Food Chemistry 271 (2019) 715–723

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

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

Jennifer Fideler[a,b], Suzanne D. Johanningsmeier[b,a,*], Måns Ekelöf[c], David C. Muddiman[c]

[a] North Carolina State University, Department of Food, Bioprocessing and Nutrition Sciences, Raleigh, NC 27695, USA
[b] U.S. Department of Agriculture, Agricultural Research Service, SEA, Food Science Research Unit, 322 Schaub Hall, Box 7624, North Carolina State University, Raleigh, NC 27695-7624, USA
[c] North Carolina State University, Department of Chemistry, Raleigh, NC 27695, USA

ARTICLE INFO

Keywords:
Lactic acid fermentation
ACE-inhibitory peptides
Isoleucine-proline-proline
Leucine-proline-proline
Valine-proline-proline
Vegetable composition

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. In this study, infrared, matrix-assisted laser desorption electrospray ionization (IR-MALDESI) mass spectrometry (MS) was employed to identify bioactive peptides in fermented cucumber. 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.

1. 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 et al., 2017; Kayser & Meisel, 1996; Koyama et al., 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

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* Corresponding author at: U.S. Department of Agriculture, Agricultural Research Service, SEA, Food Science Research Unit, 322 Schaub Hall, Box 7624, North Carolina State University, Raleigh, NC 27695-7624, USA.
E-mail address: suzanne.johanningsmeier@ars.usda.gov (S.D. Johanningsmeier).

https://doi.org/10.1016/j.foodchem.2018.07.187
Received 13 March 2018; Received in revised form 15 July 2018; Accepted 25 July 2018
Available online 31 July 2018
0308-8146/ Published by Elsevier Ltd.

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, chap. 51). 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 and 140 mM lactic acid is produced and the pH equilibrates near 3.2–3.6 (Pérez-Díaz et al., 2013, chap. 51). 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 from vinegar, 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, 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 $LC\text{-}MS^n$ 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, & Gobbetti, 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 desorped 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. Materials and methods

2.1. Chemicals and materials

Pickling cucumbers, pickling salt (sodium chloride, NaCl, ≥ 99%), and vinegar (acetic acid, 20%) were obtained from Mount Olive Pickle Company (Mount Olive, NC, USA). Calcium chloride ($CaCl_2$, ≥ 93%), hydrochloric acid (HCl, ≥ 37%), sulfuric acid (H_2SO_4 3 N) and lactic acid (≥ 85%) were purchased from Sigma-Aldrich (St. Louis, MO, USA); calcium hydroxide ($Ca(OH)_2$, ≥ 95%) was purchased from Fisher Scientific (Hampton, NH, USA); and sodium benzoate (≥ 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); and MS-grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitrogen gas (≥ 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, ≥ 98%), valine-proline-proline (VPP, ≥ 99.5%), leucine-proline-proline (LPP, ≥ 99%), arginine-tyrosine (RY, ≥ 99%), and lysine-proline (KP, ≥ 99%). Amicon Ultra-0.5 filters with a 3 kDa cutoff were purchased from Fisher Scientific (Hampton, NH, USA).

2.2. 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 while preventing fermentation with the addition of sodium benzoate.

2.3. 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 6.4×10^5 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.

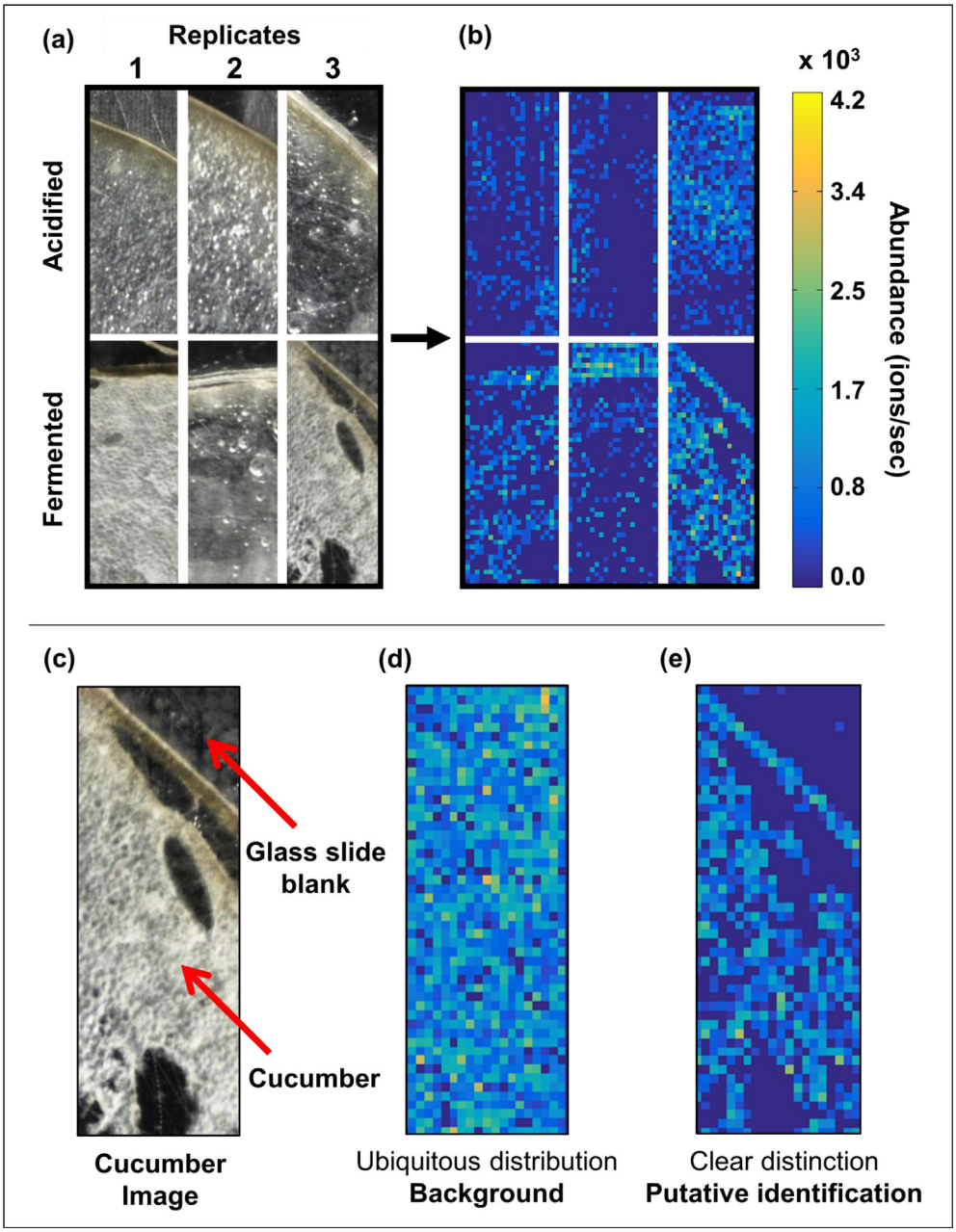

Fig. 1. MS imaging interpretation (a) Image orientation of acidified and fermented cucumber areas sampled for MS analysis (b) Abundance (AU) heat map of lys-pro $[M+H^+]^+$ (m/z 244.1656) in acidified and fermented cucumbers (c) Magnified image of fermented cucumber replicate 3 (d) MS heat map of gly-glu-phe $[M+H^+]^+$ (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).

2.4. 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 9000 × *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 (0.5–100 mM) for all analytes.

2.5. 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 to 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 × *g* for 5 min. Supernatants were filtered through 3 kDa centrifugal filters for 15 min at 11,000 × *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 390 nm/Em 475 nm 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 $\alpha = 0.05$ using JMP®, Version 13 (SAS Institute Inc., Cary, NC, 1989-2007). Results are reported as mM and mg/kg IPP equivalents.

2.6. Bioactive peptide discovery by IR-MALDESI

2.6.1. 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 h. 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 ($\lambda = 2.94$ 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 to 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 higher energy collisional dissociation (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 to 200 *m/z* and 100–400 *m/z*.

2.6.2. IR-MALDESI MS data analysis

An internal database of 86 bioactive di- and tripeptides previously discovered in foods was compiled from the scientific literature (See Supplementary Material Table S1). 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.

2.7. 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 × *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 $\alpha = 0.05$ 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.

3. Results and discussion

3.1. 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.

3.2. 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

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

Peptide (precursor *m/z*)	Retention Time (min)	Product Ion (Collision Energy)	Reference Ions (Collision Energy)	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-hoc Tukey HSD test. LOD: limit of detection. LOQ: limit of quantification.

† 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.

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.

3.3. Bioactive peptide discovery by IR-MALDESI mass spectrometry

MS data was examined for the $[M+H^+]^+$, $[M+H^+\text{-}H_2O]^+$, 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 bioactive 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, Hu, Aita, and Maruyama (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 et al., 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 et al., 2003), fish skin (Karnjanapratum et al., 2017), sourdough (Rizzello et al., 2008), wheat, kamut, and emmer (Babini, Tagliazucchi, Martini, Piu, &

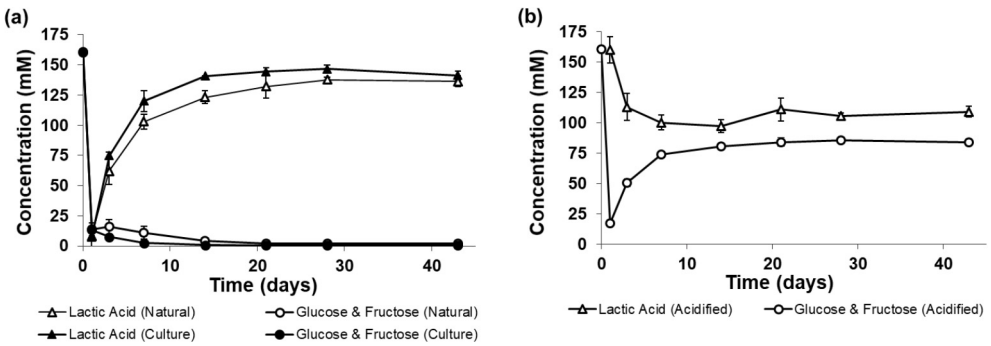

Fig. 2. Changes in sugars and lactic acid content in fermented and acidified cucumber brines (a) Fermentation biochemistry of naturally fermented (Natural) and starter culture fermented (Culture) cucumbers and (b) equilibration of components in acidified cucumber over time.

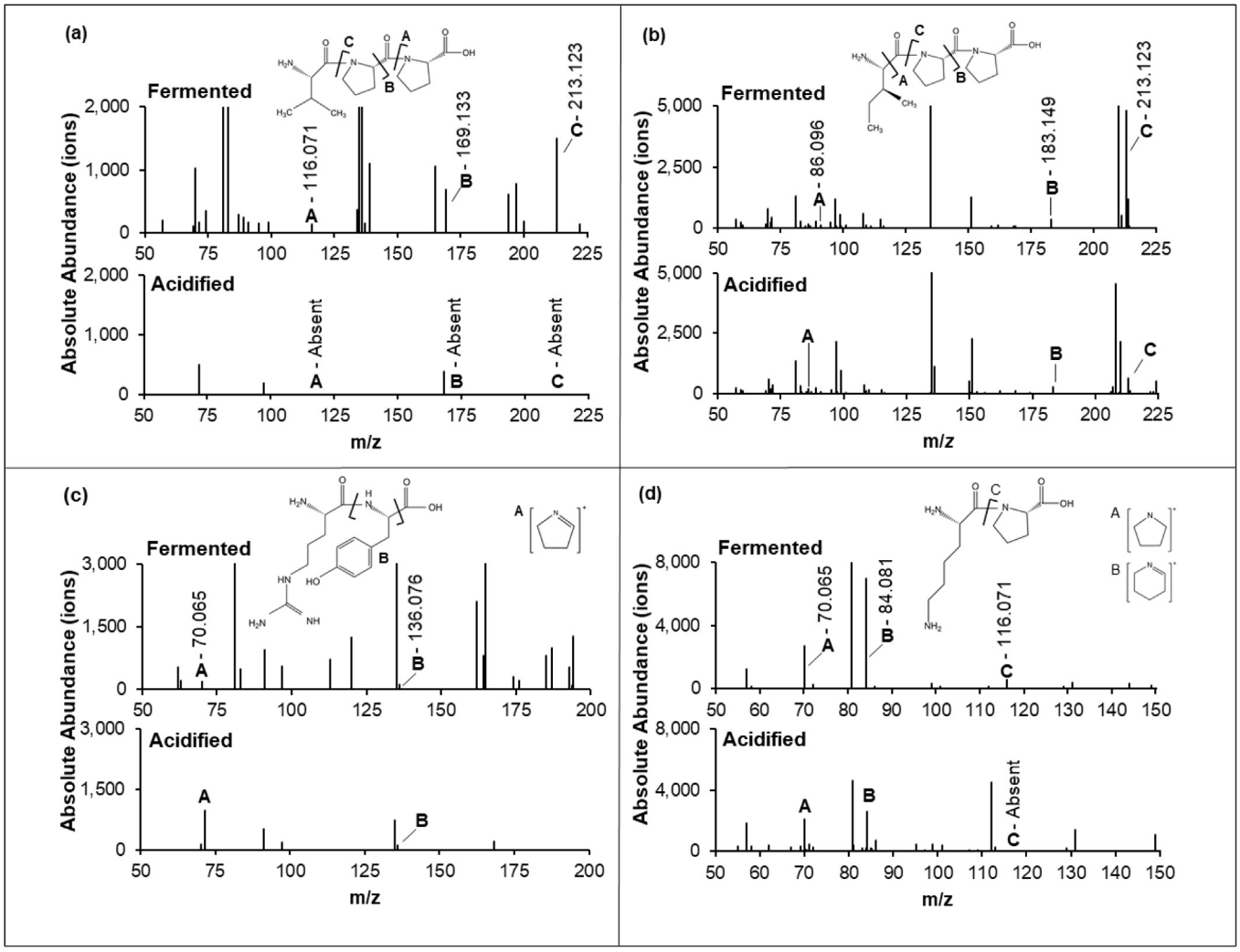

Fig. 3. MS/MS spectra for $[M+H^+]^+$ adducts of bioactive peptides in acidified and fermented cucumbers. (a) val-pro-pro *m/z* 312.2 ± 0.5, 30 NCE, (b) ile/leu-pro-pro *m/z* 326.2 ± 0.5, 30 NCE, (c) arg-tyr *m/z* 338.2 ± 0.5, 40 NCE (d) lys-pro *m/z* 244.2 ± 0.5, 20 NCE.

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 Supplementary Material Table S1.

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-MALDESI 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.

3.4. 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 cucumber samples. In the presence of organic acid, peak height was significantly

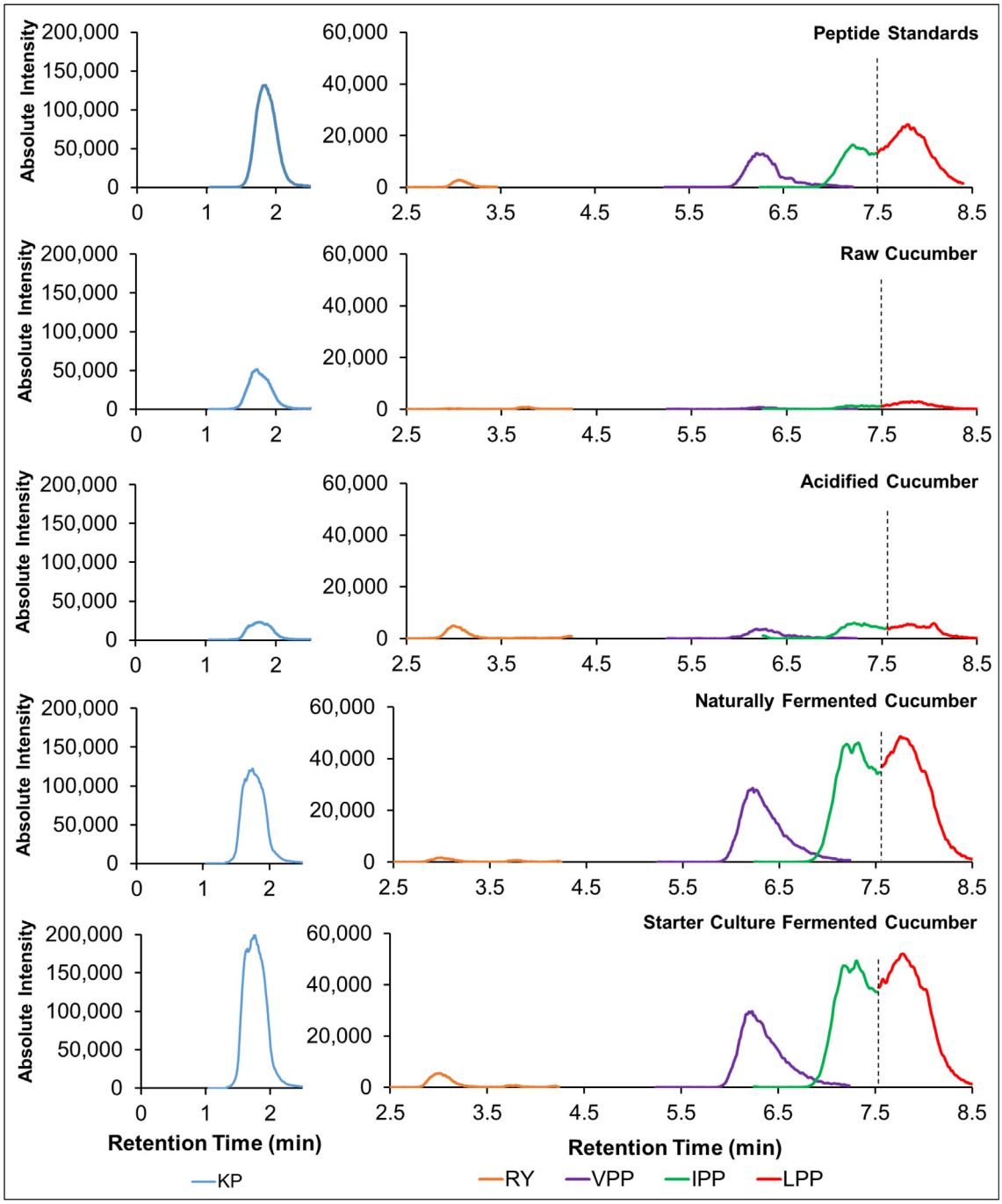

Fig. 4. Separation of bioactive peptides in raw, acidified, and fermented cucumbers on a Restek Pinnacle DB biphenyl column (100 × 2.1 mm, 1.9 µm). Column temperature was held at 45 °C with a segmented gradient of 0.2% formic acid in water and 0.2% formic acid in methanol. MRMs used for quantification 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*).

reduced for IPP ($p < 0.01$) and RY ($p < 0.05$) and increased for KP ($p < 0.05$); while 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, 2017, 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 et al. (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.

4. 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.

Acknowledgements

This study was funded by the USDA-ARS Food Science Research Unit (Raleigh, NC, USA) with partial support from Pickle Packers International (Washington, DC, USA) and D.C.M. acknowledges the National Institutes of Health (R01GM087964) for funding. We would also like to acknowledge Rong Reynolds (USDA-ARS) for laboratory support and Mrs. Sandra Parker (USDA-ARS) for administrative assistance and editorial review of the manuscript.

Conflict of interest statement

Authors declare no conflict of interests.

Authors' contribution statements

Jennifer Fideler designed and performed all experiments, analyzed data, and wrote the manuscript. Suzanne D. Johanningsmeier contributed to the conception and design of the study and advised on fermentation, LC-QQQ-MS experiments, data analysis and writing the manuscript. Måns Ekelof assisted in conducting the IR-MALDESI experiments and contributed to editing the manuscript. D.C.Muddiman advised on experimental design, data analysis and writing of the manuscript. All authors participated in reviewing the intellectual content of the manuscript and approved the final version submitted.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.07.187.

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