[M+Na]$^+$	ACE inhibitory	Sake and sake lees	Saito et al. (1994)	4.1 μM
PSY	348.155	[M+H-H$_2$O]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	16 μM
PSY	366.166	[M+H]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	16 μM
PSY	388.148	[M+Na]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	16 μM
PYP	358.176	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk	Maruyama et al. (1987a)	220 μM
PYP	376.187	[M+H]$^+$	ACE inhibitory	Milk	Maruyama et al. (1987a)	220 μM
PYP	398.169	[M+Na]$^+$	ACE inhibitory	Milk	Maruyama et al. (1987a)	220 μM
RF	304.1768	[M+H-H$_2$O]$^+$	ACE inhibitory	Sweet potato	Huang et al. (2008)	392.2 μM
RF	322.1874	[M+H]$^+$	ACE inhibitory	Sweet potato	Huang et al. (2008)	392.2 μM
RF	344.169	[M+Na]$^+$	ACE inhibitory	Sweet potato	Huang et al. (2008)	392.2 μM
RY	320.172	[M+H-H$_2$O]$^+$	ACE inhibitory	Sardine hydrolyzate, Sake and sake lees	Matsufuji et al. (1994), Saito et al. (1994)	51 μM, 10.5μM

Table E.1 (cont.)

RY	338.182	[M+H]$^+$	ACE inhibitory	Sardine hydrolyzate, Sake and sake lees	Matsufuji et al. (1994), Saito et al. (1994)	51 µM, 10.5µM
RY	360.164	[M+Na]$^+$	ACE inhibitory	Sardine hydrolyzate, Sake and sake lees	Matsufuji et al. (1994), Saito et al. (1994)	51 µM, 10.5µM
SM	219.080	[M+H-H$_2$O]$^+$	Zinc chelating	Sesame	Wang et al. (2012)	NA
SM	237.090	[M+H]$^+$	Zinc chelating	Sesame	Wang et al. (2012)	NA
SM	259.072	[M+Na]$^+$	Zinc chelating	Sesame	Wang et al. (2012)	NA
SY	251.103	[M+H-H$_2$O]$^+$	ACE inhibitory	Garlic	Suetsuna (1998)	66.3 µM
SY	269.113	[M+H]$^+$	ACE inhibitory	Garlic	Suetsuna (1998)	66.3 µM
SY	291.095	[M+Na]$^+$	ACE inhibitory	Garlic	Suetsuna (1998)	66.3 µM
TF	249.123	[M+H-H$_2$O]$^+$	ACE inhibitory	Wheat	Matsui et al. (1999), Nogata et al. (2009)	17.8 µM, 18 µM
TF	267.134	[M+H]$^+$	ACE inhibitory	Wheat	Matsui et al. (1999), Nogata et al. (2009)	17.8 µM, 18 µM
TF	289.116	[M+Na]$^+$	ACE inhibitory	Wheat	Matsui et al. (1999), Nogata et al. (2009)	17.8 µM, 18 µM
TNP	313.151	[M+H-H$_2$O]$^+$	ACE inhibitory	Porcine skeletal muscle	Arihara et al. (2001)	207.4 µM
TNP	331.161	[M+H]$^+$	ACE inhibitory	Porcine skeletal muscle	Arihara et al. (2001)	207.4 µM
TNP	353.143	[M+Na]$^+$	ACE inhibitory	Porcine skeletal muscle	Arihara et al. (2001)	207.4 µM
TVY	364.187	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk	Tauzin et al. (2002)	15 µM
TVY	382.197	[M+H]$^+$	ACE inhibitory	Milk	Tauzin et al. (2002)	15 µM
VAE	300.155	[M+H-H$_2$O]$^+$	ACE inhibitory	Buckwheat sprouts	Koyama et al. (2013)	NR
VAE	318.166	[M+H]$^+$	ACE inhibitory	Buckwheat sprouts	Koyama et al. (2013)	NR
VAE	340.148	[M+Na]$^+$	ACE inhibitory	Buckwheat sprouts	Koyama et al. (2013)	NR
VF	265.1547	[M+H]$^+$	ACE inhibitory	Wheat, Red-mold rice extracts	Matsui et al. (1999), Kuba et al. (2009)	9.2 µM, 47.9 µM
VF	287.137	[M+Na]$^+$	ACE inhibitory	Wheat, Red-mold rice extracts	Matsui et al. (1999), Kuba et al. (2009)	9.2 µM, 47.9 µM

Table E.1 (cont.)

VF	247.144	[M+H-H$_2$O]$^+$	ACE inhibitory	Wheat, Red-mold rice extracts	Matsui et al. (1999), Kuba et al. (2009)	9.2 µM, 47.9 µM
VK	228.171	[M+H-H$_2$O]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	13 µM
VK	246.181	[M+H]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	13 µM
VK	268.163	[M+Na]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	13 µM
VKP	325.223	[M+H-H$_2$O]$^+$	ACE inhibitory	Jellyfish	Li et al. (2014)	1.3 µM
VKP	343.234	[M+H]$^+$	ACE inhibitory	Jellyfish	Li et al. (2014)	1.3 µM
VKP	365.216	[M+Na]$^+$	ACE inhibitory	Jellyfish	Li et al. (2014)	1.3 µM
VNP	311.171	[M+H-H$_2$O]$^+$	ACE inhibitory	Rice	Chen et al. (2013)	6.4 µM
VNP	329.182	[M+H]$^+$	ACE inhibitory	Rice	Chen et al. (2013)	6.4 µM
VNP	351.164	[M+Na]$^+$	ACE inhibitory	Rice	Chen et al. (2013)	6.4 µM
VPP	294.181	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk, (*Lb. helveticus*)	Nakamura et al. (1995)	9 µM
VPP	312.192	[M+H]$^+$	ACE inhibitory	Milk, (*Lb. helveticus*)	Nakamura et al. (1995)	9 µM
VPP	334.174	[M+Na]$^+$	ACE inhibitory	Milk, (*Lb. helveticus*)	Nakamura et al. (1995)	9 µM
VRP	353.230	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk	Kohmura et al. (1990)	2.2 µM
VRP	371.240	[M+H]$^+$	ACE inhibitory	Milk	Kohmura et al. (1990)	2.2 µM
VRP	393.222	[M+Na]$^+$	ACE inhibitory	Milk	Kohmura et al. (1990)	2.2 µM
VSV	286.176	[M+H-H$_2$O]$^+$	ACE inhibitory	Canola	Wu et al. (2008)	0.15 µM
VSV	304.187	[M+H]$^+$	ACE inhibitory	Canola	Wu et al. (2008)	0.15 µM
VSV	326.169	[M+Na]$^+$	ACE inhibitory	Canola	Wu et al. (2008)	0.15 µM
VVG	256.166	[M+H-H$_2$O]$^+$	ACE inhibitory	Buckwheat sprouts	Koyama et al. (2013)	NR
VVG	274.176	[M+H]$^+$	ACE inhibitory	Buckwheat sprouts	Koyama et al. (2013)	NR
VVG	296.158	[M+Na]$^+$	ACE inhibitory	Buckwheat sprouts	Koyama et al. (2013)	NR
VW	286.155	[M+H-H$_2$O]$^+$	ACE inhibitory	Rapeseed, Red-mold rice extract	Marczak et al. (2003), Kuba et al. (2009)	1.6 µM
VW	304.166	[M+H]$^+$	ACE inhibitory	Rapeseed, Red-mold rice extract	Marczak et al. (2003), Kuba et al. (2009)	1.6 µM

Table E.1 (cont.)

VW	326.148	[M+Na]$^+$	ACE inhibitory	Rapeseed, Red-mold rice extract	Marczak et al. (2003), Kuba et al. (2009)	1.6 µM
VWP	383.208	[M+H-H$_2$O]$^+$	ACE inhibitory	Rice	Chen et al. (2013)	4.5 µM
VY	263.139	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk, Wheat	Rutella et al. (2016), Nogata et al. (2009)	5µM, 21 µM
VY	281.150	[M+H]$^+$	ACE inhibitory	Milk, Wheat	Rutella et al. (2016), Nogata et al. (2009)	5µM, 21 µM
VY	303.132	[M+Na]$^+$	ACE inhibitory	Milk, Wheat	Rutella et al. (2016), Nogata et al. (2009)	5µM, 21 µM
YGL	334.176	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk	Pihlanto-Leppälä et al. (2000)	409 µM
YGL	352.187	[M+H]$^+$	ACE inhibitory	Milk	Pihlanto-Leppälä et al. (2000)	409 µM
YGL	374.169	[M+Na]$^+$	ACE inhibitory	Milk	Pihlanto-Leppälä et al. (2000)	409 µM
YP	261.123	[M+H-H$_2$O]$^+$	ACE inhibitory	Milk (*Lb. helveticus* CPN4)	Yamamoto et al. (1999)	720 µM
YP	279.1340	[M+H]$^+$	ACE inhibitory	Milk (*Lb. helveticus* CPN4)	Yamamoto et al. (1999)	720 µM
YP	301.1159	[M+Na]$^+$	ACE inhibitory	Milk (*Lb. helveticus* CPN4)	Yamamoto et al. (1999)	720 µM
YPK	389.218	[M+H-H$_2$O]$^+$	ACE inhibitory	Broccoli	Lee et al. (2006)	23.7 µM
YQ	292.129	[M+H-H$_2$O]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	628 µM
YQ	310.140	[M+H]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	628 µM
YQ	332.122	[M+Na]$^+$	ACE inhibitory	Buckwheat	Li et al. (2002)	628 µM

*IC$_{50}$ = concentration that achieves 50% inhibition of ACE activity. NA = not applicable. NR = not reported.