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Evaluation of Salmonella Enteritidis and Escherichia coli O157:H7 Volatile Organic Compound Analysis for Food Safety Monitoring: a Preliminary Study

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Evaluation of *Salmonella* Enteritidis and *Escherichia coli* O157:H7 Volatile Organic Compound
Analysis for Food Safety Monitoring: a Preliminary Study

Jessica E. Lemley

A thesis submitted to the
Davis College of Agriculture, Natural Resources and Design
at West Virginia University

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in

Nutritional and Food Science

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Food Safety, Liquid Egg, Ground Beef

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Abstract

Evaluation of *Salmonella enterica* serotype Enteritidis and *Escherichia coli* O157:H7 volatile organic compounds for food safety monitoring: a Preliminary Study

Jessica E. Lemley

Food borne illness has the potential to impact every point of production from producers to consumers. Food product recalls are common due to the presence of food borne illness causing bacteria. Detection of volatile organic compounds (VOC) have the potential to reduce the number of illnesses and recalls through application of additional monitoring of food products like those most commonly recalled. The objective of this research was to 1) explore the use of closed-loop headspace analysis coupled with gas chromatography-mass spectrometry (GC-MS) on food borne illness causing bacteria and 2) examine the VOC profile of *Escherichia coli* serotype O157:H7 in both tryptic soy broth (TSB) and ground beef. *Escherichia coli* JM109, *Escherichia coli* DH5 α , and *Salmonella enterica* serotype Enteritidis ATCC 13076 were chosen to determine potential of bacteria to produce compounds not observed in their controls. A closed-loop headspace analysis was utilized to collect headspace VOCs through the use of glass absorbent filters filled with Alltech HaySep-Q™ PoraPak-Q™ that were then eluted with dichloromethane. Eluted VOC samples were analyzed by GC-MS. Results of analysis were evaluated for peak differences. Both *E. coli* strains grown in TSB produced typical indole peaks during 2 different lengths (24 hours and 5 hours) of VOC collection in addition to four and five peaks from *E. coli* JM109 and *E. coli* DH5 α , respectively, that were not observed in their controls. *S. Enteritidis* grown under optimal conditions in liquid egg produced a profile in which seven compounds were detected in the inoculated liquid egg, but not observed in control after a 6-hour VOC collection. A 2-hour collection series produced inconsistent results suggesting that

a collection of 2 hours may require a more sensitive GC-MS analysis or headspace collection.

Escherichia coli serotype O157:H7 ATCC 43985 grown in TSB and ground beef were subjected to headspace VOC collection utilizing the closed-loop system for analysis. *E. coli* O157:H7 VOC analysis displayed 34 peaks of interest with 4 of these peaks not being detected in the control of sterile TSB at retention times of 8.722, 9.402, 20.919, and 30.777. Ground beef inoculated with *E. coli* O157:H7 displayed 48 peaks of interest with 22 compounds only appearing in the bacteria inoculated ground beef and not the control of uninoculated ground beef. This study has contributed to the foundation of VOC application within food safety and can be used to guide future research utilizing this methodology.

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Chapter I

Introduction

An estimated 9.4 million illnesses and 1,351 deaths occur within the United States each year due to foodborne illness raising questions about the safety of our food (Scallan et al. 2011). The U.S. Food and Drug Administration has listed over 100 recalls on food and drink products due to actual and potential bacterial contamination in 2018 and 2019 (Affairs 2019). Multistate outbreaks and recalls can have negative consequences leaving consumers questioning for years if a product is safe to consume. A product-harm crisis such as bacterial contamination, also known as product recall, has the potential to negatively impact a company through immediate loss in sales, loss in effectiveness of marketing strategies, and a now negative outlook from consumers on any brands that are owned by the same firm or associations (Van Heerde, Helsen, and Dekimpe 2007). When prevention fails, early detection of bacteria is key in preventing illness and loss of product. Bacterial contamination detection currently takes 24 hours for a positive result and then an additional 3 days for further identification and confirmation (Zhu and Hill 2013). Specific volatile organic compounds (VOC) and their patterns and clusters have the potential to allow for early, rapid detection of bacteria possibly down to species (Veselova, Plyuta, and Khmel 2019). VOCs have the potential to be used for monitoring food products during production and manufacturing to alert for the need for further microbiological testing.

VOCs are small organic compounds that are volatile under typical temperature and pressure conditions; furthermore, these compounds are often responsible for odor. Examples of VOCs include the aroma provided by different wines, flowers and even decomposition. The VOCs released by bacteria are primary and secondary metabolites produced during metabolism and for communication purposes (Chen et al. 2017). Expected bacterial compound production

includes fatty acid derivatives, terpenes, aromatic compounds, as well as sulfur and nitrogen containing compounds (Veselova, Plyuta, and Khmel 2019). Investigation of VOCs and species specific bacterial VOCs useful for identification can become applicable in many fields including medical diagnosis, agricultural improvement, environmental contamination, food monitoring and food safety (Boots et al. 2014; Chen et al. 2017; Taiti et al. 2015; Veselova, Plyuta, and Khmel 2019; Wood et al. 2006). There are numerous ways of collecting and differentiating VOCs. The most common way reported in literature is that of headspace solid phase micro-extraction coupled with gas chromatography (SPME/GC-MS). Other collection systems include secondary electrospray ionization-mass spectrometry (SESI-MS) and proton-transfer time-of-flight mass spectrometry (PTR-ToF-MS) (Taiti et al. 2015; Zhu et al. 2010).

VOCs have been monitored as part of environmental soil science and health. Its application within food science generally falls into two categories: food monitoring and food safety. Food monitoring applications would use volatile compounds, not necessarily from bacteria, to examine the quality of the food matrix product. Examples may include products that are known for their aroma like wine, cheese and the ripening of fruit. Food safety would examine the potential use of VOCs to identify bacteria on food products thus declaring them unsafe or adulterated. Characterization of the compounds produced by bacteria is just the first step in the use of VOCs in an applicable manner to monitoring food supplies for spoilage and foodborne illness causing bacteria.

Monitoring VOCs released from food has the potential to lead to food quality monitoring of products such as milk and the post-harvest ripening of imported fruit (Decimo et al. 2018; Taiti et al. 2015). Food quality and processing has become and will continue as a critical aspect of today's consumer market. Restaurants and businesses have moved to advertise quality of

ingredients and have changed menu offerings to draw in potential consumers. VOCs are being examined as a tool in providing those quality, fresh ingredients that may be prone to spoilage due to their fresh nature. VOC detection may be used to identify potentially harmful bacteria in food products and can potentially streamline microbiological testing through bacteria identification or directing when further microbiological testing should be completed saving time and resources. For example, the FSIS of the USDA suggests sampling at least one 2-pound sample of ground beef per production lot (USDA, 2019). In the raw beef product sampling inspection methods sampling frequencies are stated to vary by production facility size as well as production volume (Inspection Methods Training n.d.). VOCs need to be examined for the potential to easily analyze food products more fully to aid in the prevention of adulterated or unsafe products being released for consumption. The aim of this research is to evaluate current studies regarding the use of VOCs to detect pathogenic bacteria associated with food products and the potential use of VOCs in detecting foodborne illness causing bacteria.

References

- Affairs, Office of Regulatory. 2019. "Recalls, Market Withdrawals, & Safety Alerts." *FDA*.
<http://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts> (July 1, 2019).
- Bhattacharjee, Paramita, Suranjan Panigrahi, Dongquig Lin, Catherine M. Logue, Julie S. Sherwood, Curt Doetkott, and Martin Marchello. 2011. "A Comparative Qualitative Study of the Profile of Volatile Organic Compounds Associated with Salmonella Contamination of Packaged Aged and Fresh Beef by HS-SPME/GC-MS." *Journal of Food Science and Technology* 48(1): 1–13.
- Boots, A W, A Smolinska, J J B N van Berkel, R R R Fijten, E E Stobberingh, M L L Boumans, E J Moonen, E F M Wouters, J W Dallinga, and F J Van Schooten. 2014. "Identification of Microorganisms Based on Headspace Analysis of Volatile Organic Compounds by Gas Chromatography–Mass Spectrometry." *Journal of Breath Research* 8(2): 027106.
- Chen, Juan, Junni Tang, Hui Shi, Cheng Tang, and Rong Zhang. 2017. "Characteristics of Volatile Organic Compounds Produced from Five Pathogenic Bacteria by Headspace-Solid Phase Micro-Extraction/Gas Chromatography-Mass Spectrometry." *Journal of Basic Microbiology* 57(3): 228–37.
- Decimo, Marilù, María C, Juan A Ordóñez, Ivano De Noni, and Milena Brasca. 2018. "Volatile Organic Compounds Associated with Milk Spoilage by Psychrotrophic Bacteria." *International Journal of Dairy Technology* 71(3): 593–600.

“Inspection Methods Training.”

<https://www.fsis.usda.gov/wps/portal/fsis/topics/inspection/workforce-training/regional-on-site-training/inspection-methods/inspection-methods> (January 25, 2020).

Scallan, Elaine, Patricia M. Griffin, Fredrick J. Angulo, Robert V. Tauxe, and Robert M.

Hoekstra. 2011. “Foodborne Illness Acquired in the United States—Unspecified Agents.” *Emerging Infectious Diseases* 17(1): 16–22.

Taiti, Cosimo, Corrado Costa, Paolo Menesatti, Stefania Caparrotta, Nadia Bazihizina, Elisa

Azzarello, William Antonia Petrucci, Elisa Masi, and Edgardo Giordani. 2015. “Use of Volatile Organic Compounds and Physicochemical Parameters for Monitoring the Post-Harvest Ripening of Imported Tropical Fruits.” *European Food Research and Technology* 241(1): 91–102.

United States Food and Drug Administration. Food Safety and Inspection Service. 2019. *Raw Beef Product Sampling*.

Van Heerde, Harald, Kristiaan Helsen, and Marnik G. Dekimpe. 2007. “The Impact of a Product-Harm Crisis on Marketing Effectiveness.” *Marketing Science* 26(2): 230–45.

Veselova, M. A., V. A. Plyuta, and I. A. Khmel. 2019. “Volatile Compounds of Bacterial Origin: Structure, Biosynthesis, and Biological Activity.” *Microbiology* 88(3): 261–74.

Wood, William L., Daniel J Higbee, Melanie Gooldy, Scott Glogowski, Rich Fitzpatrick,

Richard J Karalus, Troy D. Wood, and David J. Mangino. 2006. “Analysis of Volatile Bacterial Metabolites by Gas Chromatography--Mass Spectrometry.” *Spectroscopy* 21(6): 20–28.

Zhu, Jiangjiang, Heather D. Bean, Yin-Ming Kuo, and Jane E. Hill. 2010. "Fast Detection of Volatile Organic Compounds from Bacterial Cultures by Secondary Electrospray Ionization-Mass Spectrometry." *Journal of Clinical Microbiology* 48(12): 4426–31.

Zhu, Jiangjiang, and Jane E. Hill. 2013. "Detection of Escherichia Coli via VOC Profiling Using Secondary Electrospray Ionization-Mass Spectrometry (SESI-MS)." *Food Microbiology* 34(2): 412–17.

Chapter II

Review of Literature

Volatile Organic Compounds

This review of literature will examine resources in an effort to guide future research dedicated to the use of volatile organic compounds for the application of using VOCs as a way to detect potential bacterial contamination of food products. This review will look into VOC production from bacteria, analysis of those compounds, influences upon VOC production that may impact the use of VOC in food safety and past studies examining profiles of bacterial VOCs from food products.

VOC Production

Bacterial VOCs are metabolites released by bacteria during growth and maintenance processes through metabolic pathways (Tait et al. 2014b). Secondary metabolites that may be used for communication between cells and with the environment can also be considered bacterial volatile compounds (Chen et al. 2017; Vaishnav et al. 2017). These compounds are disseminated through the environment from the bacteria including air, liquid media or even soil (Veselova, Plyuta, and Khmel 2019). Bacterial VOCs can induce bacterial sporulation, suppress the growth of the surrounding microbial community, and even induce factors of virulence (Audrain et al. 2015; Veselova, Plyuta, and Khmel 2019). Volatiles can be defined as compounds that have a low molecular weight, less than 300 Daltons, in addition to a high vapor pressure allowing for the evaporation of the compound into the environment (Vaishnav et al. 2017). Common and well-known VOCs include the aromas produced by bacteria fermentation seen in wine along with cheese production that help to produce desired flavors (Schulz and

Dickschat 2007). VOCs in food production have been studied like that in wine production which aims to create a desirable aroma that influences the overall flavor and final product (Panighel and Flamini 2014). Generally catabolic pathways are the metabolic processes that produce these volatile compounds which include pathways like glycolysis which is the breakdown of glucose or lipolysis known as the hydrolysis of triglycerides to produce glycerol and fatty acids.

Expected Bacteria Produced Compounds

Alkenes, alcohols, ketones, terpenes, benzoids, pyrazines, acids and esters are the dominant compounds produced by bacteria (Veselova, Plyuta, and Khmel 2019). These compounds can fall into five general categories including fatty acid derivatives, terpenes, aromatic compounds, sulfur containing compounds, and nitrogen containing compounds. Fatty acid derivatives encompasses a wide range of compounds from hydrocarbons to acids and esters and are generally intermediates of fatty acid metabolism (Veselova, Plyuta, and Khmel 2019). Terpenes are compounds produced through biosynthesis pathways including the methylerythritol 4-phosphate (MEP) pathway producing monoterpenes and sesquiterpenes both of which have previously been reported as bacteria VOC (Schulz and Dickschat 2007; Veselova, Plyuta, and Khmel 2019). Metabolites that are aromatic compounds are derived from the degradation pathways of L-phenylalanine and L-tyrosine. Sulfur containing organic compounds are generally seen in fermented products such as cheese and wine with bacteria producing compounds that include dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide. There are three major pathways bacteria can use to produce sulfur containing compounds and they include dimethylsulfoniopropionate (by marine bacteria generally), methylation of inorganic sulfide (generally in freshwater habitats), and the L-methionine catabolic pathway which produces a

range of sulfur compounds by cheese ripening bacteria and lactic acid bacteria (Audrain et al. 2015; Schulz and Dickschat 2007). The last major grouping of bacteria produced volatile compounds are the nitrogen containing organic compounds. Indole is an example of a nitrogen containing VOC in which production has been well documented in *Escherichia coli* species. *E. coli* utilize the enzyme tryptophanase which can reversibly use tryptophan to produce indole, pyruvate and ammonia influenced by environmental factors (Veselova, Plyuta, and Khmel 2019). It should also be noted that bacteria can also produce inorganic compounds such as hydrogen sulfide, ammonia and nitric oxide (Audrain et al. 2015; Veselova, Plyuta, and Khmel 2019). Produced bacterial VOC tends to change with environmental influences including media and cultivation methods which in turn influence metabolic processes. Different bacterial species may also be more adept at producing certain compounds for example cyanobacteria are known to produce longer and branched hydrocarbons (Audrain et al. 2015).

VOC Collection and Analysis

In addition to the numerous compounds bacteria can produce, there are numerous ways to collect and analyze these compounds. Closed-loop stripping systems with gas chromatography-mass spectrometry (GC-MS), solid-phase microextraction (SPME) followed by GC-MS, thermal desorption, ion-molecule reaction-MS, secondary electrospray ionization-MS, proton-transfer-reaction time-of-flight MS (PTR-ToF-MS) are all methods used to capture and analyze VOCs released from bacteria that have been seen in scientific literature. In the sample of experimental papers discussed in this review, SPME with GC-MS has been the most common method. Plant studies evaluating VOCs commonly use a closed-loop system in which volatile compounds are

trapped and extracted for analysis with GC-MS or directed airflow systems (Vaishnav et al. 2017).

Generally, with all of the systems available to collect volatile organic compounds, the main principle remains the same in collection. Whether a trapping system, solid phase microextraction, or even magnetic stir-bar sorptive extraction using polydimethylsiloxane, all of these systems use an absorptive surface that interacts with and traps the volatiles released from the interested sample (Sparkman, Penton, and Kitson 2011). Once the collection of VOCs takes place, the samples are then analyzed by gas chromatography coupled with mass spectrometry.

Oftentimes with the adsorptive surfaces of VOC collection systems, the VOCs are removed and enter the GC-MS analysis system through increased temperature or elution with organic solvents to disrupt the VOC-adsorptive surface interface. Some absorptive surfaces like the closed-loop stripping apparatus filters in which the headspace is pumped through, an organic solvent is used to disrupt the interaction between the filter and the collected VOC. This produces a liquid VOC sample that can then be injected for GC-MS analysis.

GC-MS offers a way to evaluate metabolites through identification and has been used for years reliably while covering several classes of compounds (Lisec et al. 2006). GC-MS analyses, though generally coupled together in a lot of cases, are two separate processes that provide VOC analysis, also known as metabolite profiling (Lindon, Nicholson, and Holmes 2019). Gas chromatography is the first step in which the contents of the VOC sample are separated in a heated column and carrier gas such as helium or hydrogen (Sparkman, Penton, and Kitson 2011). The heated injection and heated column promotes the migration of compounds as they vaporize from the sample and begin to separate based on the volatility and polarity of the compound. Moving from the mobile phase into a stationary phase within the column, the

characteristics of the compound determine separation. The faster a compound is traveling within the column the earlier the compound will leave the column resulting in a shorter retention time for that compound appearing within the data output (Lindon, Nicholson, and Holmes 2019). Mass spectrometry, in this case, follows GC in which the separated compounds still being carried by the carrier gas are injected into the MS generally under temperature controlled conditions (Sparkman, Penton, and Kitson 2011). Generally, each MS consists of 3 parts, an ion source in which the ions are transferred into the MS, a mass analyzer which will separate ions based on their mass and charge and finally an ion detector (Sauer and Kliem 2010). Mass spectrometry produces the data output known as a mass spectra for each compound previously separated then analyzed. A chromatograph is also a common output that includes the mass spectra but graphs the analyzed compounds by intensity and retention time (Sparkman, Penton, and Kitson 2011). From there software reference libraries designed for compound identification can be used to potentially identify compounds of interest. An authentic standard for comparison can then be used for further identification of the compound (Lisec et al. 2006). Beyond compound identification, analysis can then take many forms including the quantification of compounds of interest also using a known internal standard.

Influence of Media

Of the reviewed literature, multiple influences upon the production of bacterial volatiles have become apparent including the media used to culture bacteria, the presence of a mixed culture system, and the growth phase of the bacterial community. Media may influence VOC production based on the available nutrients to the bacteria to utilize during metabolism. A study evaluating volatiles released by *Chondromyces crocatus* grown on two different media cultures

of either VY/2 agar or CY agar with the first specific to yeast and the second containing peptone from casein (Schulz, Fuhlendorff, and Reichenbach 2004). Both media were treated the same and incubated in the dark at 30°C. VOCs were collected from the headspace and analyzed by a GC-MS. Their results showed considerable variation between the two media with sesquiterpenoids more present using the peptone medium and pyrazines more associated with the yeast medium (Schulz, Fuhlendorff, and Reichenbach 2004). Though *Chondromyces crocatus* is a common bacteria species found within the soil, the differences seen within this study must be kept in mind for any food matrix and differences between products moving forward.

Different media would impact released VOCs due to the starting substrate for bacterial metabolism. Bacteria adapt to their surroundings and the resources available influencing their speed of growth, proliferation, and overall metabolism. An objective of Tait et al's "Identification of Volatile Organic Compounds Produced by Bacteria Using HS-SPME-GC-MS" was to evaluate the effect of media upon bacterial VOC profiles of *E. coli*, *S. aureus* and *Klebsiella pneumoniae*. Three broth media were used for this study including brain heart infusion, tryptone soya and enteric fermentation base. Bacteria were incubated for 18 hours at 37°C without shaking before being subjected to the headspace solid-phase microextraction gas chromatography-mass spectrometry collection and analysis (Tait et al. 2014b). Results showed comparable VOC profiles between the BHI and TS broths attributed to similar composition. VOC profile for *E. coli* and the other two bacteria were significantly different in EF when compared to the other broth types (Tait et al. 2014b). Ratiu and colleagues also looked at *E. coli* (ATCC 8739) grown in three different broth media of tryptic soy broth (TSB), Mueller Hinton (MH) medium and minimal salts (M9) medium enriched with glucose while using headspace SPME coupled with GC-MS to analyze volatile profiles (Ratiu et al. 2017). *E. coli* was

inoculated to the three broth types with an optical density 1 at 600nm. After 24 hours and for each day following optical density was recorded and remained about the same throughout the 72-hour experiment suggesting 2100×10^6 CFU ml⁻¹, 1800×10^6 CFU ml⁻¹, and 1350×10^6 CFU ml⁻¹ for TSB, MH and M9 respectively. The SPME fiber was exposed for 45 minutes to the culture at 37°C after 24, 48 and 72 hours (Ratiu et al. 2017). Results suggested that each media produced characteristic VOCs including indole production highest in TSB, pyrazines characteristic of MH with M9 producing a VOC profile including primary and secondary alcohols and ketones. Overall, different compounds can be seen to be produced in three different media tested with 24 compounds unique to one media (Ratiu et al. 2017).

The next study evaluated media's influence on VOCs in food modeling medium of meat extract, vegetable extract and apple extract media testing *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Salmonella enterica* serotype Typhimurium (Zhu and Hill 2013). Results reported the production of unique VOC profiles containing consistent compounds with similar intensities for each bacteria across the three media. No comparison was discussed between different media profiles for the same bacteria, but visual differences can be seen in the chromatographs (Zhu and Hill 2013). This study, however, shows that core peaks can be identified for bacteria across media and could be suggested for use of bacterial identification. Media influence was also explored in another publication by Zhu and Hill that looked at the growth of *Pseudomonas aeruginosa* on a variety of media including TSB, LB-Lennox, a synthetic cystic fibrosis media and MOPS media (Zhu et al. 2010). The PCA plot demonstrating growth on different media did present a cluster at the center of the plot. The VOC spectra produced on TSB showed some variability suggesting that the method of secondary electrospray ionization-mass spectrometry may be used to determine bacteria despite media (Zhu et al. 2010).

Zhu et al. 2010 still notes that different media may have the potential to impact VOC profiles. Differences in VOC profiles in food matrices potentially may be seen through the relative intensity between similar samples. An example of this in relation to food safety may be considered in beef products. The fat content of ground beef in different products may impact the potential for differing profiles of volatiles for those products.

VOC Influence of Mixed Cultures

A second potential influence to be addressed is the VOCs collected from mixed bacterial cultures. The nature of environmental samples and the ubiquity of bacteria make it unrealistic to expect only one bacteria species or strain when collecting and identifying VOCs. Once a bacterial VOC profile on a desired media has been solidified the use of mixed culture VOC collection can help to mimic a more realistic sample that could be obtained. For example, 70% of raw ground beef samples tested positive for coliforms, 17.5% for *E. coli* (not *E. coli* O157:H7), 25% *Listeria* spp. and 5% of samples for *Listeria monocytogenes* (Pao and Ettinger 2009). Pao and Ettinger also noted psychrotrophs, yeasts and molds within the local raw beef samples as well as locally frozen beef and internet ordered beef samples. Fresh ground beef has been shown to host a number of bacteria in addition to molds and yeast creating a complex food matrix that will need to be kept in mind moving forward.

To investigate complex microbial mixture environments, Zhu et al. examined the VOC profile of three different ratio mixed culture systems of *S. aureus* and *P. aeruginosa*. The VOC profile was more representative of the bacteria that had a higher presence with the 1:1 ratio displaying expected mix of VOCs from each bacteria involved. Results displaying a linear trend of VOC production suggest that for these two bacteria a mixed culture did not influence VOC

production (Zhu et al. 2010). The limitations of this study are that it was limited to two bacteria species and was completed in tryptic soy broth suggesting that more studies in different media and more varied bacteria should be tested.

Chen et al. also performed mixed culture VOC analysis consisting of *S. aureus* and *E. coli* O157:H7. 3-methyl-butannal and 3-methyl-butanoic acid both decreased in maximum signal intensities for *S. aureus* VOC production when comparing the mixed to the mono culture. Indole intensities from *E. coli* O157:H7 were similar when comparing the two experimental groups (Chen et al. 2017). This conflicts with the results presented by Zhu et al. that suggested a linear relationship of VOCs and mixed cultures. This could be due to the presence of different bacteria in question and their interactions within the growth medium. These studies discussing mixed culture influence were completed using tryptic soy broth (TSB).

VOC Influence of Growth Phase

A third impact can be considered the growth phase of the bacteria. The 4 phases of growth in which different metabolic processes would occur for bacteria are lag, log, stationary and death phases (Debnath et al. 2012). Lag phase is the slowest rate of growth where the bacteria adapt to their environment followed by exponential growth. Following the exponential growth of log phase bacteria reach stationary phase in which population numbers are sustained due to competition for available nutrients (Debnath et al. 2012). The last phase is the death phase in which nutrients are depleted leading to a decline of the bacteria population. Each of these phases would be influenced by the external environment and secondary metabolites released by the bacteria transferring to the VOCs the bacteria may release as well as affecting growth of microorganisms within the micro community (Veselova, Plyuta, and Khmel 2019).

Kunze et al. and Bunge et al. have both shown influence of growth phase upon the VOC profile recorded utilizing standard laboratory mediums. Kunze et al. used lysogeny broth to determine *E. coli* and *P. aeruginosa* growth curves establishing growth curves in which four times representative of the first three of the four growth phases. The four time points were 45, 105, 225, and 450 minutes after inoculation and 90, 270, 440, and 760 minutes for *E. coli* and *P. aeruginosa* respectively (Kunze et al. 2013). Synthetic air was used to keep environmental contamination as low as possible. Gas samples were taken for 10 seconds, analyzed using multi-capillary column-ion mobility spectrometry and substances were identified using a database that included reference data. Multiple differences were detected within the VOC profiles including 5-methylheptan-3-on, nonanal, 2-phenylacetaldehyde, and unknown P_720_16 intensities decreasing in both cultures after inoculation. Azane increased intensity in both cultures over time. For *E. coli* unknown substance P_642_48 had the highest intensity during early active growth but then continued to fall as the bacteria monoculture increased. This same compound increased regularly for *P. aeruginosa*. Kunze et al., although not continuously recording VOC profiles of growing cultures, did present results that represent an active growing culture moving through the four phases of microbial growth and that different VOC profiles are present within that culture. One limitation to this is the overlapping of phases after the VOC collection has taken place. This could influence the intensity of compounds present. To move forward with this design, but decrease the overlapping of VOC profiles more sampling points along the bacterial growth curve is suggested.

The second of the two growth phase studies by Bunge et al. openly challenges VOC studies that only include one VOC measurement collection due to the different compounds potentially produced at different phases of growth. Complex media suggested by the

microorganism retailer was used to support the growth of *E. coli*, *Shigella flexneri*, *S. enterica* serotype *enterica* and yeast *Candida tropicalis*. 12 minute long headspace VOC measurements were taken continuously for 24 hours with 36 minute interval between samples (Bunge et al. 2008). Of the 12 minute sample, the first 5 minutes was discarded to reduce the influence of carryover from the previous sample and processed using proton transfer reaction-mass spectrometry (Bunge et al. 2008). With an interval of only 36 minutes Bunge and associates were able to more accurately measure the VOC profile over a 24-hour period of microbial growth with the majority of microbial culture measurements taken during stationary phase. Compounds they were not able to identify were referred to as their mass number reported as atomic mass unit (amu). *S. enterica* serotype *enterica* saw the increase of compound 89 amu with the progression of time in all inoculated samples while compound 73 amu did not appear until incubation time reached 9 hours. *S. flexneri* developed an acetaldehyde peak before visual signs of microbial growth were reported and not seen again after 6 hours. Indole, compound 118 amu, production from *E. coli* increased quickly after 3 hours peaking near hour 6 and gradually declining. Starting optical density measured at 660 nm was 0.01 and shown as just below 1×10^8 cells per mL of broth (Bunge et al. 2008). To accurately show the different profiles over 24 hours of growth starting at a smaller dilution would provide more information on the VOC profiles in different phases of growth. This study does portray methodology that allows for continuous monitoring of released volatiles from a microorganism which can, if utilized further, provide key insight into early volatile production of bacteria. This study has also displayed that single time VOC analysis of a bacteria without the consideration of phase of bacteria may not appropriately capture VOCs one may be interested in. This concept must be kept in mind

moving forward when utilizing VOCs to potentially predict conditions of food products during production.

Raw chicken and beef strip loins were examined in 3 different studies evaluating VOC response in storage conditions for ten and four days respectively. Klein and colleagues examined VOCs from chicken inoculated with *P. fluorescens* or *E. coli* under modified atmosphere packaging (MAP) utilizing thermal desorption-gas chromatography-mass spectrometry analysis. MAP conditions contained 70% oxygen and 30% carbon dioxide and held within a standard refrigerator (Klein et al. 2018). During the evaluation of *P. fluorescens* different concentrations of compounds were seen when compared to the uninoculated chicken breast. Dimethyl disulfide (DMDS) and 3-hydroxy-2-butanone began to increase peak area after day 3 of the study and continued to rise throughout the remaining 10 days. S-methyl thioacetate did not start to increase until day 8. Pent-1-ene, ethyl acetate and dimethyl sulfide also showed increases over the 10 day study (Klein et al. 2018). For *E. coli* only 3-methylbutanal was observed starting at day 2 and seen to increase and then remain steady after day 6. 2-methyl-1-butanol, 2-methyl-1-propanol, 3-methyl-1-butanol and DMDS were first seen on day 6 of storage and increased for the remainder for the study. Dimethyl trisulfide and s-methyl thioacetate were first seen on day 7 and 8 respectively and continued to increase for the remaining days in storage (Klein et al. 2018). This 10 day study presents the change in VOCs under consumer purchased refrigerated conditions demonstrating the production of VOCs under refrigeration conditions that may transfer to food production. Sensitivity and specificity of VOC measurements will be major factors when evaluating high volumes of a food product with potential for a very low bacterial presence.

Packaged aged and fresh beef were also examined over multiple days after inoculation with an avirulent strain of *S. typhimurium* by Bhattacharjee and colleagues incubated at 20°C for four days. Inoculation of the beef was approximately 10^3 - 10^4 CFU per gram of beef and analyzed using SPME GC-MS to obtain the VOC profile (Bhattacharjee et al. 2011). There were four positively correlated compounds for fresh beef and 5 positively correlated compounds for age beef that increased with rising bacteria population. The results of this study exhibit a change in VOC profile during bacterial growth. The temperature held at 20°C represented the potential for the meat to increase up to during processing (Bhattacharjee et al. 2011). To get a more accurate representation of VOCs during production refrigeration or processing temperatures should be used to more accurately mimic food processing.

Like that of the laboratory media studies discussed previously, Chen and colleagues evaluated the VOC of *E. coli* O157:H7 on irradiated pork over 20 hours with a starting incubation of about $1.4 \log_{10}$ CFU per gram of pork. This study incubated inoculated pork at 35°C for 20 hours analyzing VOCs using headspace SPME GC-MS (Chen et al. 2016). Before the SPME fiber was inserted into the sample vial the sample was incubated at 80°C for 10 minutes and the fiber was left to collect VOCs from the headspace for 30 minutes remaining at 80°C (Chen et al. 2016). The increase in sample temperature would yield the best and high volatile intensities than remaining at 35°C. Though these measurements may be optimal they are not under conditions seen in food processing and manufacturing and must be kept in mind when suggesting this technology for further use. Indole was measured in the inoculated pork samples, but did not appear in the uninoculated control. Indole, in this study, increased with increasing incubation time with an especially sharp increase after 14 hours of incubation (Chen et al. 2016). 2-Tridecanone follows a similar trend as indole through increasing greatly around 14 hours of

incubation. 1-Nonanone and 2-heptanone begin to increase around hour 8 and 10 respectively peaking at hour 16 followed by a decline in peak area for the rest of the experiment. Both 1-hexanol and 1-octanol peaked between 10 and 12 hours of incubation followed by a decline. These compounds were not seen produced in the uninoculated pork (Chen et al. 2016). This study supports the temporality of VOCs and the fluctuations associated with different growth phases.

Bacterial volatile compounds have been seen to vary depending on multiple factors including nutrient source, the presence of other bacteria and phase of growth for the targeted microorganism. With the standardization of methodology and established volatile profiles VOC have the potential to benefit agriculture and food production through identifying pathogenic bacteria through the detection of one or several compounds released during microbial growth.

VOC Application using Food Products

Food products tested for bacterial VOCs of foodborne illness causing bacteria mainly include that of chicken (Arnold and Senter 1998; Klein et al. 2018), beef (Abdallah et al. 2013; Bhattacharjee et al. 2011; Cevallos-Cevallos, Danyluk, and Reyes-De-Corcuera 2011) and pork (Chen et al. 2016). Food modeling media have also been used to simulate potential VOC profiles (Zhu and Hill 2013). Application of VOCs in a sensor for meat production has the potential to guide testing procedures and potentially identify bacteria present within the product. This would allow for an increased monitoring over a larger amount of product than the microbiological testing of a randomly selected sample. Meat, however, is not the only food known to carry foodborne illness causing bacteria. Recently enoki mushrooms and curry powder

were recalled due to the potential for *L. monocytogenes* and *Salmonella*, respectively (Nutrition 2020a, 2020b).

Keshun Yu and colleagues investigated the VOC production from *E. coli* O157:H7 inoculated strawberries. Strawberries were inoculated with *E. coli* O157:H7 concentration of 10^7 CFU/mL and allowed to dry for 1-hour prior to headspace VOC collection at room temperature for up to 72 hours using Super-Q absorbent filter, solvent extract followed by GC-MS analysis (Yu et al. 2000). Indole proved to be a main compound seen in VOC collection using BHI broth but did not appear when evaluating the inoculated strawberries. The compounds seen were those released from the strawberry. The next step to their experiment was to evaluate the strawberry's ability to absorb volatile compounds as suggested by a previous paper (Yu et al. 2000). When testing indole, the strawberry decreased the compound by 97% over an 18-hour test period. Overall, strawberries were found to decrease between 86% and 99% of 10 volatile compounds (Yu et al. 2000). This study helps to shed some light on another way in which the media or food product tested can interact with the volatile compounds not just produce their own.

Listeria monocytogenes in different milk samples were evaluated for enzyme-generated VOC by Emma Tait and colleagues for bacterial detection. Headspace SPME with GC-MS was used to analyze enzyme-generated VOC from *Listeria* enrichment broth (LEB) inoculated with 100 μ l of $1-1.5 \times 10^7$ CFU bacterial suspension followed by the addition of 0.9mL of one of seven milk samples and enzyme substrate. The LEB, enzyme substrates, milk and bacteria mixture were incubated overnight at 37°C before being analyzed for VOCs (Tait et al. 2014a). This experiment targeted specifically the products of bacterial enzymes that would utilize 2-nitrophenyl- β -D-glucoside and 2-(3-fluorophenyl) to produce 2-nitrophenol and 3-fluoroaniline respectively (Tait et al. 2014a). Uninoculated milk did not produce these targeted VOCs but did

so in the seven milk types tested when inoculated with *L. monocytogenes*. Other bacterial species tested showed that 3 species also released the two targeted VOCs and suggests that because the two VOCs may be present in an unknown sample does not specifically point to *L. monocytogenes* as the bacteria (Tait et al. 2014a). The study utilized processes already known to about pathogenic bacteria to develop a potential system for bacterial identification. Future studies looking at specific target bacteria may utilize enzymatic knowledge to potentially move forward with selecting potential VOCs as targeted VOCs. Though this may help to direct some VOC detection for foodborne illness causing bacteria enzymatic utilization of substrates can provide false positives as in this study with the three bacteria that could also utilize the substrates to produce the targeted VOC.

Establishment of VOC profiles for food matrix and varying pathogenic bacteria can help to further the goal of utilizing VOCs for the identification of potentially harmful food products due to the presence of bacteria. Meat has been the main focus for VOC potential, but other food products like that of fruits, vegetables and beverages may also benefit from this science and developing technology. VOC monitoring in fruit and vegetable products should be approached with a food/VOC interaction in mind as seen in the study examining strawberries where VOCs were readily absorbed by the strawberries (Yu et al. 2000).

Conclusion

Many studies have worked to and achieved the objective of furthering research into VOC use to identify foodborne illness causing bacteria. To continue this trend of positive VOC movement there are several factors that should be considered when moving forward with studies. Internal and external standards are needed to provide peak area normalization and relative

comparison for data analysis as well as the solidification of compound identity. Food matrices, like that of ground beef, have background flora that are introduced during processing. This must be kept in mind when evaluating VOCs because of the potential for mixed cultures to interfere with VOC production in some cases. Further media and food testing for VOC profiles need to be completed to understand the dynamic relationship between VOC production and nutrients present to ensure food product variability would not impact VOC release in the event of food product monitoring. Moving forward, the VOC profile must be established on targeted food products then compared to the VOC profiles when conditions match that of food processing to provide the most comprehensive view of VOCs for that food product. Overall, there have been a few studies that have displayed promising results and methodology that can be built upon to pursue the use of VOC for bacterial identification in food products.

References

- Abdallah, Soad A, Laila A Al-Shatti, Ali F Alhajraf, Noura Al-hammad, and Bashayer Al-Awadi. 2013. "The Detection of Foodborne Bacteria on Beef: The Application of the Electronic Nose." *SpringerPlus* 2(1): 687.
- Affairs, Office of Regulatory. 2019. "Recalls, Market Withdrawals, & Safety Alerts." *FDA*. <http://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts> (July 1, 2019).
- Arnold, Judy W, and Samuel D Senter. 1998. "Use of Digital Aroma Technology and SPME GC-MS to Compare Volatile Compounds Produced by Bacteria Isolated from Processed Poultry." *Journal of the Science of Food and Agriculture* 78(3): 343–48.
- Audrain, Bianca, Mohamed A. Farag, Choong-Min Ryu, and Jean-Marc Ghigo. 2015. "Role of Bacterial Volatile Compounds in Bacterial Biology." *FEMS Microbiology Reviews* 39(2):222-33.
- Bhattacharjee, Paramita, Suranjan Panigrahi, Dongquig Lin, Catherine M. Logue, Julie S. Sherwood, Curt Doetkott, and Martin Marchello. 2011. "A Comparative Qualitative Study of the Profile of Volatile Organic Compounds Associated with Salmonella Contamination of Packaged Aged and Fresh Beef by HS-SPME/GC-MS." *Journal of Food Science and Technology* 48(1): 1–13.
- Boots, A W, A Smolinska, J J B N van Berkel, R R R Fijten, E E Stobberingh, M L L Boumans, E J Moonen, E F M Wouters, J W Dallinga, and F J Van Schooten. 2014. "Identification of Microorganisms Based on Headspace Analysis of Volatile Organic Compounds by Gas Chromatography–Mass Spectrometry." *Journal of Breath Research* 8(2): 027106.

- Bunge, M., N. Araghipour, T. Mikoviny, J. Dunkl, R. Schnitzhofer, A. Hansel, F. Schinner, A. Wisthaler, R. Margesin, and T. D. Mark. 2008. "On-Line Monitoring of Microbial Volatile Metabolites by Proton Transfer Reaction-Mass Spectrometry." *Applied and Environmental Microbiology* 74(7): 2179–86.
- Cevallos-Cevallos, Juan Manuel, Michelle D. Danyluk, and José I. Reyes-De-Corcuera. 2011. "GC-MS Based Metabolomics for Rapid Simultaneous Detection of Escherichia Coli O157:H7, Salmonella Typhimurium, Salmonella Muenchen, and Salmonella Hartford in Ground Beef and Chicken." *Journal of Food Science* 76(4): M238–46.
- Chen, Juan, Junni Tang, Cheng Tang, Hui Shi, Huaiyin Gu, and Xiaozhu Lu. 2016. "Growth and Volatile Compounds of Escherichia Coli O157:H7 on Irradiated Pork." *Journal of Food Quality* 39(6): 766–72.
- Chen, Juan, Junni Tang, Hui Shi, Cheng Tang, and Rong Zhang. 2017. "Characteristics of Volatile Organic Compounds Produced from Five Pathogenic Bacteria by Headspace-Solid Phase Micro-Extraction/Gas Chromatography-Mass Spectrometry." *Journal of Basic Microbiology* 57(3): 228–37.
- Debnath, Mousumi, G. B. Prasad, Prakash S. Bisen, and Prakash S. Bisen. 2012. *Microbes: Concepts and Applications*. Somerset, UNITED STATES: John Wiley & Sons, Incorporated. <http://ebookcentral.proquest.com/lib/wvu/detail.action?docID=848522> (June 5, 2020).

Decimo, Marilù, María C, Juan A Ordóñez, Ivano De Noni, and Milena Brasca. 2018. “Volatile Organic Compounds Associated with Milk Spoilage by Psychrotrophic Bacteria.”

International Journal of Dairy Technology 71(3): 593–600.

“Inspection Methods Training.”

<https://www.fsis.usda.gov/wps/portal/fsis/topics/inspection/workforce-training/regional-on-site-training/inspection-methods/inspection-methods> (January 25, 2020).

Klein, Daniel, Stephan Maurer, Ulrike Herbert, Judith Kreyenschmidt, and Peter Kaul. 2018.

“Detection of Volatile Organic Compounds Arising from Chicken Breast Filets Under Modified Atmosphere Packaging Using TD-GC/MS.” *Food Analytical Methods* 11(1): 88–98.

Kunze, Nils, Stephan Maurer, Ulrike Herbert, Judith Kreyenschmidt, and Peter Kaul. 2013.

“Detection and Validation of Volatile Metabolic Patterns over Different Strains of Two Human Pathogenic Bacteria during Their Growth in a Complex Medium Using Multi-Capillary Column-Ion Mobility Spectrometry (MCC-IMS).” *Applied Microbiology and Biotechnology* 97(8): 3665–76.

Lindon, John C., Jermy K. Nicholson, and Elaine Holmes. 2019. *The Handbook of Metabolic Phenotyping*. Amsterdam, Netherlands: Elsevier.

<http://search.ebscohost.com/login.aspx?direct=true&scope=site&db=nlebk&AN=172465>
1 (January 27, 2020).

Lisec, Jan, Nicolas Schauer, Joachim Kopka, Lothar Willmitzer, and Alisdair R. Fernie. 2006.

“Gas Chromatography Mass Spectrometry–Based Metabolite Profiling in Plants.” *Nature Protocols* 1(1): 387–96.

Nutrition, Center for Food Safety and Applied. 2020a. “H&C Food Inc. Recalls Enoki

Mushroom Because of Possible Health Risk.” *U.S. Food and Drug Administration*.

<https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts/hc-food-inc-recalls-enoki-mushroom-because-possible-health-risk> (June 10, 2020).

Nutrition, Center for Food Safety and Applied. 2020b. “New Hoque and Sons, Inc. Recalls

Radhuni Curry Powder Because of Possible Health Risk.” *U.S. Food and Drug*

Administration. <https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts/new-hoque-and-sons-inc-recalls-radhuni-curry-powder-because-possible-health-risk> (June 11, 2020).

Panighel, Annarita, and Riccardo Flamini. 2014. “Applications of Solid-Phase Microextraction and Gas Chromatography/Mass Spectrometry (SPME-GC/MS) in the Study of Grape and Wine Volatile Compounds.” *Molecules* 19(12): 21291–309.

Pao, S., and M. R. Ettinger. 2009. “Comparison of the Microbial Quality of Ground Beef and Ground Beef Patties from Internet and Local Retail Markets.” *Journal of Food Protection* 72(8): 1722–26.

Ratiu, Ileana-Andreea, Tomasz Ligor, Victor Bocos-Bintintan, Hossam Al-Suod, Tomasz

Kowalkowski, Katarzyna Rafińska, and Bofus Buszewski. 2017. “The Effect of Growth

- Medium on an Escherichia Coli Pathway Mirrored into GC/MS Profiles.” *Journal of Breath Research* 11(3): 036012.
- Sauer, Sascha, and Magdalena Kliem. 2010. “Mass Spectrometry Tools for the Classification and Identification of Bacteria.” *Nature Reviews Microbiology* 8(1): 74–82.
- Scallan, Elaine, Patricia M. Griffin, Fredrick J. Angulo, Robert V. Tauxe, and Robert M. Hoekstra. 2011. “Foodborne Illness Acquired in the United States—Unspecified Agents.” *Emerging Infectious Diseases* 17(1): 16–22.
- Schulz, Stefan, and Jeroen S. Dickschat. 2007. “Bacterial Volatiles: The Smell of Small Organisms.” *Natural Product Reports* 24(4): 814.
- Schulz, Stefan, Jens Fuhlendorff, and Hans Reichenbach. 2004. “Identification and Synthesis of Volatiles Released by the Myxobacterium Chondromyces Crocatus.” *Tetrahedron* 60(17): 3863–72.
- Sparkman, O. David, Zelda Penton, and Fulton G. Kitson. 2011. *Gas Chromatography and Mass Spectrometry: A Practical Guide*. San Diego, United States: Elsevier Science & Technology. <http://ebookcentral.proquest.com/lib/wvu/detail.action?docID=670206> (July 20, 2020).
- Tait, Emma, John D. Perry, Stephen P. Stanforth, and John R. Dean. 2014a. “Bacteria Detection Based on the Evolution of Enzyme-Generated Volatile Organic Compounds: Determination of Listeria Monocytogenes in Milk Samples.” *Analytica Chimica Acta* 848: 80–87.

- Tait, Emma, John D. Perry, Stephen P. Stanforth, and John R. Dean. 2014b. "Identification of Volatile Organic Compounds Produced by Bacteria Using HS-SPME-GC-MS." *Journal of Chromatographic Science* 52(4): 363–73.
- Taiti, Cosimo, Corrado Costa, Paolo Menesatti, Stefania Caparrotta, Nadia Bazihizina, Elisa Azzarello, William Antonia Petrucci, Elisa Masi, and Edgardo Giordani. 2015. "Use of Volatile Organic Compounds and Physicochemical Parameters for Monitoring the Post-Harvest Ripening of Imported Tropical Fruits." *European Food Research and Technology* 241(1): 91–102.
- United States Food and Drug Administration. Food Safety and Inspection Service. 2019. *Raw Beef Product Sampling*.
- Vaishnav, Anukool, Ajit Varma, Narendra Tuteja, and Devendra Choudhary. 2017. "Characterization of Bacterial Volatiles and Their Impact on Plant Health Under Abiotic Stress." In *Volatiles and Food Security*, Springer Nature Singapore Pte Ltd., 15–23.
- Van Heerde, Harald, Kristiaan Helsen, and Marnik G. Dekimpe. 2007. "The Impact of a Product-Harm Crisis on Marketing Effectiveness." *Marketing Science* 26(2): 230–45.
- Veselova, M. A., V. A. Plyuta, and I. A. Khmel. 2019. "Volatile Compounds of Bacterial Origin: Structure, Biosynthesis, and Biological Activity." *Microbiology* 88(3): 261–74.
- Wood, William L., Daniel J Higbee, Melanie Gooldy, Scott Glogowski, Rich Fitzpatrick, Richard J Karalus, Troy D. Wood, and David J. Mangino. 2006. "Analysis of Volatile Bacterial Metabolites by Gas Chromatography--Mass Spectrometry." *Spectroscopy* 21(6): 20–28.

- Yu, Keshun, Thomas R. Hamilton-Kemp, Douglas D. Archbold, Randall W. Collins, and Melissa C. Newman. 2000. "Volatile Compounds from Escherichia Coli O157:H7 and Their Absorption by Strawberry Fruit." *Journal of Agricultural and Food Chemistry* 48(2): 413–17.
- Zhu, Jiangjiang, Heather D. Bean, Yin-Ming Kuo, and Jane E. Hill. 2010. "Fast Detection of Volatile Organic Compounds from Bacterial Cultures by Secondary Electrospray Ionization-Mass Spectrometry." *Journal of Clinical Microbiology* 48(12): 4426–31.
- Zhu, Jiangjiang, and Jane E. Hill. 2013. "Detection of Escherichia Coli via VOC Profiling Using Secondary Electrospray Ionization-Mass Spectrometry (SESI-MS)." *Food Microbiology* 34(2): 412–17.

Chapter III

Volatile organic compound methodology development using *Escherichia coli* DH5, *Escherichia coli* JM109, and *Salmonella enterica* serotype Enteritidis

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Abstract

The sensitivity of a closed-loop headspace collection and analysis of volatile organic compounds (VOC) produced from *Escherichia coli* JM109, *Escherichia coli* DH5 α , and *Salmonella enterica* serotype Enteritidis ATCC 13076 was determined using gas chromatography-mass spectrometry (GC-MS). Glass volatile collection trap filters with Alltech HaySep-Q™ PoraPak-Q™ chemical absorbent were eluted with dichloromethane before analysis with GC-MS. *E. coli* strains produced typical and well known compound indole detected during different collection lengths. Four peaks were detected from *E. coli* JM109 with peak retention times 4.389, 20.341, 20.984 (indole), and 22.525. *E. coli* DH5 α presented five peaks at 4.378, 6.909, 9.191, 20.967 (indole), and 33.277. These peaks were not identified in the control of sterile tryptic soy broth. The VOC profile was collected analyzed from *S. Enteritidis* grown optimally in liquid egg to further explore the use of VOC analysis for food safety. Seven compounds were detected in a 6-hour collection that were not seen in the uninoculated liquid egg control. A 2-hour collection series of *S. Enteritidis* produced mixed results with unique, but inconsistent compounds as well as a lack of most peaks identified in the 6-hour collection. These mixed results suggest that the collection system may not be sensitive enough to be utilized for 2-hour collections of *S. Enteritidis* in liquid egg.

Keywords: closed-loop headspace collection, volatile organic compounds, gas chromatography-mass spectrometry, *Escherichia coli*, indole, *Salmonella* Enteritidis, liquid egg

Introduction

Product recalls, illness and death occurs yearly for food products within the United States (Scallan et al. 2011). Recalls can have lasting impacts upon a company including loss of sales, decreased effectiveness of marketing, and an overall negative consumer outlook on the company and any associated companies (Van Heerde, Helsen, and Dekimpe 2007). Early detection of adulterated products prior to reaching the consumer has the potential to lower the number of recalls, illness and deaths seen. Exploring volatile organic compounds (VOC) may provide an opportunity to develop technology for detection of compounds released by bacteria with applications in food safety. Bacterial VOCs are primary and secondary metabolites released from bacteria during metabolism (Chen et al. 2017). Compounds generally released by bacteria can fall into five general categories including fatty acid derivatives, terpenes, aromatic compounds, sulfur containing compounds, and nitrogen containing compounds (Veselova, Puta, and Khmel 2019).

Like the numerous compounds bacteria are capable of producing, there are also numerous ways to collect those compounds including headspace collection. Independent of the trapping system used, VOC collection systems typically use an absorptive surface that interacts with and traps the volatiles released from the interested sample (Sparkman, Penton, and Kitson 2011). The VOCs are then released from these collection systems usually with either heat or an organic solvent. Common headspace collection systems include the use of the solid-phase microextraction fiber (Arnold and Senter 1998; Bhattacharjee et al. 2011; Cevallos-Cevallos, Danyluk, and Reyes-De-Corcuera 2011; Chen et al. 2016, 2017; Tait et al. 2014). One study that evaluated headspace of four bacteria used desorption tubes that contained carbograph 1TD/Carbopack X from Markes International to trap VOC (Boots et al. 2014). Like the previous study, another study which examined strawberries utilized filters that contained Super-Q

absorbent from Alltech which allowed VOC to be trapped over an 18-hour period before eluting the absorbent with a hexane and methylene chloride solution (Yu et al. 2000). This methodology coupled with gas chromatography-mass spectrometry (GC-MS) has been used successfully to identify volatiles in plants and microbial-plant interactions (Vaishnav et al. 2017).

GC-MS is an established, sensitive method for evaluating collected metabolites (Lindon, Nicholson, and Holmes 2019). Once a sample is injected into a GC unit, oftentimes at a high temperature, the compounds are separated along a column. Those separated compounds are then introduced into the mass spectrometer which separates the ions of a compound providing the final data output as a mass spectra (Lisec et al. 2006). GC-MS has been ranked highly in its ability to produce quality results with libraries, like the NIST spectrum reference library, for analysis of results (Lindon, Nicholson, and Holmes 2019). The aim of this study was to investigate the potential use of VOC headspace collection utilizing a closed-loop stripping apparatus coupled with GC-MS analysis of bacterial VOCs and application in food borne illness causing bacteria.

Materials and Methods

Inoculum Prep

Escherichia coli JM109 and *Escherichia coli* DH5α were both retrieved from laboratory stock frozen in glycerol and stored at -80°C. Frozen cultures were aseptically transferred into sterile tryptic soy broth (TSB) and incubated at 37°C for 24 hours. Stock tryptic soy agar (TSA) streak plates were made from the inoculated TSB for each *E. coli* JM109 and *E. coli* DH5α. *Salmonella enterica* serotype Enteritidis ATCC13076 was obtained from the American Type Culture Collection. The dehydrated pellet was aseptically rehydrated following ATCC guides using TSB. Once established, bacteria stock cultures in TSB were stored at 4°C. At least twice a

month all bacteria stock cultures were transferred to fresh, sterile TSB and TSA and grown at 37°C for 24 hours before storage again at 4°C to maintain cultures.

24 hours prior to VOC collection, the stock culture was used to inoculate 50mL TSB with a 10 µL loop of the selected bacteria. The inoculated broth was then placed on a 150 rpm shaker for 24 hours and incubated at 37°C. For studies involving *S. Enteritidis* the 24-hour culture was diluted using 0.1 % Buffered Peptone Water (BPW) to desired starting concentration. Once diluted, the tube was centrifuged at 5,000 rpm for 10 minutes and supernatant was discarded leaving the remaining bacteria pellet for use. At the start of each VOC collection bacteria concentration was determined using the spread plating technique utilizing a tenfold dilution into 0.1% BPW onto either TSA for *E. coli* strains or xylose lysine deoxycholate (XLD) agar plates for *S. Enteritidis*.

VOC Collection

Early Methodology of *E. coli* VOC Sample Collection

24 hours after inoculation of 50 mL TSB with desired *E. coli* the culture was transferred to a sterile 250 mL glass beaker. 50 mL of uninoculated TSB was added to a separate sterile beaker for use as a control. Both beakers were placed into the closed-loop, headspace stripping apparatus for VOC collection as viewed in Figure 1. This collection utilized glass volatile collection trap filters with Alltech HayeSep-Q™ PoraPak-Q™ chemical absorbent. The top right of Figure 2 displays the glass volatile collection trap utilized. This collection took place at room temperature under a sterile biosafety cabinet for 24 and 5 hours as displayed in Figure 1. For the two, 24 hour VOC collections of *E. coli* JM109 and DH5α, the starting bacterial concentrations reported as mean ± standard deviation were 9.01±0.12 log CFU mL⁻¹ TSB and

8.81±0.19 log CFU mL⁻¹ TSB respectively. 8.93±0.07 log CFU mL⁻¹ TSB was the starting concentration for the third VOC collection of DH5a that lasted for only 5-hours.

Final VOC Collection System

Volatiles released by the bacteria and media were collected using a closed-loop stripping apparatus held at 37°C to provide optimal growth conditions for the bacteria. Glass volatile collection trap filters with Alltech HayeSep-Q™ PoraPak-Q™ chemical absorbent were used to trap volatile compounds released from samples. Filters were cleaned between each use and prior to first use after purchase by running 5 volumes of dichloromethane to remove any potential VOCs from previous VOC collections. Teflon tubing was used to connect air pumps and desiccators to avoid the potential absorbance of VOCs that could occur with typical plastic tubing.

S. Enteritidis in Liquid Egg VOC Sample collection

Large grade A Kroger brand eggs were purchased from Kroger Patterson Drive, Morgantown WV one day prior to the expected VOC collection. On the day of the expected VOC collection, eggs were dipped into 70% ethanol and allowed to dry under UV within a sterile hood. Eggs were flipped 30 minutes into drying to expose all sides to UV. Once dry, 5 eggs were cracked and the contents were added to a Whirl-Pak® bag. The bag was stomached for 60 seconds to mix the egg thoroughly. 190mL of liquid egg was then measured into a control and experimental flask each. 1mL of the mixed egg from the experimental flask was added to the *S. Enteritidis* bacteria dilution tube for resuspension of the bacteria pellet then used to inoculate the flask. A sample of liquid egg in the experiment was plated onto XLD media prior to test to confirm *Salmonella* spp. were not present.

40 mL of liquid egg from each flask was transferred aseptically into a sterile 250mL beaker equipped with a stir bar. Each beaker was placed into the closed-loop headspace VOC collection apparatus for 6-hours for collection under gentle agitation using a stir plate. Starting bacterial concentration reported as of the 6-hour VOC collection was $4.02 \pm 0.08 \log \text{CFU mL}^{-1}$ of egg.

A second VOC collection of *S. Enteritidis* in liquid egg followed the same starting conditions as the 6-hour collection. This collection took place over 8-hours with the VOC filter being replaced every two hours. During the filter replacement after 2 hours, the 40 mL of liquid egg control and experimental samples were also replaced. The replaced samples came from the initially inoculated 190 mL liquid egg at the start of the VOC collection period. For the duration of the experiment, the inoculated and uninoculated control of liquid egg were kept in an incubator shaker at 37°C and 150 rpm. Bacteria concentration was taken from the initial 190 mL inoculated flask at the time of the filter and egg replacement. Three replicates were completed for this study. Starting bacterial concentration reported as mean $\pm$ standard deviation for the 8-hour study was $4.92 \pm 0.19 \log \text{CFU mL}^{-1}$ of egg.

Analysis

VOC Analysis

VOC filters were removed from the system after the collection period and eluted with 260 μ L of dichloromethane into labeled auto sampler vials and stored in a freezer until processed. These VOC GC-MS samples and blank samples of dichloromethane were analyzed by a TRACE 1310 gas chromatograph system coupled with a TSQ 8000 Triple Quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). 2 μ L of each sample were injected, volatilized at 220°C in a SSL injector module and separated using a TraceGOLD TG-5MS GC column (30

m length, 0.25 mm I.D., 0.25 μ m film; Thermo Fisher Scientific, Pittsburgh, PA, USA). Column temperature was held at 40°C for 3 minutes and then ramped at 5°C/min to 120°C, 10°C/min to 180°C and 20°C/min to 300°C which was held constant for 2 minutes. Helium was used as a carrier gas flowing at 1.3 mL/min. Masses were scanned utilizing a full-scan mode with the abundance or peak area of each measured under total ion chromatogram (TIC).

Chromatograph Interpretation

After analysis by the GC-MS was completed and the dichloromethane background was subtracted, chromatograph intensity and retention time was exported in Microsoft Excel and visually examined for peaks in all samples. Peaks were noted and tentatively identified using NIST 09 standard reference database (Stein et al. 2011 ; *Xcalibur* 2.2 2011). Peaks that were not confidently identified are labeled as unknown.

Results and Discussion

VOC Collection System Progression

The VOC collection system was developed from methodology for collecting VOCs from plants through collaboration (Gutensohn and Dudareva 2016). The first collections took place at room temperature with 24-hour bacteria cultures to get an understanding of potential compounds that could be used to uniquely identify each bacterium (Figure 1). *E. coli* JM109 and DH5 α were first chosen to work with due to their non-pathogenic properties prior to utilizing biosafety level 2 designated organisms. Obstacles faced and surpassed include condensation within collection desiccators, utilization of stir plates for gentle agitation, and creating optimal growth conditions for bacteria by moving the system into a large incubator. Optimal growth conditions for bacteria were eventually chosen to ensure optimal VOC output from metabolic processes. Optimal VOC output will provide insight into compounds when studying VOC at

food processing temperatures. Figure 2 displays the final set up of the dedicator and stir plate that was held within an incubator to control temperature and ensure gentle agitation.

VOC Collections of *E. coli* DH5α and *E. coli* JM109

Four peaks were detected within the collection period for *E. coli* JM109 by their peak retention time; tentative identification includes lupeol acetate at 4.389, delta-dodecanolactone at 20.341, indole at 20.984 and an unknown compound at 22.525 (Table 1). Five peaks were detected within the collection period for *E. coli* DH5α by their peak retention time; tentative identification includes pleiocarpamine at 4.378 and 6.909, 3-oxo-α-ionone at 9.191, indole at 20.967 and (x)-pentatriacont-17-ene at 33.277 (Table 2). These compounds were identified in the inoculated TSB but not the sterile control TSB. Table 3 displays the 10 peaks of interest from the 5-hour collection of *E. coli* DH5α. Five of these 10 peaks were identified as compounds of interest in the sterile control, but was not observed to have a peak in exported excel chromatographs. An unknown compound at 10.981, indole at 20.937, and trielaidin at 33.810 and 34.107 were all observed in the inoculated broth, but did not identify using the NIST reference library within the control.

Indole was observed in all three collections of both *E. coli* DH5α and JM109 strains with retention time peaks of 20.98, 20.97 and 20.94 as seen in Figures 4, 5, and 6. Indole VOC production from *E. coli* species has been seen in multiple studies on food products and different media including TSB (Arnold and Senter 1998; Boots et al. 2014; Bunge et al. 2008; Chen et al. 2016, 2017; Kunze et al. 2013; Ratiu et al. 2017; Tait et al. 2014; Yu et al. 2000; Zhu et al. 2010). Using headspace SPME coupled with GC-MS Tait and others reported *Escherichia coli* NCTC 10418 producing indole in both tryptone soya broth and brain heart infusion broth, but not in difco enteric fermentation base broth (Tait et al. 2014). Two different ATCC type *Escherichia*

coli O157:H7 and a non pathogenic *E. coli* ATCC 15597 were reported to produced indole at higher concentrations than other observed compounds after being grown in brain heart infusion broth then transferred to plate count agar for headspace VOC collection (Yu et al. 2000). Also utilizing TSB, like this experiment, Arnold and Senter reported only *E. coli* produced indole of the 7 bacteria analyzed for VOCs (Arnold and Senter 1998). These studies support our results of the two *E. coli* strains producing indole. Indole is produced from *E. coli* through the tryptophan pathway in which tryptophan is converted to indole, pyruvate, and ammonia (Han et al. 2011). Steady production of indole during the growth of *E. coli* is not seen, but instead the highest amount of indole produced occurs between logarithmic and stationary growth phases (Gaimster and Summers 2015). It would make sense that we are seeing a high intensity of indole with the VOC sample due to the 24-hour culture age in which we do not expect a great increase of growth.

VOC Collections of *S. Enteritidis* in Liquid Egg

The preliminary collection of VOCs produced by *E. coli* strains were taken when bacterial concentrations were approximately $8.9 \pm 0.10 \log \text{CFU ml}^{-1}$ of TSB. The successful detection of compounds from *E. coli* VOC collections at high bacteria concentrations progressed to the next step of decreasing bacterial concentration for *S. Enteritidis* collections to see if volatiles could still be collected. With the potential influence of growth phase, as seen in our *E. coli* collections, capturing VOCs during active growth may lead to different compounds as well as in a closer to the bacteria concentration that may be seen in contaminated food products. Liquid egg was also used instead of standard laboratory media to further investigate the potential for application within food safety. The 6-hour VOC collection of *S. Enteritidis* in liquid egg had a bacteria concentration of $4.02 \pm 0.08 \log \text{CFU ml}^{-1}$ liquid egg. Table 4 displays the seven

compounds seen in inoculated liquid egg and not in the uninoculated liquid egg. No previous studies have seen these compounds, and to our knowledge there have been no VOC studies of *S. Enteritidis* in liquid egg. Media has the ability to influence VOCs that are produced through limiting the availability of nutrients bacteria need for metabolic activities (Arnold and Senter 1998; Bhattacharjee et al. 2011; Cevallos-Cevallos, Danyluk, and Reyes-De-Corcuera 2011; Chen et al. 2017; Ratiu et al. 2017; Tait et al. 2014; Zhu et al. 2010). Tait and associates examined the VOC profile of three different bacteria grown in each of the three media chosen and reported that there were similar VOC profiles between the brain heart infusion broth and tryptone soya broths but presented significantly different in the enteric fermentation base broth (Tait et al. 2014). Ratiu and colleagues also examined the influence of media on VOC production for *E. coli* grown in tryptic soy broth, Mueller Hinton broth and a minimal salts (M9) broth enriched with glucose. Reported results suggest that each *E. coli* inoculated media produced unique compounds when compared between the three (Ratiu et al. 2017). With the documented influence of media upon bacterial VOC production it is not surprising that *S. Enteritidis* inoculated liquid egg has produced compounds that were not seen in the literature reviewed.

The unique peaks detected within the 6-hour VOC collection of *S. Enteritidis* in liquid egg were compared to the chromatographs produced during the 2-hour collection series to see if these compounds could be identified. Table 6 displays the starting bacteria concentration for each 2-hour collection with the first collection starting at $4.92 \pm 0.19 \log \text{CFU ml}^{-1}$. Peak 4.164 tentatively identified as 1,1,2,2-tetrafluoroethane in the 6-hour collection did not appear in any of the 2-hour collection series. Peaks at the retention time of 4.480 and 5.49 did appear in two of the three replicates with different tentative identifications. Peak 7.518 tentatively

identified as 2-nitrobutane did identify as the same compound in all three replicate controls of uninoculated liquid egg. This suggests that this compound may not be unique to *S. Enteritidis* VOC production. The software used to identify the peaks was unable to recognize the peaks at 11.596 or 11.722 in the 6-hour collection (Stein et al. 2011 ; *Xcalibur* 2.2 2011). The peak at 11.249 was seen in one of the three replicates but had a different compound identification. The lack of identifiable peaks from the 6-hour collection to the 2-hour collection series suggests that the VOC collection system in place may not be sensitive enough to utilize for such a short collection period. Table 6 does identify inconsistent compounds that may be relative with future studies in a more sensitive collection system. These compounds were not seen in the control of uninoculated liquid egg.

Conclusion

This study has met its aim of determining that this methodology can be used reliably to collect VOC from two difference *E. coli* strains as seen through the regular collection of indole during a short collection period. Specific VOCs were also produced by *S. Enteritidis* grown in liquid egg, but more replicates are needed to identify and confirm consistent compounds. To fully capture and identify the signature VOCs produced by bacteria a more sensitive system or a modification of analysis by the GC-MS is needed.

Some limitations that occurred during this study include tentative identification utilizing the NIST reference library, multiple same-compound identifications, and the potential that compounds chemically converted upon injection. Tentative identification using a reference library can only provide a probability that a compound matches the reference library. Identification must further be confirmed utilizing external standards and compared to the compound in question. Due to the probability that a compound matches the reference library it is

possible for multiple compounds of interest to identify as the same compound. The GC-MS separates compounds by size and then by charge so these compounds are not the same compound but may have very similar structures with slight differences that would cause them to show up at a different retention time and will need to be confirmed through the use of external standards with additional VOC samples. For future studies, GC-MS analysis may want to include tandem sampling to increase the ability to identify compounds seen in systems like *S. Enteritidis* in egg.

References

- Arnold, Judy W, and Samuel D Senter. 1998. "Use of Digital Aroma Technology and SPME GC-MS to Compare Volatile Compounds Produced by Bacteria Isolated from Processed Poultry." *Journal of the Science of Food and Agriculture* 78(3): 343–48.
- Bhattacharjee, Paramita, Suranjan Panigrahi, Dongquig Lin, Catherine M. Logue, Julie S. Sherwood, Curt Doetkott, and Martin Marchello. 2011. "A Comparative Qualitative Study of the Profile of Volatile Organic Compounds Associated with Salmonella Contamination of Packaged Aged and Fresh Beef by HS-SPME/GC-MS." *Journal of Food Science and Technology* 48(1): 1–13.
- Boots, A W, A Smolinska, J J B N van Berkel, R R R Fijten, E E Stobberingh, M L L Boumans, E J Moonen, E F M Wouters, J W Dallinga, and F J Van Schooten. 2014. "Identification of Microorganisms Based on Headspace Analysis of Volatile Organic Compounds by Gas Chromatography–Mass Spectrometry." *Journal of Breath Research* 8(2): 027106.
- Bunge, M., N. Araghipour, T. Mikoviny, J. Dunkl, R. Schnitzhofer, A. Hansel, F. Schinner, A. Wisthaler, R. Margesin, and T. D. Mark. 2008. "On-Line Monitoring of Microbial Volatile Metabolites by Proton Transfer Reaction-Mass Spectrometry." *Applied and Environmental Microbiology* 74(7): 2179–86.
- Cevallos-Cevallos, Juan Manuel, Michelle D. Danyluk, and José I. Reyes-De-Corcuera. 2011. "GC-MS Based Metabolomics for Rapid Simultaneous Detection of Escherichia Coli O157:H7, Salmonella Typhimurium, Salmonella Muenchen, and Salmonella Hartford in Ground Beef and Chicken." *Journal of Food Science* 76(4): M238–46.

- Chen, Juan, Junni Tang, Cheng Tang, Hui Shi, Huaiyin Gu, and Xiaozhu Lu. 2016. “Growth and Volatile Compounds of Escherichia Coli O157:H7 on Irradiated Pork.” *Journal of Food Quality* 39(6): 766–72.
- Chen, Juan, Junni Tang, Hui Shi, Cheng Tang, and Rong Zhang. 2017. “Characteristics of Volatile Organic Compounds Produced from Five Pathogenic Bacteria by Headspace-Solid Phase Micro-Extraction/Gas Chromatography-Mass Spectrometry.” *Journal of Basic Microbiology* 57(3): 228–37.
- Debnath, Mousumi, G. B. Prasad, Prakash S. Bisen, and Prakash S. Bisen. 2012. *Microbes: Concepts and Applications*. Somerset, UNITED STATES: John Wiley & Sons, Incorporated. <http://ebookcentral.proquest.com/lib/wvu/detail.action?docID=848522> (June 5, 2020).
- Gaimster, Hannah, and David Summers. 2015. “Regulation of Indole Signalling during the Transition of E. Coli from Exponential to Stationary Phase” ed. Marie-Joelle Virolle. *PLOS ONE* 10(9): e0136691.
- Gutensohn, M., and N. Dudareva. 2016. “Chapter Thirteen - Tomato Fruits—A Platform for Metabolic Engineering of Terpenes.” In *Methods in Enzymology, Synthetic Biology and Metabolic Engineering in Plants and Microbes Part B: Metabolism in Plants*, ed. Sarah E. O’Connor. Academic Press, 333–59.
<http://www.sciencedirect.com/science/article/pii/S0076687916001245> (June 25, 2019).
- Han, Thi Hiep, Jin Hyung Lee, Moo Hwan Cho, Thomas K. Wood, and Jintae Lee. 2011. “Environmental Factors Affecting Indole Production in Escherichia Coli.” *Research in Microbiology* 162(2): 108–16.

- Klein, Daniel, Stephan Maurer, Ulrike Herbert, Judith Kreyenschmidt, and Peter Kaul. 2018. "Detection of Volatile Organic Compounds Arising from Chicken Breast Filets Under Modified Atmosphere Packaging Using TD-GC/MS." *Food Analytical Methods* 11(1): 88–98.
- Kunze, Nils, Stephan Maurer, Ulrike Herbert, Judith Kreyenschmidt, and Peter Kaul. 2013. "Detection and Validation of Volatile Metabolic Patterns over Different Strains of Two Human Pathogenic Bacteria during Their Growth in a Complex Medium Using Multi-Capillary Column-Ion Mobility Spectrometry (MCC-IMS)." *Applied Microbiology and Biotechnology* 97(8): 3665–76.
- Lindon, John C., Jeremy K. Nicholson, and Elaine. Holmes. 2019. *The Handbook of Metabolic Phenotyping*. Amsterdam, Netherlands: Elsevier.
<http://search.ebscohost.com/login.aspx?direct=true&scope=site&db=nlebk&db=nlabk&AN=1724651> (January 27, 2020).
- Lisec, Jan, Nicolas Schauer, Joachim Kopka, Lothar Willmitzer, and Alisdair R. Fernie. 2006. "Gas Chromatography Mass Spectrometry–Based Metabolite Profiling in Plants." *Nature Protocols* 1(1): 387–96.
- Ratiu, Ileana-Andreea, Tomasz Ligor, Victor Bocos-Bintintan, Hossam Al-Suod, Tomasz Kowalkowski, Katarzyna Rafińska, and Bofus Buszewski. 2017. "The Effect of Growth Medium on an Escherichia Coli Pathway Mirrored into GC/MS Profiles." *Journal of Breath Research* 11(3): 036012.

- Scallan, Elaine, Patricia M. Griffin, Fredrick J. Angulo, Robert V. Tauxe, and Robert M. Hoekstra. 2011. "Foodborne Illness Acquired in the United States—Unspecified Agents." *Emerging Infectious Diseases* 17(1): 16–22.
- Sparkman, O. David, Zelda Penton, and Fulton G. Kitson. 2011. *Gas Chromatography and Mass Spectrometry: A Practical Guide*. San Diego, United States: Elsevier Science & Technology. <http://ebookcentral.proquest.com/lib/wvu/detail.action?docID=670206> (July 20, 2020).
- Stein, S., Y. Mirokhin, G. Mallard, A. Mikaia, P. Neta, D. Sparkman, E. White, X. Yang, V. Zaikin, and D. Zhu. (2011). NIST Mass Spectral Search Program (2.0g) [Computer software]. FairCom Corporation.
- Tait, Emma, John D. Perry, Stephen P. Stanforth, and John R. Dean. 2014. "Identification of Volatile Organic Compounds Produced by Bacteria Using HS-SPME-GC–MS." *Journal of Chromatographic Science* 52(4): 363–73.
- Vaishnav, Anukool, Ajit Varma, Narendra Tuteja, and Devendra Choudhary. 2017. "Characterization of Bacterial Volatiles and Their Impact on Plant Health Under Abiotic Stress." In *Volatiles and Food Security*, Springer Nature Singapore Pte Ltd., 15–23.
- Van Heerde, Harald, Kristiaan Helsen, and Marnik G. Dekimpe. 2007. "The Impact of a Product-Harm Crisis on Marketing Effectiveness." *Marketing Science* 26(2): 230–45.
- Veselova, M. A., V. A. Plyuta, and I. A. Khmel. 2019. "Volatile Compounds of Bacterial Origin: Structure, Biosynthesis, and Biological Activity." *Microbiology* 88(3): 261–74.

Xcalibur 2.2 (2.2 SP1.48). (2011). [Computer software]. Thermo Fisher Scientific Inc.

Yu, Keshun, Thomas R. Hamilton-Kemp, Douglas D. Archbold, Randall W. Collins, and Melissa C. Newman. 2000. "Volatile Compounds from *Escherichia Coli* O157:H7 and Their Absorption by Strawberry Fruit." *Journal of Agricultural and Food Chemistry* 48(2): 413–17.

Zhu, Jiangjiang, Heather D. Bean, Yin-Ming Kuo, and Jane E. Hill. 2010. "Fast Detection of Volatile Organic Compounds from Bacterial Cultures by Secondary Electrospray Ionization-Mass Spectrometry." *Journal of Clinical Microbiology* 48(12): 4426–31.

Tables and Figures

Table 1. *E. coli* JM109 VOC compounds observed from a 24-hour VOC collection in tryptic soy broth. These compounds were not identified in the control of sterile tryptic soy broth.

Compounds have been displayed with observed peak retention time (RT). Starting bacterial concentration was a mean and standard deviation of 9.01 ± 0.12 log CFU mL⁻¹ tryptic soy broth.

Peak RT	Tentative Compound ID
4.389	Lupeol acetate
20.341	Delta-dodecanolactone
20.984	Indole
22.525	Unknown

Table 2. *E. coli* DH5 α VOC compounds observed from a 24-hour VOC collection in tryptic soy broth. These compounds were not observed in the control of tryptic soy broth. Compounds have been displayed with observed peak retention time (RT). Starting bacterial concentration was a mean and standard deviation of 8.81 $\pm$ 0.19 log CFU mL⁻¹ tryptic soy broth.

Peak RT	Tentative Compound ID
4.378	Pleiocarpamine
6.909	Pleiocarpamine
9.191	3-Oxo-a-ionone
20.967	Indole
33.277	(z)-Pentatriacont-17-ene

Table 3. *E. coli* DH5 α VOC compounds observed from a 5-hour VOC collection in tryptic soy broth. These compounds were not observed in the control of tryptic soy broth. Compounds have been displayed with observed peak retention time (RT). Starting bacterial concentration was a mean and standard deviation of 8.93 $\pm$ 0.07 log CFU mL⁻¹ tryptic soy broth.

Peak RT	Tentative Compound ID
4.372	Pleiocarpamine*
7.984	Pleiocarpamine*
10.981	Unknown
15.716	1,5-Diphenyl-3-(3-cyclopentylpropyl)pentane
20.937	Indole
29.590	1-Heptatriacontanol*
31.036	1-Heptatriacontanol*
32.648	1-Heptatriacontanol*
33.810	Trielaidin
34.107	Trielaidin

* Indicates that this compound, though seen as a peak within the inoculated tryptic soy broth and not in the control of sterile broth, this compound was identified for the control at the same retention time.

Table 4. VOC compounds seen from *S. Enteritidis* inoculated liquid egg that did not appear in the control of liquid egg. Starting bacteria concentration of this VOC collection was 4.02 ± 0.08 log CFU ml⁻¹ liquid egg. Compounds have been displayed with observed peak retention time (RT).

Peak RT	Tentative ID
4.164	1,1,2,2-Tetrafluoroethane
4.480	1,1,2,2-Tetrafluoroethane
5.491	4-Amino-1-pentanol
7.518	2-Nitrobutane
11.249	2-Amino-5-Methylhexane
11.596	5-(Cyclohexylmethyl)pyrrolidin-2-one
11.722	Unknown

Table 5. The bacteria concentration reported as mean and standard deviation (STDEV) of log CFU mL⁻¹ of *S. Enteritidis* in liquid egg every 2 hours of the VOC collection series for 8 hours.

Hour	Mean log CFU/mL	STDEV
0	4.92	0.19
2	5.06	0.51
4	6.03	0.87
6	8.06	0.60
8	8.44	0.85

Table 6. Unique, inconsistent compounds that appeared in *S. Enteritidis* inoculated liquid egg 2-hour series that did not appear in the control of uninoculated egg. Compounds have been displayed with observed peak retention time (RT).

Peak RT	Tentative ID	Sample Hour
7.147	Diacetone alcohol	0
15.722	2,3,4-Trimethyloxetane	0
18.554	2-Ethyl-3,5-dimethylpyrazine	0
24.127	2-Butyl-1-methylpyrrolidine	0
4.494	1-Chloro-1,2,3-butanetriene	2
28.576	4-Aminopentan-1-ol	2
6.929	2-Cyclopropylethanol	4
10.167	(3,3--Dimethylbutyl)oxirane	4
7.797	2-Nitrobutane	6
14.787	2-Methyl-1-Nitropropane	6
16.174	Unknown	6
20.411	2,5-Di(tert-amyl)hydroquinone	6
22.617	Unknown	6

Figure 1. The early methodology for collecting VOCs from 24-hour cultures utilizing a closed-loop headspace VOC collection system held at room temperature under a biosafety cabinet. This system utilizes a glass filter to trap headspace VOCs and Teflon tubing to prevent potential interruption of VOC collection.

Figure 2. The closed-loop headspace collection system with a close up of the glass volatile collection trap filter with Alltech HayeSep-Q™ PoraPak-Q™ chemical absorbent at the top right. The Pyrex glass desiccator was placed on a standard stir plate with a glass beaker and stir bar to provide gentle agitation. A rubber seal, hollow metal rod and Teflon tubing were used to connect this system to the air pump and power supply. This system was utilized within a large incubator for temperature control.

Figure 3. 24-hour VOC collection of uninoculated and *E. coli* JM109 tryptic soy broth. Starting bacteria concentration is reported as a mean $\pm$ standard deviation of 9.01 ± 0.12 log CFU mL⁻¹ of tryptic soy broth.

Figure 4. 24-hour VOC collection of uninoculated and *E. coli* JM109 tryptic soy broth with the with the tentatively identified compound lupeol acetate labeled.

Figure 5. 24-hour VOC collection of uninoculated and *E. coli* JM109 tryptic soy broth with the tentatively identified compound delta-dodecanolactone compound labeled.

Figure 6. Chromatograph from the 24-hour VOC collection of uninoculated and *E. coli* DH5 α inoculated tryptic soy broth. Starting bacteria concentration is reported as a mean $\pm$ standard deviation of 8.81 ± 0.19 log CFU mL⁻¹ of tryptic soy broth. The tentatively identified indole is labeled.

Figure 7. Chromatograph from the 24-hour VOC collection of uninoculated and *E. coli* DH5 α inoculated tryptic soy broth with tentatively identified compound 3-oxo- α -ionone labeled.

Figure 8. Chromatograph from the 24-hour VOC collection of uninoculated and *E. coli* DH5 α inoculated tryptic soy broth with tentatively identified (x)-pentatriacont-17-ene labeled.

Figure 9. Chromatograph from the 5-hour VOC collection of uninoculated and *E. coli* DH5 α inoculated tryptic soy agar. *E. coli* TSB was inoculated 24 hours prior to the start of the VOC collection. Starting bacteria concentration is reported as a mean $\pm$ standard deviation of 8.93 ± 0.07 log CFU mL⁻¹ of tryptic soy broth. The tentatively identified compound indole was labeled.

Figure 10. Chromatograph from the 5-hour VOC collection of uninoculated and *E. coli* DH5 α inoculated tryptic soy agar. Tentatively identified compound 1-heptatriacontanol peaks are denoted with a *.

Figure 11. Chromatograph from the 5-hour VOC collection of uninoculated and *E. coli* DH5 α inoculated tryptic soy agar. Tentatively identified compound 1,5-diphenyl-3-(3-cyclopentylpropyl)pentane has been labeled.

Figure 12. Chromatograph from a 6-hour VOC collection of *S. Enteritidis* inoculated liquid egg coupled with an uninoculated sample of liquid egg. Bacteria concentration at the start of this collection is reported as mean $\pm$ standard deviation of 5.22 ± 0.66 log CFU mL⁻¹ of liquid egg.

Figure 13. Chromatograph from a 6-hour VOC collection of *S. Enteritidis* inoculated liquid egg coupled with an uninoculated sample of liquid egg with 1,1,2,2-tetrafluorethane, 4-amino-1-pentanol all labeled. The peaks labeled *1 and *2 are 2-nitrobutane and 5-(cyclohexylmethyl)pyrrolidine-2-one respectively.

Chapter IV

Volatile Organic Compounds from *Escherichia coli* Grown in Ground Beef and Tryptic Soy Broth

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Abstract

Volatile organic compounds (VOC) have been investigated for use of bacterial identification across different media and applications. Utilizing a headspace closed-loop stripping VOC collection coupled with gas chromatography-mass spectrometry (GC-MS), this study aimed to evaluate the VOC profile of *Escherichia coli* serotype O157:H7 ATCC 43985 grown in both tryptic soy broth and ground beef. Fresh beef was ground in house under sanitary conditions to reduce the presence of background flora. Glass volatile collection trap filters with Alltech HaySep-Q™ PoraPak-Q™ chemical absorbent were eluted with dichloromethane before being analyzed with a GC-MS. *E. coli* O157:H7 grown in TSB produced a profile of 34 peaks of interest with four of these peaks not being detected in the control of sterile TSB at retention times 8.722, 9.402, 20.919, and 30.777. When grown in ground beef a profile of 48 peaks of interest was determined with 22 compounds appearing only in *E. coli* O157:H7 inoculated ground beef. Tentative identification of detected compounds was performed using the NIST 09 reference library. Multiple compounds have been seen in previous studies solidifying the use of this methodology for VOC collection with more steps that can be taken in the future to increase identification abilities.

Key words: volatile organic compounds, *Escherichia coli* O157:H7, food microbiology, ground beef, headspace gas chromatography-mass spectrometry

Introduction

Despite the safe food supply the United States is known for there have already been over 100 recalls up through the end of June, 2020 posted by the Food and Drug Administration and an increasing instance of shiga like toxin producing *Escherichia coli* as reported by the Center for Disease Control and Protection (Affairs 2020; Tack et al. 2020). Bacteria volatile organic compounds (VOCs) may have the potential to help mediate illness and recalls. Use of VOCs have been examined for applications in medical diagnosis, agriculture improvement, environmental contamination, food monitoring and food safety (Boots et al. 2014; Chen et al. 2017; Tahiti et al. 2015; Veselova, Puta, and Khmel 2019; Wood et al. 2006). Though influences like media, mixed culture and growth phase have been seen in the literature, there are still gaps in knowledge when looking at VOC application within the food industry.

VOCs are primary and secondary metabolites that are released by bacteria during growth and proliferation (Chen et al. 2017). Common classes of compounds that bacteria release include fatty acid derivatives, terpenes, aromatic compounds, sulfur containing compounds, and nitrogen containing compounds (Veselova, Puta, and Khmel 2019). Generally, these compounds are produced by catabolic pathways in which compounds are broken down for use (Veselova, Plyuta, and Khmel 2019). There are various ways to collect VOCs including headspace stripping apparatus and headspace solid phase microextraction. These systems utilize a surface that interacts with the VOCs trapping them (Sparkman, Penton, and Kitson 2011). The trapped VOCs are released through the use of organic solvents or heat which disrupt that VOC-surface interaction. Collections are largely paired with gas chromatography-mass spectrometry (GC-MS) for analysis. Headspace closed-loop stripping apparatus coupled with analysis by GC-MS

has been successfully used to identify volatiles in plants and microbial-plant interactions (Vaishnav et al. 2017).

Exploring this technology and science for improved food safety VOCs have been collected from samples including chicken (Arnold and Senter 1998; Klein et al. 2018), beef (Abdallah et al. 2013; Bhattacharjee et al. 2011; Cevallos-Cevallos, Danyluk, and Reyes-De-Corcuera 2011) and pork (Chen et al. 2016). VOCs have the potential to guide food production monitoring through identifying VOC biomarkers that are released from bacteria (Veselova, Plyuta, and Khmel 2019). Application and development of this science could lead to the increased monitoring of products like ground beef and thus lowering potential for recalls and food borne illness in consumers.

The purpose of this study is to detect VOCs produced by *Escherichia coli* by utilizing a closed-loop VOC collection apparatus followed by analysis and identification with gas chromatography-mass spectrometry in both ground beef and tryptic soy broth (TSB). The aim of this study is to characterize the specific VOC profile of *Escherichia coli* serotype O157:H7 and potentially discover any compounds that may be of interest for food safety applications. This preliminary study will be used to help direct future research through offering insights into limitations and modifications that could improve future collection, analysis and identification of VOCs from food products.

Materials and Methods

Preparation of Inoculum

Escherichia coli serotype O157:H7 ATCC 43895 was obtained from the American Type Culture Collection. The dehydrated pellet was rehydrated following ATCC procedures using Tryptic Soy Broth (TSB) and aseptic technique. Once established, bacteria were stored at 4°C in

TSB. At least twice a month, bacteria cultures were transferred to new media. One 10 μ L loop from the previous bacteria culture was used to inoculate sterile TSB and grown at 37°C for 24 hours before storage again at 4°C to maintain working stock culture.

24 hours prior to VOC collection or growth study, a single 10 μ L loop of stock culture was used to inoculate 10 mL TSB. The inoculated TSB was placed on a shaker set to 150 rpm for 24 hours incubated at 37°C. The 24-hour bacteria culture was added to 0.1% buffered peptone water (BPW) using a 10-fold dilution technique to obtain a desired starting concentration of around 10^4 cells per mL of media. Once diluted, the tube was centrifuged at 5,000 rpm for 10 minutes and supernatant was discarded leaving the remaining bacteria pellet for use. A control dilution tube of 10mL BPW was included in the centrifuge. The supernatant was discarded from both the bacteria containing tube and the uninoculated control tube. To ensure there was minimal differences between the control and experimental groups, the uninoculated control tube was treated in the same manner as the bacteria containing tube. Bacteria concentration was determined using the spread plating technique prior to VOC collection. A tenfold dilution into 0.1% buffered peptone water (BPW) was performed prior to plating onto tryptic soy agar (TSA) plates.

Preliminary Growth Study

After discarding the supernatant, the bacteria pellet was resuspended in 1mL of TSB prior to the inoculation of three flasks of 189mL sterile TSB for a final total volume of 190 mL. The starting concentration for this growth study was $5.45 \pm 0.5 \log \text{CFU ml}^{-1}$ TSB. Inoculated flasks were stored in an incubator shaker set to 37°C at 150 rpm for 10 hours. At the time of inoculation and every hour following for 10 hours bacteria concentration was determined using 10-fold dilutions into 0.1% BPW and spread plating technique onto TSA. TSA plates were

incubated, inverted for 24-48 hours at 37°C prior to counting colony forming units (CFU). 10 hours was the timeframe chosen to capture the growth that would take place during VOC collection from a starting concentration around 5 log CFU mL⁻¹. This experiment was performed with three replicates that included control plates testing media prior to inoculation.

Figure 1 displays the growth exhibited by *E. coli* O105:H7 inoculated TSB. The growth study was started with a bacteria concentration of 5.45±0.51 log CFU mL⁻¹ TSB and finished with 9.48±0.35 log CFU mL⁻¹ TSB for a total growth of about 4 log. This growth study was done with this starting concentration and for the length of time to capture the growth during *E. coli* O157:H7 VOC collection. The growth of 4 log suggests active growth and thus the VOC collection took place during logarithmic phase of growth (Debnath et al. 2012). As seen in previous VOC studies, the growth phase can impact the production and types of volatiles that are being released (Bunge et al. 2008; Kunze et al. 2013).

Sample preparation and VOC Collection

Tryptic Soy Broth 1mL of TSB was added to each dilution tube and used to resuspend the bacteria pellet and as a control. Each dilution tube was used to inoculate experimental and control flasks of 189mL of sterile TSB to be used for VOC collection. Starting bacteria concentration of VOC collection was 6.07±0.52 log CFU mL⁻¹.

Ground Beef A large chuck roast was purchased from Kroger Patterson Drive, Morgantown WV within the use by date. Utensils and a kitchen grinder were dipped in 70% ethanol and placed in a sterile biosafety cabinet under UV light until dry and flipped over after 30 minutes to expose all surfaces to UV light. The outer 1.5 cm of the roast was removed and discarded. The remaining roast was ground in the sterile biosafety cabinet and frozen to be used later. Ground

beef was thawed the day of the VOC collection. 380g of beef was split between the control and the experimental group. Centrifuged control and *E. coli* O157:H7 dilution tubes were each resuspended in 1mL of 0.1% BPW and then added to each of the 190g of beef. Both control and inoculated beef were mixed by hand for 10 minutes. Starting bacteria concentration was $5.68 \pm 0.15 \log \text{CFU g}^{-1}$.

VOC Collection Volatiles released by the bacteria and media were collected using a closed-loop stripping apparatus held at 37°C to provide optimal growth conditions for the bacteria. Glass volatile collection trap filters with Alltech HayeSep-Q™ PoraPak-Q™ chemical absorbent were used to trap volatile compounds released from samples. Filters were cleaned between each use by running 5 volumes of dichloromethane to remove any potential VOCs from previous trials. Teflon tubing was used to connect air pumps and desiccators to avoid the potential absorbance of VOCs that could occur with typical plastic tubing.

40mL of inoculated and uninoculated TSB were placed into sterile glass beakers equipped with magnetic stir bars. The beakers were placed into the VOC desiccator for collection lasting 6 hours. Gentle agitation was provided from the stir plate located underneath the desiccator. For tests involving beef, 20g of inoculated beef was added to 3 sterile petri dishes to produce 3 experimental groups and 20g of uninoculated control beef was added to a 4th petri dish. The petri dishes were placed into outwardly labeled desiccators to collect VOCs for 6 hours at 37°C.

After 6 hours, the VOC filters were removed from the system and eluted with 260μL of dichloromethane into labeled autosampler vials. VOC GC-MS samples were stored in a freezer until prepped for analysis. Prior to analysis by GC-MS, 20μL from a 1:40 dilution of

naphthalene was added to each control and replicate of both VOC beef and broth samples.

Naphthalene was chosen for the internal standard after preliminary analysis determined that no peaks of interest would be interrupted by using this standard.

Analysis

Preliminary Growth Study CFU plate counts were transformed into log CFU mL⁻¹ TSB. Log CFU mL⁻¹ TSB were used to produce a mean with standard deviation and graphed with Microsoft Excel.

GC-MS Protocol A TRACE 1310 gas chromatograph system coupled with a TSQ 8000 Triple Quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). 2 µL of each sample were injected, volatilized at 220°C in a SSL injector module and separated using a TraceGOLD TG-5MS GC column (30 m length, 0.25 mm I.D., 0.25 µm film; Thermo Fisher Scientific, Pittsburgh, PA, USA). Column temperature was held at 40°C for 3 minutes and then ramped at 5°C/min to 120°C, 10°C/min to 180°C and 20°C/min to 300°C which was held constant for 2 minutes. Helium was used as a carrier gas flowing at 1.3 mL/min. Masses were scanned utilizing a full-scan mode with the abundance or peak area of each measured under total ion chromatogram (TIC). In addition to the sample, blank samples of dichloromethane were included with each run to be used to subtract the background of dichloromethane prior to analysis.

Chromatograph Interpretation After analysis by the GC-MS was completed and the background of dichloromethane was removed, chromatograph intensity and retention time was exported in Microsoft Excel and visually examined for peaks in all samples. Peaks were noted and

tentatively identified using NIST 09 standard reference database (Stein et al. 2011; *Xcalibur* 2.2 2011). Peaks that were identifiable were labeled as unknown. Tentative compound identification was assigned if 2 or more samples identified the same compounds. For peaks with two identifications that can be applicable due to probability comparison both identifications were included. Peak area was exported from the chromatograph using *Xcalibur* 2.2 software. Normalization was done through the division of the peak area of the internal standard, divided again by the number of hours of the VOC collection, six, and finally divided by the average log bacteria concentration to produce area VOC/hour/log CFU. Only the starting bacteria concentration was collected for the 6-hour collection in tryptic soy broth. This starting concentration was used in peak area unlike the averaged log CFU of the starting and ending log CFU of VOC collected from ground beef.

Results and Discussion

Broth VOC Collection

Thirty-four peaks of interest were determined when comparing the chromatographs. Of those thirty-four peaks, four peaks were not detected by the software within the control: an unknown compound at both retention times of 8.722 and 20.919, 2,5-dimethyl pyrazine at 9.402, and 1-heptatriacotanol at 30.777. Two of these four compounds, 2,5-dimethyl pyrazine (retention time 9.402) and 1-heptatriacotanol (retention time at 30.777), only samples appeared in all three inoculated samples. 2,5-Dimethyl pyrazine has been identified in headspace collections with bacteria growth on TSB previously. (Cevallos-Cevallos, Danyluk, and Reyes-De-Corcuera 2011). In a previous study, 2,5-dimethyl pyrazine was found to be produced from enriched media due to the Maillard reaction (Cevallos-Cevallos, Danyluk, and Reyes-De-Corcera 2011). Cevallos-Cevallos and others found a significant difference in concentration between

their control and *Escherichia coli* K12 samples which is consistent with our current findings in that our bacteria samples produced this compound, but was lacking in our control. (Cevallos-Cevallos, Danyluk, and Reyes-De-Corcuera 2011). 1-Heptatriacontanol, a 37 long carbon chain molecule, was tentatively identified four times within this study, yet is not reported in the literature. Due to the nature of GC-MS analysis in addition to the tentative identification utilizing the NIST reference library it is likely that one of these identified compounds could be 1-heptatriacontanol. Other peaks tentatively identified as 1-heptatriacontanol may have similar structures that the reference library would identify as 1-heptatriacontanol slight differences causing them to show up at different retention times.

Of the thirty-four peaks of interest identified by this study, eight of these compounds were also identified in previous studies including 2,5-dimethyl pyrazine discussed earlier. Benzaldehyde was seen to have a peak area of 1.82×10^{-4} for the control and 1.30×10^{-3} , 4.6010^{-4} and 2.18×10^{-4} . One sample did show a higher peak area than the control which is not supported by the decrease shown in all organisms by (Boots et al. 2014; Yu et al. 2000). Dimethyl trisulfide had a smaller peak area in the control than that of the experimental groups. Ratiu et al. and Yu et al. found the presence of dimethyltrisulfide in samples tested with Ratiu et al. displaying an increase from *Staphylococcus aureus* and Yu et al. showing no significant difference between their *E. coli* strains and the control due to high variability in samples. 2-Ethyl-3,5-dimethylpyrazine followed a similar trend as benzaldehyde in which 1 experimental group showed a higher peak area than that of the control and other two samples. Cevallos-Cevallos and colleagues saw varied peak areas across their mixed, *Salmonella typhimurium* and *E. coli* O157:H7 cultures. 2-Nonanone is variable across literature in the bacteria that present this VOC (Boots et al. 2014; Chen et al. 2016, 2017; Ratiu et al. 2017; Yu et al. 2000; Zhu et al.

2010). The TSB control had the lowest peak area value within this study with the 3 inoculated samples producing more. 1-Nonanal, another compound commonly seen within literature produced by *E. coli* strains and an uninoculated control tested, was produced with similar peak areas for both control and inoculated samples (Cevallos-Cevallos, Danyluk, and Reyes-De-Corcuera 2011; Klein et al. 2018; Kunze et al. 2013; Ratiu et al. 2017). Butylated hydroxytoluene (BHT) was similar in that peak area was relatively close in value when evaluated in this study. BHT was also observed in both the control and *S. typhimurium* inoculated beef samples (Bhattacharjee et al. 2011). One compound expected, but not present was that of indole. Indole was not identified within this sample as seen in previous studies. This could be due to the active growth phase these VOC were captured during where indole is seen mostly when the growth phase becomes more stationary (Gaimster and Summers 2015).

Beef VOC Collection

Forty eight peaks of interest were determined when comparing chromatographs of the control and three *E. coli* O157:H7 inoculated beef samples. Of these compounds, twenty two were observed within the inoculated samples but not the control of uninoculated beef and can be seen in Table 2. Peak area of sample 2 was considered much smaller than the other two samples and controls. This is due to a smaller amount of the internal standard being administered to the GC-MS sample vial prior to analysis through the GC-MS. Due to this, peak area is not as comparable as the VOC collection of TSB. The peak was still recognized by the software utilized during analysis and potentially identified using the NIST reference library (Stein et al. 2011; *Xcalibur* 2.2 2011).

From this VOC collection on beef only two tentatively identified compounds were seen in literature: dodecane at a retention time of 18.831 that appeared in the control and 3 inoculated

samples and peak 25.402 as either an unknown compound or butylated hydroxytoluene (BHT) that appeared as tentative identifications and only within inoculated sample. Though BHT did not appear in the control for this study it was reported in both uninoculated fresh and aged beef samples in which the authors suggested the source of this compound was potentially the Styrofoam trays used (Bhattacharjee et al. 2011). Their results suggest that this compound, because it was not seen in the control, is the second unknown identification. Dodecane was identified in both *E. coli* and *Pseudomonas aeruginosa* cultivated in lysogeny broth medium (Kunze et al. 2013). Many compounds seen within this study remain unknown. Increased replicates that may produce better profiles could lead to increased tentative identification for these compounds. This study only used 20 grams of ground beef per sample and control. Increasing this to 40 grams or higher could also help to solidify compounds and tentative identification through presumably increasing peak area of compounds overcoming interference that may influence tentative compound identification.

Conclusions

Analysis limitations within this study include the limited identification of compounds using an experimental reference library which produces theoretical results. Tentative identifications need to be further confirmed using external standards to ensure the identification of the compound. This study included both unknown compounds and tentatively identified compounds. There were also times that the same compound identification appeared in more than one peak. Though the compounds may be related through similar backbone structures, these are not the same compound due to the GC-MS separation of compounds first by size and then by charge. Samples analyzed by the GC-MS in this study were stored in a freezer up to two months before analysis. During those two months chemicals had the potential to interact with each other

and the air within the sample potentially altering compounds detected. The samples were also injected into the GC-MS at a temperature of 220°C which has the potential to alter compounds viewed after analysis.

Experimental limitations include the lack of growth curve of *E. coli* O157:H7 and thus an average of the starting and ending concentration was used to divide peak area to provide VOC peak area per log CFU for ground beef. This does not accurately incorporate the non linear growth of bacteria and is thus a limitation to this study in both the beef and TSB VOC collections. Another limitation that may be present is the interaction between VOCs released from *E. coli* O157:H7 and the ground beef. In a previous study where the VOCs interacted with the sample where VOC compounds were absorbed by the food product (Yu et al. 2000). Ground beef, with the lipid content, may prevent the volatilization of some compounds due to the compound's interactions with the lipid present. In addition to the interaction between bacteria produced VOCs there is also potential for the beef to absorb VOCs from the packaging and slowly release this over time especially with the influence of increased temperature. A final limitation to note would be that the VOC trap was held inside the incubator used to control the temperature for this experiment. This particular VOC filter is often used at room temperature and thus may not allow for the complete capture of VOCs.

Despite limitations, this study met the aim of evaluating the VOC profile of *E. coli* O157:H7 grown in both TSB and ground beef. In both experiments there were compounds seen in only the inoculated samples depicting the potential for VOC collection as a tool to monitor food production. This study lays the foundation for future studies further identifying and solidifying consistent compound produced from *E. coli* O157:H7.

Future Study Directions

This study is a great start in the evaluation of VOC with food safety application in mind. Moving forward, more replications are needed with a more sensitive system to obtain higher intensities of compounds for better identification using the NIST reference library. This may also be obtained using larger inoculated samples as the present study only sampled 20 g of ground beef. Cold injection, injecting more than 2 μ L, or utilizing tandem analysis settings for GC-MS analysis can also help with identification of the true compounds. For future replicates at increased temperatures it should be considered to move the filter outside of the incubator so it is not held at a higher temperature potentially influencing the VOC collection.

In addition to specific measures that can be different, once a VOC profile at optimal temperature is solidified moving the system into a temperature controlled system mimicking processing temperatures can give greater insight into if these VOC will be produced in amounts that can be recognized at a lower temperature. Moving beyond ground beef, meat products are not the only products that can carry foodborne illness causing bacteria and thus may benefit from VOC monitoring. Extending this study to fruit and vegetables would help provide a more overarching picture of VOC potential within the food production industry.

References

- Affairs, Office of Regulatory. 2020. "Recalls, Market Withdrawals, & Safety Alerts." FDA. <https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts> (July 7, 2020).
- Bhattacharjee, Paramita, Suranjan Panigrahi, Dongquig Lin, Catherine M. Logue, Julie S. Sherwood, Curt Doetkott, and Martin Marchello. 2011. "A Comparative Qualitative Study of the Profile of Volatile Organic Compounds Associated with Salmonella Contamination of Packaged Aged and Fresh Beef by HS-SPME/GC-MS." *Journal of Food Science and Technology* 48(1): 1–13.
- Boots, A W, A Smolinska, J J B N van Berkel, R R R Fijten, E E Stobberingh, M L L Boumans, E J Moonen, E F M Wouters, J W Dallinga, and F J Van Schooten. 2014. "Identification of Microorganisms Based on Headspace Analysis of Volatile Organic Compounds by Gas Chromatography–Mass Spectrometry." *Journal of Breath Research* 8(2): 027106.
- Bunge, M., N. Araghipour, T. Mikoviny, J. Dunkl, R. Schnitzhofer, A. Hansel, F. Schinner, A. Wisthaler, R. Margesin, and T. D. Mark. 2008. "On-Line Monitoring of Microbial Volatile Metabolites by Proton Transfer Reaction-Mass Spectrometry." *Applied and Environmental Microbiology* 74(7): 2179–86.
- Cevallos-Cevallos, Juan Manuel, Michelle D. Danyluk, and José I. Reyes-De-Corcuera. 2011. "GC-MS Based Metabolomics for Rapid Simultaneous Detection of Escherichia Coli O157:H7, Salmonella Typhimurium, Salmonella Muenchen, and Salmonella Hartford in Ground Beef and Chicken." *Journal of Food Science* 76(4): M238–46.

- Chen, Juan, Junni Tang, Cheng Tang, Hui Shi, Huaiyin Gu, and Xiaozhu Lu. 2016. "Growth and Volatile Compounds of Escherichia Coli O157:H7 on Irradiated Pork." *Journal of Food Quality* 39(6): 766–72.
- Chen, Juan, Junni Tang, Hui Shi, Cheng Tang, and Rong Zhang. 2017. "Characteristics of Volatile Organic Compounds Produced from Five Pathogenic Bacteria by Headspace-Solid Phase Micro-Extraction/Gas Chromatography-Mass Spectrometry." *Journal of Basic Microbiology* 57(3): 228–37.
- Debnath, Mousumi, G. B. Prasad, Prakash S. Bisen, and Prakash S. Bisen. 2012. *Microbes: Concepts and Applications*. Somerset, UNITED STATES: John Wiley & Sons, Incorporated. <http://ebookcentral.proquest.com/lib/wvu/detail.action?docID=848522> (June 5, 2020).
- Gaimster, Hannah, and David Summers. 2015. "Regulation of Indole Signalling during the Transition of E. Coli from Exponential to Stationary Phase" ed. Marie-Joelle Virolle. *PLOS ONE* 10(9): e0136691.
- Klein, Daniel, Stephan Maurer, Ulrike Herbert, Judith Kreyenschmidt, and Peter Kaul. 2018. "Detection of Volatile Organic Compounds Arising from Chicken Breast Filets Under Modified Atmosphere Packaging Using TD-GC/MS." *Food Analytical Methods* 11(1): 88–98.
- Kunze, Nils, Stephan Maurer, Ulrike Herbert, Judith Kreyenschmidt, and Peter Kaul. 2013. "Detection and Validation of Volatile Metabolic Patterns over Different Strains of Two Human Pathogenic Bacteria during Their Growth in a Complex Medium Using Multi-

- Capillary Column-Ion Mobility Spectrometry (MCC-IMS).” *Applied Microbiology and Biotechnology* 97(8): 3665–76.
- Ratiu, Ileana-Andreea, Tomasz Ligor, Victor Bocos-Bintintan, Hossam Al-Suod, Tomasz Kowalkowski, Katarzyna Rafińska, and Bofus Buszewski. 2017. “The Effect of Growth Medium on an Escherichia Coli Pathway Mirrored into GC/MS Profiles.” *Journal of Breath Research* 11(3): 036012.
- Sparkman, O. David, Zelda Penton, and Fulton G. Kitson. 2011. *Gas Chromatography and Mass Spectrometry: A Practical Guide*. San Diego, United States: Elsevier Science & Technology. <http://ebookcentral.proquest.com/lib/wvu/detail.action?docID=670206> (July 20, 2020).
- Stein, S., Y. Mirokhin, G. Mallard, A. Mikaia, P. Neta, D. Sparkman, E. White, X. Yang, V. Zaikin, and D. Zhu. (2011). NIST Mass Spectral Search Program (2.0g) [Computer software]. FairCom Corporation.
- Tack DM, Ray L, Griffin PM, et al. 2020. “Preliminary Incidence and Trends of Infections with Pathogens Transmitted Commonly Through Food — Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 2016–2019.” *MMWR. Morbidity and Mortality Weekly Report* 69. <https://www.cdc.gov/mmwr/volumes/69/wr/mm6917a1.htm> (July 6, 2020).
- Taiti, Cosimo, Corrado Costa, Paolo Menesatti, Stefania Caparrotta, Nadia Bazihizina, Elisa Azzarello, William Antonia Petrucci, Elisa Masi, and Edgardo Giordani. 2015. “Use of Volatile Organic Compounds and Physicochemical Parameters for Monitoring the Post-

- Harvest Ripening of Imported Tropical Fruits.” *European Food Research and Technology* 241(1): 91–102.
- United States Food and Drug Administration. Food Safety and Inspection Service. 2019. *Raw Beef Product Sampling*.
- Vaishnav, Anukool, Ajit Varma, Narendra Tuteja, and Devendra Choudhary. 2017. “Characterization of Bacterial Volatiles and Their Impact on Plant Health Under Abiotic Stress.” In *Volatiles and Food Security*, Springer Nature Singapore Pte Ltd., 15–23.
- Veselova, M. A., V. A. Plyuta, and I. A. Khmel. 2019. “Volatile Compounds of Bacterial Origin: Structure, Biosynthesis, and Biological Activity.” *Microbiology* 88(3): 261–74.
- Wood, William L., Daniel J Higbee, Melanie Gooldy, Scott Glogowski, Rich Fitzpatrick, Richard J Karalus, Troy D. Wood, and David J. Mangino. 2006. “Analysis of Volatile Bacterial Metabolites by Gas Chromatography--Mass Spectrometry.” *Spectroscopy* 21(6): 20–28.
- Xcalibur 2.2 (2.2 SP1.48). (2011). [Computer software]. Thermo Fisher Scientific Inc.
- Yu, Keshun, Thomas R. Hamilton-Kemp, Douglas D. Archbold, Randall W. Collins, and Melissa C. Newman. 2000. “Volatile Compounds from Escherichia Coli O157:H7 and Their Absorption by Strawberry Fruit.” *Journal of Agricultural and Food Chemistry* 48(2): 413–17.

Zhu, Jiangjiang, Heather D. Bean, Yin-Ming Kuo, and Jane E. Hill. 2010. "Fast Detection of Volatile Organic Compounds from Bacterial Cultures by Secondary Electrospray Ionization-Mass Spectrometry." *Journal of Clinical Microbiology* 48(12): 4426–31.

Tables and Figures

Table 1. VOC peak identification and peak area presented as peak area/hour/Log CFU mL⁻¹ for E coli O157:H7 grown in tryptic soy broth (TSB). Compounds have been displayed with observed peak retention time (RT).

Peak RT	Tentative ID	Peak Area VOC/Hour/Log CFU mL ⁻¹ TSB			
		Control	S1	S2	S3
4.538	Isooctyl Alcohol	4.10E-05	5.08E-03	1.31E-04	4.75E-05
6.552	Hexamethylcyclotrisiloxane	1.16E-04	2.09E-04	X	X
8.722	Unknown	X	4.73E-04	3.32E-04	X
9.402	2,5-Dimethyl Pyrazine	X	4.87E-04	1.59E-04	6.63E-05
11.049	Benzaldehyde	1.82E-04	1.30E-03	4.60E-04	2.18E-04
11.304	Dimethyl Trisulfide	3.86E-05	2.73E-03	6.02E-04	1.29E-04
12.283	Octamethyl cyclotetrasiloxan	5.22E-04	X	3.31E-04	8.04E-04
12.457	Unknown	3.15E-04	7.01E-04	4.09E-04	4.08E-04
13.198	2-Chlorcyclohexanol	1.41E-04	2.54E-04	1.65E-04	2.00E-04
13.313	Unknown	1.54E-03	6.43E-05	8.23E-04	2.11E-03
13.868	Phenylacetaldehyde	8.47E-04	2.24E-03	6.74E-04	1.17E-03
14.161	Unknown	1.93E-05	3.81E-04	1.44E-04	6.73E-05

14.647	Unknown	8.13E-05	4.01E-04	2.14E-04	2.20E-04
14.831	3,7-Dimethyloctan-1,7-diol	3.45E-04	4.66E-04	6.15E-04	4.97E-04
15.028	2-Ethyl-3,5-dimethylpyrazin	5.17E-04	1.34E-03	8.21E-04	9.17E-04
15.426	2-Nonanone	1.07E-05	1.58E-03	3.93E-04	1.14E-04
15.651	Unknown	4.01E-04	5.24E-05	2.18E-04	1.02E-03
15.828	1-Nonanal	2.36E-03	1.40E-03	1.42E-03	2.71E-03
17.569	Unknown	2.09E-04	1.49E-04	1.63E-04	2.65E-04
17.804	Unknown	2.47E-04	1.09E-04	2.30E-04	2.89E-04
19.362	Unknown	1.81E-04	5.95E-05	3.32E-04	X
19.729	1,2-Benzisothiazole or Benzothiazole	1.47E-03	1.37E-03	1.68E-03	1.73E-03
20.919	Unknown	X	9.46E-04	1.54E-03	X
20.984	Unknown	1.35E-03	X	X	1.54E-03
21.600	Unknown	1.88E-03	1.81E-03	2.79E-03	2.82E-03
21.960	Unknown	2.01E-03	4.19E-04	1.54E-03	3.51E-03
25.402	Butylated Hydroxytoluene	1.75E-02	1.77E-03	2.09E-02	3.69E-02
26.879	2,6-Bis(1,1-dimethylethyl-4-	8.28E-02	2.54E-03	2.04E-03	1.18E-01

(1-oxopropyl)phenol or 4-sec-
butyl-2,6-di-tert-butylphenol

27.175	2,5-di-tert-Amylbenzoquinone	1.28E-02	2.96E-04	3.40E-02	6.65E-02
28.932	1-Heptatriacotanol	1.31E-03	X	X	6.67E-04
29.613	2,5-Di-tert-Pentylhydrochinon	1.90E-02	3.30E-03	2.29E-02	4.57E-02
30.777	1-Heptatriacotanol or Phorbol	X	5.72E-03	2.33E-03	5.55E-03
31.290	1-Heptatriacotanol	1.34E-03	3.58E-03	4.27E-03	2.48E-03
33.164	1-Heptatriacotanol	1.13E-04	2.74E-02	X	X

Peak area is given as VOC produced/hour/Log CFU mL⁻¹.

Table 2. VOC peak identification and peak area presented as peak area/hour/Log CFU g⁻¹ ground beef for E coli O157:H7 grown in ground beef. Compounds have been displayed with observed peak retention time (RT).

Peak RT	Tentative ID	Peak Area VOC/Hour/Log CFU g ⁻¹ Beef			
		Control	S1	S2	S3
6.185	Unknown	6.61E-06	3.99E-05	3.95E-13	1.59E-04
13.321	Unknown	8.60E-05	2.23E-04	1.07E-11	3.52E-04
15.654	Unknown	X	1.22E-04	2.10E-11	3.47E-04
16.300	Unknown	2.30E-05	3.37E-05	3.46E-12	7.67E-05
16.413	Unknown	5.51E-05	X	8.48E-12	1.75E-04
16.865	Unknown	X	1.05E-04	1.10E-11	2.81E-04
17.243	1,1- Bis(dodecyloxy)hexadecane	X	X	3.79E-12	1.34E-04
17.375	Unknown	5.30E-04	3.62E-03	7.20E-11	2.99E-03
17.913	1,1- Bis(dodecyloxy)hexadecane	3.11E-05	4.61E-05	5.96E-12	1.19E-04
18.831	Dodecane	7.71E-05	5.77E-06	3.39E-13	2.41E-05
19.256	Unknown	X	X	1.94E-11	4.31E-04

19.470	Unknown	X	X	6.33E-12	1.26E-04
19.974	Unknown	1.11E-05	1.97E-05	9.14E-12	2.39E-04
20.304	2-Methylhexadecan-1-ol	3.42E-05	8.93E-05	4.72E-12	1.97E-04
20.379	Delta-Dodecanolactone or 2-Oxohexamethylenimine	X	X	2.28E-10	1.60E-03
20.807	Unknown	X	X	4.51E-11	1.46E-03
21.443	Unknown	X	5.26E-04	1.92E-10	9.56E-03
21.831	Unknown	2.16E-05	1.53E-05	3.37E-11	1.30E-03
22.403	Unknown	X	X	5.70E-11	2.54E-03
22.508	Unknown	X	8.03E-05	4.30E-11	1.97E-03
22.559	Unknown	X	5.35E-05	3.51E-11	1.54E-03
22.668	2,6,10-trimethyltetradecane	1.27E-04	1.21E-04	4.18E-11	1.27E-04
22.753	2,6,10-trimethyltetradecane	8.36E-06	6.00E-05	8.36E-11	4.15E-03
22.889	2-Bromododecane or 4- Methyldocosane	X	1.32E-04	7.46E-11	3.19E-03
23.001	2,6,10-trimethyltetradecane	2.09E-04	1.74E-04	1.10E-10	4.90E-03
23.436	Tetradecane or 2,6,10- trimethyltetradecane	2.43E-04	4.50E-04	3.05E-10	1.59E-02

23.515	Unknown	X	3.70E-05	3.72E-11	1.87E-03
23.722	Unknown	1.27E-05	2.93E-05	2.99E-11	1.46E-03
24.042	Unknown	2.43E-04	2.95E-04	5.77E-11	2.69E-03
24.324	Unknown	9.23E-06	1.99E-04	3.38E-11	1.86E-03
24.637	1-Heptatriacotanol OR 2,6,10-trimethyltetradecane	X	X	8.46E-11	4.41E-03
24.668	Unknown	3.46E-04	3.47E-04	X	X
24.790	tert-Hexadecanethiol	7.27E-04	1.20E-03	9.77E-11	4.04E-03
25.402	Unknown or Butylated hydroxytoluene	X	1.39E-03	1.27E-10	4.28E-03
25.498	1-Heptatriacotanol	X	X	1.04E-11	3.18E-04
25.739	Unknown	1.88E-04	X	5.59E-11	2.23E-03
25.804	1-Heptatriacotanol or Tert- hexadecyl mercaptan	X	X	2.98E-11	7.58E-04
25.875	Unknown	X	X	3.93E-11	1.38E-03
25.950	Unknown	X	X	6.67E-11	2.36E-03
26.049	1-Heptatriacotanol	X	X	3.02E-11	X

26.096	1-Heptatriacotanol	4.42E-04	5.00E-04	4.54E-11	3.35E-03
26.385	Unknown	X	X	1.60E-10	7.31E-03
26.868	4-sec-Butyl-2,6-di-tert- butylphenol OR 2,6- Bis(1,1-dimethylethyl-4-(1- oxopropyl)phenol	1.14E-03	9.33E-04	2.38E-09	4.67E-02
27.175	2,5-di-tert- Amylbenzoquinone	2.61E-04	3.21E-03	4.31E-11	1.15E-03
28.573	Unknown	1.66E-04	1.61E-04	8.25E-11	2.38E-03
29.617	2,5-di(tert- amyl)Hydroquinone	1.13E-03	5.28E-03	9.78E-11	1.63E-03
32.906	4-methoxybut-1-ene	1.39E-05	6.59E-05	5.11E-12	X

Peak area is given as VOC produced/hour/Log CFU g⁻¹.

Figure 1. Growth of *E. coli* O157:H7 in tryptic soy broth over 10 hours at 37°C and shaken at 150 rpm. Starting and ending bacteria concentration reported as mean $\pm$ standard deviation of 5.45 ± 0.51 and 9.48 ± 0.35 log CFU ml⁻¹ of tryptic soy broth, respectively.

Figure 2. VOC chromatograph from the control of uninoculated tryptic soy broth and sample 1 of *E. coli* O157:H7 inoculated tryptic soy broth collected for 6 hours. Starting bacteria concentration reported as mean $\pm$ standard deviation of 6.07 ± 0.52 log CFU mL⁻¹ tryptic soy broth. The internal standard naphthalene present at retention time of 18.45 is labeled IS on the graph.

Figure 3. VOC chromatograph from the control of uninoculated tryptic soy broth and sample 2 of *E. coli* O157:H7 inoculated tryptic soy broth collected for 6 hours. Starting bacteria concentration reported as mean $\pm$ standard deviation of 6.07 ± 0.52 log CFU mL⁻¹ tryptic soy broth. The internal standard naphthalene present at retention time of 18.45 is labeled IS on the graph.

Figure 4. VOC chromatograph from the control of uninoculated tryptic soy broth and sample 3 of *E. coli* O157:H7 inoculated tryptic soy broth collected for 6 hours. Starting bacteria concentration reported as mean $\pm$ standard deviation of 6.07 ± 0.52 log CFU mL⁻¹ tryptic soy broth. The internal standard naphthalene present at retention time of 18.45 is labeled IS on the graph.

Figure 5. VOC chromatograph from the control of uninoculated tryptic soy broth and all 3 samples of *E. coli* O157:H7 inoculated tryptic soy broth collected for 6 hours. This chromatograph is showing peaks from all samples and control tentatively identified as 2,5-dimethyl pyrazine.

Figure 6. VOC chromatograph from the control of uninoculated tryptic soy broth and all 3 samples of *E. coli* O157:H7 inoculated tryptic soy broth collected for 6 hours. This chromatograph is displaying the peak from all samples and control tentatively identified as 1-heptatriacotanol marked with an *.

Figure 7. VOC chromatograph from the control of uninoculated beef and sample 1 of *E. coli* O157:H7 inoculated tryptic soy broth collected for 6 hours. Starting and ending bacteria concentration reported as mean $\pm$ standard deviation of 5.68 ± 0.15 log CFU g⁻¹ and 8.17 ± 0.23 log CFU g⁻¹ of beef respectively. The internal standard naphthalene present at retention time of 18.45 is labeled IS on the graph.

Figure 8. VOC chromatograph from the control of uninoculated beef and sample 2 of *E. coli* O157:H7 inoculated tryptic soy broth collected for 6 hours. Starting and ending bacteria concentration reported as mean $\pm$ standard deviation of 5.68 ± 0.15 log CFU g⁻¹ and 8.17 ± 0.23 log CFU g⁻¹ of beef respectively. The internal standard naphthalene present at retention time of 18.45 is labeled IS on the graph.

Figure 9. VOC chromatograph from the control of uninoculated beef and sample 3 of *E. coli* O157:H7 inoculated tryptic soy broth collected for 6 hours. Starting and ending bacteria concentration reported as mean $\pm$ standard deviation of 5.68 ± 0.15 log CFU g⁻¹ and 8.17 ± 0.23 log CFU g⁻¹ of beef respectively. The internal standard naphthalene present at retention time of 18.45 is labeled IS on the graph.

Figure 10. VOC chromatograph from the control of uninoculated beef and all 3 samples of *E. coli* O157:H7 inoculated beef collected for 6 hours. This chromatograph is displaying the peak from all samples and control tentatively identified as dodecane.

Figure 11. VOC chromatograph from the control of uninoculated beef and all 3 samples of *E. coli* O157:H7 inoculated beef collected for 6 hours. This chromatograph is displaying the peak from all samples and control tentatively identified as butylated hydroxytoluene or as an unknown compound.

Figure 12. Closed-loop headspace VOC collection system. Uninoculated and inoculated ground beef samples during a 6 hour VOC collection.