ABSTRACT

Title of Dissertation:	UNDERSTANDING THE INTERACTIONS OF COLD ATMOSPHERIC PLASMA AND THE PLANT STRESS RESPONSE TO IMPROVE FRESH PRODUCE SAFETY
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Cold atmospheric plasma (CAP) is a surface modification technology that produces oxidative species that inactivate microorganisms. This technology has been shown to be an effective sanitization technology for use on a variety of food products and leafy greens are a uniquely promising application. CAP products, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), UV light, and ozone, are environmental stressors with antimicrobial effects that plants already encounter in their natural environment, and fresh produce will produce a stress response when treated by CAP and this stress response is not limited to directly treated tissue. The stress responses of baby spinach, red leaf lettuce, baby kale and live basil were evaluated post-CAP treatment. In all evaluated fresh produce CAP treatment resulted in a significant ($p<0.05$) increase in ROS. This increase in reactive oxygen species was not limited to the plant tissues directly treated by CAP. In live basil, ROS generation was detected on indirectly treated

leaves sharing the same stem, and in spinach the increase in ROS levels continued for 12 hours post-treatment. To further understand how the elevated ROS levels affected plant tissues the flavonoid content was evaluated in baby spinach, red leaf lettuce and baby kale. Baby spinach CAP treatment resulted in a significant increase in flavonoids at 50w and 120w power level. Flavonoids in red leaf lettuce were only significantly affected in directly treated leaf tissue. In baby kale, CAP treatment significantly reduced flavonoid content in both direct and indirectly treated leaf tissue. To understand the differences between the two leafy greens species and the effect of ozone and UVB treatment, two products treated with CAP, were also evaluated on baby spinach and baby kale. The two non-CAP treatments resulted in the opposite effects in baby spinach and baby kale, and an examination into the response of the stress hormones salicylic acid and jasmonic acid was inconclusive. The novel interactions of this sanitization treatment with the plant stress response may provide useful opportunities to improve both the food safety and nutritional quality of fresh produce. In addition to the research project, I composed a needs assessment survey instrument to evaluate food safety compliance among small and very small food processors in the northeastern United States in cooperation with the Northeast Center for the Advancement of Food Safety. The survey instrument was sent to food safety professionals and regulators in the region. Results of the survey revealed that the food safety concepts that processors struggled to learn were often those cited by regulators during inspections.

UNDERSTANDING THE INTERACTIONS OF COLD ATMOSPHERIC PLASMA AND THE PLANT STRESS RESPONSE TO IMPROVE FRESH PRODUCE SAFETY

by

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Dedication

I dedicate this dissertation to my parents Dr. Percy and Shireen Gilbert, and my husband Nathan Eckman.

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CAP	Cold atmospheric plasma
CFU	Colony Forming Unit
DBD	Dielectric Barrier Discharge
DMSO	Dimethyl Sulfoxide
DPBA	Diphenylborinic-2-aminoethyl ester
FSC	Food safety communicator
HPOP	Human Pathogens on Plants
PCHF	Preventive Controls for Human Foods
PCQI	Preventive Controls qualified individual
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RONS	Reactive oxygen and nitrogen species
SMD	Surface microdischarge
STEC	Shiga toxin-producing *E. coli*

Chapter 1: Review of Relevant Literature

Introduction

Many health agencies promote the consumption of fruits and vegetables, the World Health Organization (WHO) suggests consumption of about one pound of fruits and non-starchy vegetables per day (*WHO | Increasing Fruit and Vegetable Consumption to Reduce the Risk of Noncommunicable Diseases*, n.d.). Adequate sanitization is required for minimally processed foods, such as fresh produce, in order to protect food safety and consumer health from foodborne microbial contaminants. While the overall consumption of fruits and vegetables has not significantly increased from 1970 to 2005 in the United States, the types of produce consumed has changed (Bennett et al., 2018). Raw produce accounts for an increasing share of foodborne illness outbreaks since the overall number of outbreaks decreased by 38% between 1998 and 2013, while produce outbreaks only decreased by 19% in the same timeframe.(Bennett et al., 2018). Produce related outbreaks resulted in 10% of illnesses, 18% of diseases, and 24% of deaths in this timeframe indicating that severe disease often results from produce foodborne illness outbreaks (Bennett et al., 2018). Contamination of fresh produce can occur throughout the production chain from contact with contaminated surfaces, water, workers (human pathogens have been shown to survive on gloves), and wildlife intrusion onto farms and. Contamination in fields is of particular concern (field trimming and coring of certain types of lettuce and current washing practices may enhance both pathogen internalization and cross contamination(Machado-Moreira et al., 2019). The short shelf life of many fresh produce products can make outbreak investigations difficult, and from 1998 to 2013, the most implicated raw produce type was vegetable row crops, where 230 of 235 outbreaks were caused by leafy greens (Bennett et al., 2018). Sprouts are another produce category of concern. From 1998 to 2013, outbreaks linked to sprouted seeds accounted for nearly a third of all deaths related to outbreaks identified in an analysis performed by Bennet and coworkers (2018). A 2020 report published by the United States Centers for Disease Control and Prevention stated that leafy greens were the second leading cause of STEC illness outbreaks in the United States (Marshall et al., 2020). Foodservice related outbreaks predominated in leafy greens, and both sprouts and leafy greens outbreaks are often widespread, suggesting pathogen contamination may have occurred in the pre-harvest or processing environment (Machado-Moreira et al., 2019).

To address the unique safety challenges presented by fresh produce several strategies have been implemented in the United States and other countries to improve fresh produce safety. In the U.S., the passage of the Food Safety Modernization act of 2011 included the Produce Safety Rule that is specifically designed for the safe cultivation of fresh produce commonly consumed raw as well as sprouts(Draft Guidance for Industry: Reducing Microbial Food Safety Hazards in the Production of Seed for Sprouting, FDA.). However, many challenges remain for ensuring the safety of fresh produce. Chief among them is the uncertainty surrounding the interaction of human pathogens with plants (HPOP).

Like all living things, plants have extensive interactions with the innumerable microorganisms found in the natural environment. Unlike in animals, the behavior of foodborne illness causal agents are more difficult to characterize in plants. Plant species are biologically radically different from animals and the vertebrates that serve as reservoirs for many pathogens. High levels of variation exist within similar product types and within species themselves. This can include the many different vegetables found within the same genus or family, as well as more changes to the plant genotype through selective breeding to produce products that may look the same to the consumer but may be radically different biologically. Achieving greater leafy greens safety will require a greater understanding of the plant, the plant holobiont defined as the biomolecular network of the host plus its associated microbes (Borderstein and Theis, 2015), as well as the plant stress response system. Understanding how the holobiont and the plant stress response system interact will be key to reducing the foodborne illness risk associated with these products. This knowledge will also allow growers and processors to avoid the use of antimicrobial processes that diminish the perceived freshness of the products. Cold atmospheric plasma (CAP) is a food processing technology that may be well suited for this type of application. Understanding how CAP interacts with leafy greens is necessary for wider adoption of this technology.

Cold atmospheric plasma

CAP is one promising technology that has the potential to improve the food safety of both sprouts and leafy greens. Plasma, often termed the fourth state of matter, has been explored for a variety of applications across many industries. First produced by heating pressurized gases, it has been used extensively in semiconductor manufacturing and in the field of materials science(Conrads & Schmidt, 2000). Currently there are many methods to produce plasma and several methods that can be produced at atmospheric temperature and pressure. Plasmas were first generated using neutral gases such as argon. But in antimicrobial applications, gases that are more difficult to ionize, such as oxygen, are more useful (Conrads & Schmidt, 2000; Niemira, 2012). Dielectric barrier discharge (DBD) plasma devices are one such CAP generation set up used extensively in foods. In this system, the DBD electrode serves as the charged electrode, while the substrate serves as the ground electrode. When a high voltage current runs through the electrode, plasma species are generated in the space between the two electrodes. The DBD systems can generate large amounts of gaseous discharge at large volumes at atmospheric pressure. This makes them useful for a number of sanitization and sterilization applications. These devices were first evaluated as a low-cost method to sterilize reusable metical tools (Laroussi, 2002). In DBD systems, the gas temperature in these applications remains relatively low and unlike ultraviolet light (UV) based sanitization systems "line of sight" is not required for efficacy (Herrmann et al., 1999; Laroussi et al., 2002). Plasmas generated in conventional methods do not ionize all atoms in the gas. When gas species combine, the energy is released as UV light, the high energy molecules also react with food substrate and release the stored energy into pathogens. The energy released is dependent on gas chemical composition, density, and temperature, and plasma inactivates microbes by three main mechanisms:

1. Chemical interaction of radicals, reactive species, and charged particles with cell membranes

2. Damage to membranes and organelles due to UV radiation
3. UV DNA damage

In foods, major parameters to be considered for plasma-based sanitization systems include water activity, protein and fat content, and the general product shape (Niemira, 2012). These factors influence the type of plasma generation system best suited for a particular product. There are three main categories of plasma systems used on foods: 1) remote treatment cold plasma systems; 2) direct treatment plasma systems; and 3) electrode contact treatment systems. In remote treatment plasma systems, the flow or feed gas is manipulated by magnetic fields and the power source is placed away from the food product. This can result in some of the more short-lived reactive species being quenched by the time it reaches the food product, but it allows for a wider variety of differently sized and shaped products to be treated. In direct plasma treatment, the electrode is placed directly on or very close, closer than 1 cm, from the food product. These systems allow for highly effective short-lived species to be applied to foods, but wetter products will burn due to electrical shorting, and the high dosage of reactive species can cause protein coagulation and the development of off flavors and undesirable appearances. Additionally, since the power source is much closer to the product, these systems can be more challenging to build, operate, and maintain. The final type of plasma system used in foods is the electrode contact system, where the food substrate is placed between two charged electrodes. This system results in the best exposure reactive species, but size and product composition must be controlled to prevent point discharges and heat buildup in the product. Fortunately, due to the usefulness of air-based plasma systems in antimicrobial applications, the largest cost associated with plasma-based sanitization is the electrical demands of the power supply. In many applications, the charge gas (air) will be free (Niemira, 2012).

Several factors affect the efficacy of CAP treatment on foods; however, they fall into three broad categories: processing parameters, environmental factors, and microbial properties. Processing parameters in DBD plasma systems typically involve both the voltage released onto the substrate as well as the pulse frequency of the system. In CAP treatment, environmental factors describe the attributes of the substrate, or ground electrode in a DBD system. The pH and other attributes of the food matrix such as nutrient content can influence treatment efficacy. In most prior research, CAP treatments are much more effective on agar plates and glass slides than on food products. In addition to the water activity of the substrate, the relative humidity of the environment can also enhance treatment efficacy. On yeast strains, CAP treatments were found to be more effective when using charge gasses with higher relative humidity, due to greater generation of hydroxyl radicals (Liao et al., 2017). Lastly, microbial properties can greatly affect the efficacy of CAP treatment, a 2015 study by Yong and coworkers demonstrated that *Listeria monocytogenes* on sliced cheese had a D-value of 1.19 minute compared to 0.19 minute for *E. coli* and 0.7 minute for *S. enterica*. Other studies showed no significant difference between gram positive and gram negative strains, but they used higher intensities and treatment times likely not suitable for a high moisture, high protein, and high fat product like cheese. There is no consensus on the plasma resistant properties of gram positive versus gram negative bacteria, however a 2016 study using an argon fed plasma jet showed a strong differential between gram positive and gram negative microorganisms (Liao et al., 2017; Mai-Prochnow et al., 2016). The researchers reported a strong correlation (R^2=0.8596 after 1 minute treatment) between cell wall thickness and inactivation rate (Mai-Prochnow et al., 2016).

The exact mechanism of microbial inactivation by CAP has not been fully characterized, however a 2009 study by Dobrynin and coworkers suggested that the role of UV DNA damage could be ignored as they found the application of CAP treatment through UV blocking MgF_2 shield resulted in no significant difference in antimicrobial efficacy(Dobrynin et al., 2009). Lipid oxidation of polyunsaturated fatty acids has also been suggested as a mechanism for inactivation, but it is thought to be more relevant to gram negative bacteria, which undergo extensive deformation in response to treatment(Liao et al., 2017; Luan et al., 2019). It has also been suggested that in gram positive microorganisms like *Staphylococcus aureus*, ROS pass more easily through the cell wall and membrane and trigger organelle damage that cannot be as easily observed through SEM imaging (Thirumdas et al., 2018). There are also concerns that lipid oxidation does not fully inactivate pathogens, but instead moves cells into an injured viable but not culturable (VBNC) state. Protein modulation has also been suggested as a mechanism of inactivation. Dobrynin and coworkers (2009) demonstrated that CAP treatment led to roughly 50% reduction in trypsin activity with 1 J cm^{-2} CAP treatment and complete inhibition of enzymatic activity at 4 J cm^{-2} treatment. Other results indicated that at least 20 J cm^{-2} energy is needed for protein destruction, so lower powered treatments may be sufficient to disrupt the secondary structure and inhibit functionality. Other suggested mechanisms include inducing apoptosis in microbial cells due to ROS activity as well as electroporation, which can be seen in SEM images of gram negative cells treated with high intensity or longer time CAP treatments (Liao et al., 2017; Luan et al., 2019).

Leafy Greens Safety

Leafy greens have substantial food safety challenges. In September of 2006, an STEC outbreak in fresh spinach resulted in 200 cases with 3 deaths across 26 states. In this outbreak, 51% of sickened individuals were hospitalized, 16% developed hemolytic uremic syndrome, and the three deaths included two elderly women and one two-year-old child. In the outbreak investigation, 11 of 13 spinach bags collected were manufactured in the same facility on the same day per lot code data, other two bags did not have lot codes (CDC, n.d.). A number of factors could have contributed to the severity of this outbreak. However, investigation data was not sufficiently comprehensive to draw conclusions between water sources and cause of outbreaks. Groundwater is a common irrigation water source in the California central coast and improperly dug wells can be a route for contamination. Groundwater can also interact with surface water, which is more favorable for bacterial contamination. High levels of rain that year also led to increased usage of surface water (Gelting et al., 2011). Additionally, the outbreak strain from 2006 spinach outbreak was linked to feral swine, local cattle, surface water, and soil. Both the local cattle and feral swine had the exact reference strain detected in their feces. The most likely route of contamination was thought to be from feral swine intrusion.

In addition to wild animal intrusion, irrigation water from surface sources (i.e., ponds or lakes) can be a source of zoonotic pathogens that may contaminate fresh produce that likely will be consumed raw. In the United States, the passage of the 2011 Food Safety Modernization Act (FSMA) charged the FDA with developing the Produce Safety Rule intended to improve the safety of produce consumed raw. However, preharvest water testing standards keep getting extended due to difficulty and confusion to implement for growers. In addition to irrigation water sources, there are multiple other hazards in farm environments. Water distribution systems can also contribute to contamination, and extension agents have suggested irrigation water treatment as a useful solution for growers (Wall et al., 2019). Additionally, there has been a shift in produce pathogen interactions, with many pathogens being implicated for the first time, such as the 2015 *Listeria monocytogenes* outbreak resulting from caramel apples. Several factors may be responsible for this shift, such as: increasingly global production chains, the increased usage of reusable plastic bins rather than single use containers, as well as highly sensitive testing methods that detect pathogens that had previously been missed. Pathogens can survive for long periods in the farm environment, often establishing themselves in little niches in processing facilities and packinghouses. Previously established "die off periods" determined for the use of natural fertilizers such as manure (Harris et al, 2013) may instead be transitions to viable but non-culturable (VBNC) periods. Post-harvest, produce washing efficacy is difficult to validate as sanitizer concentration and sanitizer contact time can be highly variable from lot to lot and within the washing systems (Murray et al., 2017). Cross contamination a major concern in produce washing, water is often recycled for produce washing to save resources and further enforces the need to use sanitizers. Sanitizer efficacy must be monitored as it can decline with the presence of dirt, debris, and time; therefore, hurdle technology or combined treatments may be needed (López-Gálvez et al., 2021).

Due to the uncertainty surrounding routes of entry for human pathogens, understanding of how human pathogens survive and persist on plants has been a challenge. Conditions in the soil, water, and on the plants themselves are substantially different from that of the intestines of warm blooded animals (Zamora et al., 2021a). Bacteria are the predominant microorganisms found on plant leaves followed by filamentous fungi and yeasts, but far less numerous than the concentrations found on the roots and in the soil, phyllosphere organisms are typically present at approximately 2 log CFU/cm^2. These microbial populations do not originate from a single source and may be introduced through the air, soil, water, and interactions with insects and other wildlife.

CAP and the plant stress response

In initial studies using a surface microdischarge (SMD) plasma device, we found that *E. coli* not directly exposed to could still be inactivated. Furthermore, on the spinach leaf completely covered by one leaf compared to a leaf directly exposed to the electrode (Luan et al., 2019) no significant difference in inactivation was observed. These results were exciting, as one of the major limitations of fresh produce processing is that sanitizers added to wash water can only remove surface pathogens and have no impact on internalized microorganisms (Wall et al., 2019). The primary focus of the Luan et al., 2019 study was evaluation of the effect CAP treatment had on the cell membrane of *E. coli* as a model gram-negative pathogen. This dissertation focuses on an in-depth evaluation of the results of our layered inactivation experiment and the effects CAP treatment on plant tissue.

How do the reactive products generated by CAP treatment interact with plant tissue ?

The first question that arose after completing the layered inactivation experiment was: *How do the highly reactive species generated by a DBD plasma electrode interact with plant leaf tissue?* Ideally, the reactive gaseous species would pass through the spinach leaf tissue and be quenched by existing antioxidants while inactivating internalized pathogens that do not have as elaborate abiotic stress defense mechanisms as the plant. This result would be ideal for the toxicological safety of produce products treated with CAP and could hopefully lead to a rapid adoption of this technology throughout the industry. However, prior research indicates that CAP products likely have complex reactions with plant tissues (Laroussi, 2002; Niemira, 2012; Thirumdas et al., 2018). Understanding these interactions is key to evaluating and ensuring produce quality and safety from both biological and potential chemical hazards. DBD plasma treatment produces several highly reactive radical species and intense ultraviolet light in the space between the charged electrode and the substrate. These chemical and physical products can trigger abiotic stress responses in spinach, these stress responses could also be affected by the presence of certain pathogens and trigger biotic stress responses as well(Cui et al., 2015; Dangl & Jones, 2001; Jones & Dangl, 2006). To further understand how these stress responses can affect product quality and safety, CAPs effect on spinach will be examined through its effect on the phenylpropanoid pathway, the salicylic acid (SA) pathway, and the jasmonic acid (JA) pathway.

Phenolic compounds are thought to have arisen to help protect land plants from environmental stresses, chief among them, UV light and the benzene ring structure allows them to generate free radicals (Cheynier et al., 2013). Phenolic compounds produced by plants serve as natural antimicrobials and are considered beneficial for human health (Gyawali & Ibrahim, 2014; Liu, 2003). Outside of plants, phenolic compounds have been combined with physical treatments such as light to act as sanitizers in wash water (Cossu et al., 2016; Gilbert et al., 2018). The phenylpropanoid pathway can be elicited through certain chemical and physical treatmentssuch as, (J et al., 2015; Matsuda et al., 2005; Ni et al., 1996; Rasmussen & Dixon, 1999). Singh (2016) found that chitosan treatment also enhanced phenylpropanoid content in spinach as shown through the activity of Phenylalanine Ammonia Lyase (PAL), a critical enzyme in the phenylpropanoid pathway. At low concentrations (0.01 mg/ml), chitosan significantly increased total chlorophyll, sugar, protein, phenolics and flavonoids. These results suggest activation/manipulation of pathway may create more nutritious product (Singh, 2016). This enzyme has been proposed to have broad spectrum disease resistance without yield cost and identifying genes could be useful for pre-harvest applications (Yadav et al., 2020). In plants, salicylic acid (SA) mediates host responses upon pathogen infection, and aids in the development of disease resistance(Lefevere et al., 2020). SA can be synthesized from either phenylalanine or benzoic acid through either the isochorismate synthase (ICS) or the PAL pathways (An & Mou, 2011; Lefevere et al., 2020). In Arabidopsis, the

ICS pathway originating in the chloroplast is responsible for the majority of SA synthesis when the plant is treated with UV light or infected with a pathogen (An & Mou, 2011; Dempsey et al., 2011). In the plant immune system, SA may trigger DNA damage in absence of genotoxic agent to induce effector triggered immune responses. This damage then enhances the SA mediated defense gene expression (Yan et al., 2013), this could be impacted either positively or negatively in CAP treated plant tissue. SA also has extensive crosstalk with other plant hormones, including Jasmonic acid (JA) especially at the low temperatures required for maintaining leafy green and sprout quality post-harvest (Li et al., 2020; Yang et al., 2019). JA is thought to be a core signal in the phytohormone signaling network, engaging in synergistic crosstalk with ethylene, an important ripening hormone, while SA activity is suppressed by JA and ethylene (Li et al., 2020; Yang et al., 2019). SA activity at low temperatures enhances immunity to *Pseudomonas syringae* via biosynthesis and signaling genes which could help extend product shelf life (Li et al., 2020). Both SA and JA have been examined as elicitors to help improve plant nutritional quality and stress resistance (Alavi-Samani et al., 2015; Farhangi-Abriz & Ghassemi-Golezani, 2018; Złotek et al., 2016). JA synthesis is often triggered by mechanical damage, and its synthesis often relies on cell surface receptors. JA signaling can also be triggered by systemic acquired resistance at an uninjured site in the plant as well. Synthesis pathways generally require molecules in the chloroplast, peroxisome and cytoplasm; therefore, CAP treatment should induce some pathway response (Ruan et al., 2019)

How can these interactions be modified to enhance antimicrobial effect ?

CAP is well established as an antimicrobial treatment and its effects on bacteria are better understood than its effects on fresh produce quality (Laroussi, 2002; Laroussi et al., 1999, 2002, 2003; Niemira, 2012; Niemira et al., 2018). CAP treatment has also been shown to have varying levels of efficacy on different microorganisms which may present unique challenges in fresh produce safety (Mai-Prochnow et al., 2016). While *Salmonella* and Shiga toxin producing *E.* coli (STEC) are the leading causes of sprout and leafy greens foodborne illness outbreaks, *Listeria monocytogenes* is cause for concern(Marik et al., 2020; Prado-Silva et al., 2015; Zhu et al., 2017). *Listeria monocytogenes* is the leading cause of reported bacterial foodborne illness deaths, with a mortality rate of 20%. The majority of listeria outbreaks linked to produce that has been cut, processed or sprouted but most produce is grown in environments where listeria may be present providing opportunities for pre-harvest contamination and handling practices and storage may amplify populations (Marik et al., 2020; Roth et al., 2018; Zhu et al., 2017). Unlike *Salmonella* and *E. coli*, *Listeria* species can grow at refrigeration temperatures required for sprouts and leafy greens storage and transport and tends to be more resistant to antimicrobial treatments than the two other pathogens (Marik et al., 2020; Zhu et al., 2017). A 2015 meta-analysis of sanitization publications performed by Prado-Silva and coworkers showed that *Listeria monocytogenes* had a mean inactivation rate of 0.79 log(CFU/mL) when treated with sodium hypochlorite, compared to 2.27 log (CFU/mL) for *Salmonella* and 1.40 log(CFU/mL) for *E. coli*. Other microorganisms of concern include gram-negative *Pseudomonas* species. Also capable of growing at refrigeration temperatures, *Pseudomonas* are primarily spoilage organisms, but can be an opportunistic pathogen in immunocompromised individuals, and some species such as *P. syringae* are plant pathogens (Bakker et al., 2007; Fatima & Anjum, 2017; Hurley et al., 2014; Pieterse et al., 2014). Unlike the zoonotic microorganisms *Salmonella* and *E. coli*, *Pseudomonas* spp. interact more extensively with the plant immune system and can modify plant immune responses to CAP treatment. *Pseudomonas* species such as *P. syringae* and *P. cannabina* pv Alisalensis have been shown to interfere with stomatal immunity which may enhance internalization of pathogens, which could be especially problematic in pre-harvest applications of CAP, PAW, and PANS (Hurley et al., 2014; Sakata et al., 2021). *Pseudomonas fluorescens*, however, is a beneficial microorganism that has been shown to interact with the plant immune system to enhance stress resistance via induced systemic resistance (ISR) (Bakker et al., 2007). Understanding how these organisms interact with CAP treated produce will be essential to ensuring the efficacy of this antimicrobial technology.

Chapter 2: Objective One, the Stress Response of Leafy Greens To Dielectric Barrier Discharge Plasma Treatment

Introduction

In fresh produce, contamination can occur throughout the production chain via surfaces, water, wildlife intrusion onto farms, and workers. Contamination in field is of particular concern, field trimming and coring of certain types of lettuces and current washing practices may enhance both pathogen internalization and cross contamination (Machado-Moreira et al., 2019).The short shelf life of many fresh produce products can make outbreak investigations difficult, and from 1998 to 2013 the most implicated raw produce type was vegetable row crops, where 230 of 235 outbreaks were caused by leafy greens (Bennett et al., 2018). A 2020 report published by the United States Centers for Disease Control and Prevention stated that leafy greens were the second leading cause of STEC illness outbreaks in the United States (Marshall et al., 2020). Foodservice related outbreaks predominated in leafy greens, and outbreaks are often widespread, suggesting pathogen contamination may have occurred in the pre-harvest or processing environment (Machado-Moreira et al., 2019).

Cold atmospheric plasma is a surface modification technology that shows extensive promise in biomedical and biological applications. Often called the fourth state of matter, plasma exists in a higher energy state than gases containing radicals, free electrons, and molecular fragments. Plasmas were first generated in laboratory settings in the early 1900's by heating pressurized gas, now high voltage electrons can generate plasmas at room temperature at atmospheric pressure (Kogelschatz, 2003). Plasmas are typically generated from a variety of gases using different types of electrodes that provide the energy necessary to create high energy plasma products. One such electrode type, the dielectric barrier discharge (DBD) electrode has tremendous potential as a sanitization technology for leafy greens.

With the DBD electrode, the substrate serves as the ground electrode and a single charged electrode is attached to a high voltage power supply. When the charged electrode is powered up, plasma is generated from the gas in the space in between the charged and ground electrode. In food sanitization, the highly charged plasma products generated can then inactivate microorganisms and inhibit some enzymatic activity (Laroussi, 2002; Laroussi et al., 2003; Xiang et al., 2018b). The DBD plasma electrode also has several attributes that may make it uniquely suited to leafy green sanitization. Leafy greens are high in moisture, making them a more "electrically active" ground electrode, and are also low in fat preventing much of the oxidative rancidity that can arise in plasma treated foods that have high fat contents. The structure of leafy greens also makes them ideal for plasma treatment as treatment efficacy decreases rapidly with penetration depth and their thinness makes this limitation much less of a concern. DBD electrodes also are well suited to leafy greens treatments because the air present in the production facility can be used as a charge gas and no pressurized gases will need to be purchased, keeping potential costs low. Additionally, the close contact of the plasma products with the leafy greens during a DBD plasma treatment may reduce water usage in leafy greens processing and reduce or eliminate the likelihood of cross contamination.

Since leafy greens and other fresh produce are still respiring post-harvest, CAP treatment could potentially result in the internalization of potentially harmful products as well as the formation of potentially harmful compounds in the plant tissues. While respirating, the plant stress response mechanism remains active post-harvest in leafy greens. The CAP treatment is likely to trigger the stress response in unique ways, however the response may yield benefits as well as risks. Typically, CAP generates both reactive oxygen and nitrogen species (RONS), which are key signaling molecules in plant metabolism and they play a prominent role in plant stress response. Determining how CAP modifies plant tissues could play a significant role in ensuring the safety, efficacy, and acceptance of this promising antimicrobial technology.

While cold plasma has not been approved yet for use in foods sold in the United States, other sanitization treatments that utilize oxidation to achieve microbial inactivation are already approved and in use. Plasma produces several products that can lead to oxidative damage and cell death in micro-organisms (Laroussi, 2002; Laroussi et al., 2003; Xiang et al., 2018b). These products have the potential to negatively impact other tissues as oxidative and free radical damage is very general. Plasma devices emit several reactive gases including reactive oxygen species (ROS) and reactive nitrogen species (RNS). and an increased level in the environment could activate several plant response pathways. Nitric oxide and hydrogen peroxide, RONS, are also important molecules in plant signaling (Hou et al., 2011; Marcec et al., 2019; Mittler, 2017). Concentrated doses of these molecules could also contribute to the formation of undesirable molecules in leafy greens or potentially destroy some nutrients and phytochemicals, especially those that serve as antioxidants.

Understanding these interactions is key to evaluating and ensuring produce quality and safety from both biological and potential chemical hazards. The chemical and physical plasma products can trigger abiotic stress responses in spinach, these stress responses could also be affected by the presence of certain pathogens and trigger biotic stress responses as well (Cui et al., 2015; Dangl & Jones, 2001; Jones & Dangl, 2006, 2021).

Typically, ROS are present at low levels in plant cells as byproducts of normal metabolic processes such as electron transport. Plants utilize enzymatic and non-enzymatic systems to prevent ROS toxicity, but at these sub-toxic levels these molecules are utilized extensively as signaling molecules for enzymatic and non-enzymatic processes. The ROS are generated in multiple organelles in the plant cell, including the peroxisome, where many ROS related enzymes are produced, as well as in mitochondria and chloroplasts. The plant cell wall and cell membrane harbor structures where ROS are produced. Hydrogen peroxide is one of the predominant forms of ROS and is regarded as the principal ROS for signaling because it can diffuse through cell membranes via aquaporins, thus, making it a useful signaling molecule to trigger a systemic response to stimuli(Hasanuzzaman et al., 2020; Kohli et al., n.d.; Lee et al., 2023).

Reactive nitrogen species (RNS), such as nitric oxide, are also important signaling molecules in plants and can reversibly suppress some plant metabolic processes such as electron transport in ATP production and photosynthesis. In addition, NO has been found to play a role in stomatal opening and closing, working in concert with free calcium ions ROS, influenced by cytosol pH. Additional cross-talk has been observed between NO and ROS signaling pathways(Baudouin & Hancock, 2014a; Kohli et al., n.d.; Neill et al., 2003a).

Leafy greens are well suited for CAP sanitization treatments. Their high moisture content and thin structure allows for or encourages greater penetration of CAP products and their low fat content reduces the likelihood of off flavors resulting from treatment (Niemira, 2012). However since leafy screens and other fresh produce are still respiring post-harvest, CAP treatment could result in the internalization of potentially harmful products as well as the formation of harmful compounds in the plant tissues. In addition to respirating, the plant stress response mechanism remains active post-harvest in leafy greens. Since plants lack the ability to flee from negative stimuli, they have elaborate signaling systems that allow them to quickly adapt to biotic and abiotic stressors (Dangl & Jones, 2001; Jones & Dangl, 2006). Cold atmospheric plasma is likely to trigger the stress response in unique ways, however the response may yield benefits as well as risks. CAP generates both reactive oxygen and nitrogen species, which are key signaling molecules in plant metabolism and play a prominent role in plant stress response. Determining it and how CAP modifies plant tissues could play a significant role in ensuring the safety, efficacy, and acceptance of this promising antimicrobial technology.

The purpose of this manuscript is to investigate the causes of any changes to plant tissues resulting from CAP treatment and to determine if the changes are triggered by the treatment alone or by the plant defense responses to the plasma treatment. Determining if plasma products are internalized in spinach tissue and if their internalization triggers further physiological changes in the food product is essential to determining if the processing technology is safe for approval and use in human foods.

Materials and Methods

Fresh Produce

Baby Spinach, baby kale and red leaf lettuce were purchased from a local retailer and stored at 4℃ until analysis. Sweet basil plants were also purchased from a local grocery store and stored in a portable growth chamber on a 12-hour light cycle until treatment (ViaGrow, East Point GA, USA).

Plasma Device

A bench top 30kv plasma device was purchased from advanced plasma solutions (Malvern PA, USA) and this power source and dielectric barrier discharge electrodes from the supplier were used for all experiments. All experiments with the plasma device were carried out in a fume hood as a precaution against excess ozone exposure or accidental excessive electrical arcing. Plant samples were treated with the DBD electrode after being placed on an adjustable stage and clamped in place using a magenta box lid (Plantmedia, Dublin OH, USA) that had been cut to allow the charged electrode to interact with the substrate.

When samples were treated, the fine adjustment of the stand was used to ensure that the treated sample was between 5 mm and 2.5 mm from the ground electrode, samples were not completely flattened for treatment to prevent physical injury to the plant tissue from contributing to the stress response readings. The samples were treated at three different power levels, settings 1, 3, and 5 which corresponded to 50w treatment, 90w treatment and 120w treatment respectively. Once treatment has completed, the power source was turned off before the sample was removed from under the DBD electrode. The electrode setup is shown below with basil.

Rapid Quantification of reactive oxygen species (ROS)

The fluorescein assay was used to determine the generation of ROS samples after treatment. Fluorescein is a fluorescent dye that loses fluorescence when it binds to reactive oxygen species. Fluorescein is a probe commonly used for the hydroxy radical antioxidant capacity or HORAC assay, and oxidizes in the presence of hydrogen peroxide leading to a loss of fluoresence. The greater loss of fluoresence compared to control indicates a greater concentration of hydrogen peroxide (Gomes et al., 2005). A decrease in fluorescence compared to an untreated control indicates an increase in reactive oxygen species concentration. This method was chosen since the ROS levels in leaves could be measured rapidly after treatment with little additional handling of leaves after the directly treated tissue is separated from the remaining portion of the plant.

After sanitization treatment, the directly treated portion of each leaf was separated, and the direct and indirectly treated portions were weighed, then added to a whirl-pak bag (Whirl Pak, Pleasant Prairie WI, USA) containing 5mL of 1 μM fluorescein solution and homogenized in a stomacher (Seward Stomacher 80, West Sussex, UK) on the high setting for two minutes. After homogenization, three 100 μL aliquots per sample were placed in an opaque 96 well plate and read for fluorescence at excitation wavelength of 485 nm and an emission wavelength of 525 nm. Fluorescence readings were performed using a Spectramax 5xe plate reader (Molecular Devices, Sunnyvale CA, USA) Fluoresence values were normalized compared to control using the following equation.

$$
\begin{aligned}
&normalized\ fluoresence \\
&\quad = 100 \times [(fluorescence\ sample \times \frac{mass\ control + 5mL}{mass\ control}) \\
&\quad - (fluorescence\ sample \times \frac{mass\ sample + 5mL}{mass\ sample})]
\end{aligned}
$$

Rapid Quantification of Flavonoids

The diphenylborinic-2-aminoethyl ester (DPBA) assay was used to measure the total flavonoid content in samples after treatment. The DPBA spontaneously forms a fluorescent complex with most flavonoids, making the assay very useful for rapid quantification of these metabolites (Filippi et al., 2015). A standard curve was first generated using Epigallocatechin gallate (Thermo Scientific Chemicals, Fairlawn NJ, USA) at concentrations of 0.5, 1, 2.5, 5, 7.5 and 10 mMol in a 2% DPBA in dimethylsulfoxide (DMSO) solution.

After sanitization treatment, the directly treated portion of each leaf was separated, and the directly and indirectly treated portions were weighed, and added to a whirl pak bag (Whirl Pak, Pleasant Prairie WI, USA) containing 5mL of 2% DPBA solution in dimethyl sulfoxide and homogenized in a stomacher (Seward Stomacher 80,West Sussex, UK) on the high setting for two minutes. After homogenization, three 100 µL aliquots per sample were placed in an opaque 96 well plate and read for fluorescence at an excitation wavelength of 400 nm and emission wavelength of 465 nm.

Statistical analysis

All experiments were performed in triplicate and statistically evaluated using a paired *t*-test (α =0.05) in Microsoft Excel.

Results

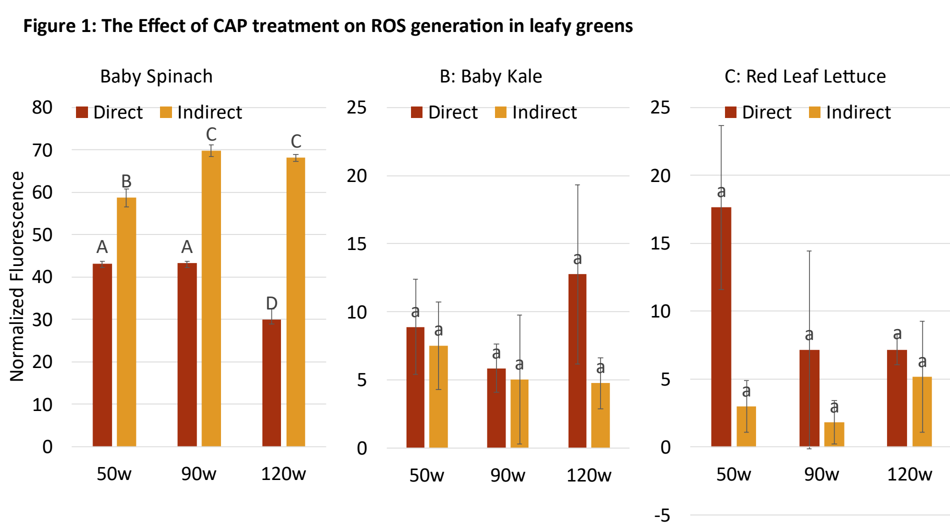


Figure 1: The ROS response in leafy greens to CAP treatment at three power levels, significant ($p<0.05$) differences were observed between directly and indirectly treated

spinach. Significant differences between directly and indirectly treated lettuce and kale were not observed due to the more substantial reduction in fluorescence

ROS generation

The changes in reactive oxygen species levels in leafy greens were dependent on species, with spinach experiencing far lower levels of reactive oxygen species generation than in red leaf lettuce and baby kale (Figure 1). Red leaf lettuce and baby kale both had much stronger ROS response to CAP treatment, with fluorescence reduced by over 80%, while the strongest reduction in spinach, the 120w direct treatment resulting in a less than 60% reduction in fluorescence. In addition to no significant differences being observed between directly and indirectly treated lettuce and kale tissue and no significant differences observed between power levels.

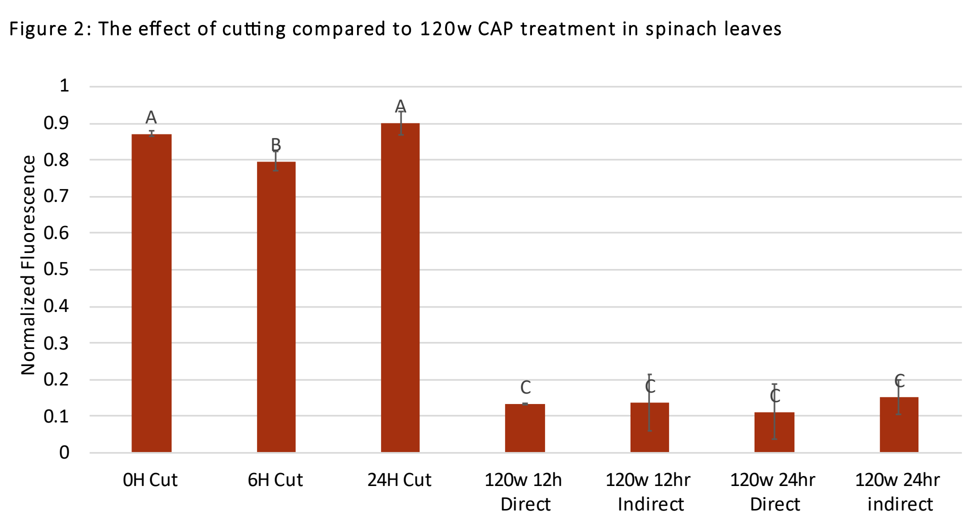


Figure 2: Comparison of ROS response in cut spinach compared to CAP treated spinach. CAP treatment results a significantly higher ($p<0.05$) level of ROS generation than cutting. A further increase in ROS content of CAP treated spinach occurs between 0 and 12 hours, and this does not appear to reverse.

Interestingly, a similar result is observed in 120w CAP treated spinach after 12 hours post treatment (Figure 2). ROS activity in plant leaf tissue continues well after treatment and remains high, and this contrasts with the ROS response triggered by cutting spinach leaves, which results in a much lower reduction in fluorescence levels compared to CAP treatment.

Flavonoid Content

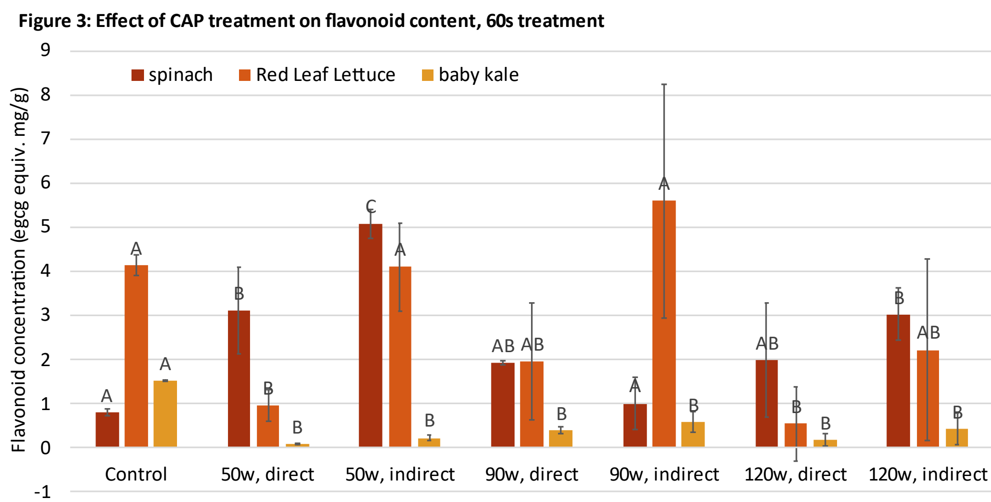


Figure 3: Flavonoid response to CAP treatment of leafy greens. CAP treatment had a highly species-specific effect on the flavonoid content of leafy greens. In red leaf lettuce spinach, significant ($p<0.05$) differences in flavonoid content were observed between directly and indirectly treated leaf tissue. In baby kale a significant reduction in flavonoids in both directly and indirectly treated tissue was observed.

Flavonoids are a common stress product in plants (Brown et al., 2005; Panche et al., 2016), and they have the additional benefit of serving as nutraceuticals for consumers ((Hosseinzade et al., 2019; Ielpo et al., 2000; Pérez-Cano & Castell, 2016; Serafini et al., 2010). An increase in flavonoid content in the plant tissue suggests that CAP treatment triggers additional stress response activities rather than the tissue internalizing ROS generated by the electrode. Significant differences in the flavonoid response were observed across the three species based on the flavonoid response, with lettuce showing the most “localized” response to the CAP treatment, with a decrease in flavonoids on indirectly treated tissue observed at the highest power level, 120w. Additionally, flavonoid content in directly treated lettuce had a dose dependent response, with 90w direct treatment resulting in elevated flavonoid content compared to 120w and 50 w while still being reduced compared to both the control and indirectly treated leaf tissue at 90w and 50 w. Spinach had a somewhat unexpected response to cap treatment.

Directly treated spinach tissue experienced a significant increase in flavonoid content at all power levels. The flavonoid response was further significantly increased in indirectly treated spinach leaf tissue at the 50w power level. At 120w power, indirectly treated spinach leaf tissue was increased at a similar level to directly treated tissue, but at 90w power, indirectly treated tissue did not significantly increase flavonoid content to compared to control, suggesting a complex power dependent relationship between flavonoid content and the composition of plasma output products at different power levels.

A different response was observed in kale, where CAP treatment at all power levels significantly reduced the flavonoid content, and at all power levels this reduction of flavonoids was equally as high in indirectly treated tissue.

Discussion

The products produced by DBD plasma inactivate microorganisms via oxidative damage, and these oxidative products also have effects on the substrate during treatment (Laroussi et al., 2002; Niemira, 2012; Thirumdas et al., 2018).. While these effects will require additional scrutiny as the technology develops for use in sanitizing foods, results observed in spinach indicate that CAP treatment may yield additional benefits to human health (Cui et al., 2015; Dangl & Jones, 2001; Jones & Dangl, 2006). The high level of exposure to RONS as well as UV light significantly increased flavonoid levels in DBD treated spinach and fluorescein measurements at 120w indicate that the treatment triggers a more systemic response to quench the radicals without exceeding the quenching capacity of the treated spinach leaves. However, the treatment appears to overwhelm the quenching capacity of kale and red leaf lettuce, leading to a sharp reduction in flavonoids present in kale. These results indicate that more research into modifications in electrode design to change output products or modifications to how the treatment is applied may be needed to apply this antimicrobial treatment to different species of leafy greens to inactivate micro-organisms without diminishing the nutritional value of the products.

Encouragingly, prior research in DBD treated spinach suggests that leafy greens do not need to be directly exposed to a plasma source to experience microbial reduction. Prior research showed *E. coli* not directly exposed to a surface microdischarge (SMD) plasma device could still be inactivated, with no significant difference in inactivation on a spinach leaf completely covered by one leaf compared to a leaf directly exposed to the electrode (Luan et al., 2019). The results observed in this study suggest that

DBD plasma may also inactivate internalized pathogens in plants, a result currently only possible with the nonthermal processing technology, irradiation (Wall et al., 2019). DBD plasma is a potentially less costly technology to incorporate into post-harvest handling. However, there are some potential limitations to the technology that may need to be addressed before widespread adoption of the technology.

In kale, DBD plasma treatment likely overwhelmed the plant's quenching capacity, reducing the levels of flavonoids in both directly and indirectly treated tissues in addition to sharply increasing the levels or ROS inside of the plant tissues. Unquenched radicals could further damage plant tissues, and this could promote the survival of pathogens not inactivated by CAP treatment. This potential concern may not be limited to plant species that may be overwhelmed by oxidative damage, as plasma products such as ozone and light are known to influence stomatal openings, which allow pathogens to enter plant tissues without damage (Cortleven et al., 2022). Flavonoids, other phenolic compounds, and additional hydrogen peroxide generated in response to DBD treatment could potentially inactivate these internalized pathogens, but pathogens that survived the initial treatment may be more resistant to oxidative stress. Further characterization of plant and pathogen interactions and responses will allow leafy greens processors to prevent negative outcomes and provide valuable information about the ideal time in leafy green production to deploy this antimicrobial technology. The leafy green substrates treated in this study were purchased from local retailers and their respiratory pathways were still active after undergoing the commercial triple washing process that incorporates oxidizing antimicrobials such as chlorinated and ozonated water. Kale, and other leafy greens that may experience a reduction of flavonoids and very high levels of ROS generation, may benefit more from having CAP treatment applied prior to the washing stage or even pre-harvest. While the complexity of the response of plant tissues to CAP treatment will require further investigation, the results of this study indicate that the potential benefits of this technology make it well suited to further exploration for use in leafy greens.

Chapter 3: Objective Two, Characterizing the Stress Response of Leafy Greens To CAP Treatment

Introduction

The unique attributes of the DBD electrode present unique opportunities in fresh produce processing. Unlike other styles of electrodes such as the plasma jet, the substrate serves as the ground electrode and only one charged electrode is connected to the power supply. Based on the results of previous study, the plant stress response to CAP differed significantly based on species, with kale suffering nutrient losses because of the treatment. To examine further, this study compares the effects of UVB treatment, and ozonated water treatment with 120w CAP treatment on spinach and kale.

Many highly reactive products generated in high concentrations by DBD plasma electrodes are already well known to plants and they have evolutionary defenses to protect against them such as UVB and ozone. UVB radiation is damaging to land plants so mechanisms for mitigating damage to macromolecules and organelles is incorporated into the metabolism of many land plants (Kreslavski et al., 2021). Flavonoids play a role in these defenses. Light treatment also has extensive impacts on the nutraceutical content of plants (Thoma et al., 2020). However, these defenses may be overwhelmed by the unique concentrations of these products, additionally, some of the reactive products such as H_2O_2 and NO also serve as signaling molecules, and their introduction at such relatively high concentrations could result in metabolic effects in addition to antimicrobial effect. These familiar compounds applied in an unnatural manner may yield benefits such as those seen in spinach treated with a DBD electrode. Light treatment has extensive impacts on the nutraceutical content of plants (Roeber et al., 2022; Thoma et al., 2020). The DBD electrodes emit light in the VUV to UVB range(100 -315 nm) (Samukawa et al., 2012) the role of UV in microbial inactivation due to CAP treatment is uncertain, but has been shown to play a greater role in low pressure plasma applications (Nasiru et al., 2021; Niemira, 2012).

Highly toxic in plants, ozone has extensive applications in manufacturing as well as food processing because of its reactivity (Sachadyn-Król & Agriopoulou, 2020; Sharma et al., 1996; Tamaoki, 2008; Xu et al., 2015). The dielectric barrier discharge electrode was first created for the purpose of ozone generation and the compound is one of the reactive products produced in CAP generation (Eliasson et al., 1987; Kogelschatz, 2003). Ozone, while common in the upper atmosphere is typically only found in the troposphere due to pollution and can be highly damaging to plants, however in leafy greens processing ozonated water is often used as a sanitizer in the triple washing process (Lis et al., 2018) To prevent damage, plants will expel what ozone they can through their stomata, which may modify the resident microflora on the leaf surface (Erickson, 2012). However, the opened stomata are a potential source of pathogen internalization, and it remains unseen how the increased activity to expel the ozone will effect internalized pathogens.

Characterizing the behavior of the substrate in response to cap treatment is necessary for improving the antimicrobial efficacy of this process, which while effective, to get to a consistent 5 log CFU/g inactivation that could potentially resolve the food safety issues associated with fresh produce eaten raw(Niemira et al., 2018; Nwabor et al., 2022; *Potential for Infiltration, Survival, and Growth of Human Pathogens within Fruits and Vegetables | FDA*, n.d.; Ziuzina et al., 2014)

Materials and Methods

Fresh Produce

Baby Spinach, baby kale were purchased from a local retailer and stored at 4℃ until analysis.

Plasma Device

A bench top 30kv plasma device was purchased from advanced plasma solutions (Malvern PA, USA) and this power source and dielectric barrier discharge electrodes from the supplier were used for all experiments. All experiments with the plasma device were carried out in a fume hood as a precaution against excess ozone exposure or accidental excessive electrical arcing. Plant samples were treated with the DBD electrode after being placed on an adjustable stage and clamped in place using a magenta box lid (Plantmedia, Dublin OH, USA) that had been cut to allow the charged electrode to interact with the substrate.

When samples were treated, the fine adjustment of the stand was used to ensure that the treated sample was between 5mm and 2.5 mm from the ground electrode, samples were not completely flattened for treatment to prevent physical injury to the plant tissue from contributing to the stress response readings. All samples were treated at the 120w power level, or setting 5 on the instrument. Once treatment has completed, the power source was turned off before the sample was removed from under the DBD electrode. For live basil plants treated with CAP, the fine adjustment stand was placed atop a larger adjustable stand to avoid excessive bending of the basil stem to which the treated leaf was attached. While the power output during plasma treatment remained steady over the treatment times used in the experiments, the power source took roughly 1 s to reach a steady output after being powered on. The treatment time began after the brief warming-up period.

UVB treatment

For UVB treatments, a UVB chamber (Spectroline XL-1000, Melville NY, USA) was used. Before all treatments, the UV chamber was powered on and run for 5 min to reduce the variations in output power. For each replicate, three spinach or kale leaves were placed on aluminum foil with the adaxial side facing upward. Half of the leaf obscured along the midrib of the leaves. Samples were treated for 30 and 60 s.

Ozone Treatment

A household ozone sanitizer purchased from an online retailer was used for ozone treatments (Ivation 600mg/h, Edison NJ, USA). As a safety precaution, ozone treatments were carried out in water rather than in air. 200mL of sterile 0℃ water was added to an autoclaved magenta box. The approximate total ozone concentration was 0.52 mM for 30s treatment and 1.04 mM for 60s treatment. For each replicate, three spinach or kale leaves were added to the water for ozone treatment. In the fume hood, a pumice ozone diffuser was added to the water for treatment and the ozone generator powered on for treatment.

Rapid Quantification of Flavonoids

The diphenylborinic-2-aminoethyl ester (DPBA) assay was used to measure the total flavonoid content in samples after treatment. DPBA will spontaneously form a fluorescent complex with most flavonoids, making the assay very useful for rapid quantification of these metabolites (Filippi et al., 2015). A standard curve was first generated using Epigallocatechin gallate (Thermo Scientific Chemicals, Fairlawn NJ, USA) at concentrations of 0.5, 1, 2.5, 5, 7.5 and 10 mMol in a 2% DPBA in DMSO solution.

After sanitization treatment, the directly treated portion of each leaf was separated, and the directly and indirectly treated portions were weighed, and added to a whirl pak bag (Whirl Pak, Pleasant Prairie WI, USA) containing 5mL of 2% DPBA solution in dimethyl sulfoxide and homogenized in a stomacher (Seward Stomacher 80,West Sussex, UK) on the high setting for two minutes. After homogenization, three 100 µL aliquots per sample were placed in an opaque 96 well plate and read for fluorescence at an excitation wavelength of 400 nm and emission wavelength of 465 nm.

HPLC analysis of Salycilic and Jasmonic acid

Plant stress response hormones were evaluated using an HPLC method based on one developed by Wen and coworkers (2005). After treatment, directly and indirectly treated portions of spinach and kale leaves were freeze dried (Harvest Right, Salt

Lake City UT, USA), weighed and phenol containing acids were extracted using the following method. Dried samples were weighed then crushed and added to a 1.5mL centrifuge tube with 0.8 mL of 80% methanol solution (20% water 80% methanol v/v) mixed with a vortex and centrifuged at 10000 rpm for 3 minute using an Eppendorf 5430R centrifuge at room temperature (Eppendorf Hamburg, Germany). The supernatant was collected, and 0.6 mL of methanol was added to the residue, mixed using a vortex and centrifuged, this step was performed twice. 1.5 mL of the collected supernatant was transferred to a 15 mL centrifuge tube. To the methanol extract, 0.6 mL of 2M HCl was added and mixed using a vortex, then 0.9 mL of saturated NaCl followed by vortex mixing, and then 0.9 mL of a 1:1 ethyl ether, ether acetate solution was added and mixed using a vortex. The top organic phase was collected and evaporated under nitrogen. The dried organic phase was redissolved in 0.85 mL methanol and 0.05 mL acetic acid. Samples, and the two standards, jasmonic acid (Sigma Aldrich) and salicylic acid (AcRos organics) were filtered using a 0.45 μM syringe filter and transferred to an HPLC vial for analysis.

An Arc-HPLC equipped with a 2998 PDA detector (Waters, Milford MA, USA) was used for the phenolic acid analysis with an Eclipse XDB-C18 column (Aglient, Santa Clara, CA USA). A 0.2% solution of trifluoroacetic acid (TFA) in water was used for mobile phase A, and for mobile phase B, a 0.2% TFA solution in methanol was used. The initial gradient reported by Wen and coworkers was modified slightly, the initial gradient of mobile phase b was set to 20% and increased to 22.0% at 5 min, 25% at 10 min, 30.0% at 20 min, 40.0% at 25 min, 50.0% at 30 min, 65.0 at 35 min, and 80.0% at 40 min. After 43 min, the gradient of mobile phase B decreased from 80%

to 20% at 45 min, and the run terminated at 47 min. The flow rate was 1.0 mL/min, and the detection wavelength was 280 nm. For the standards, salicylic acid and JA were dissolved in 0.85 mL methanol and 0.05 mL acetic acid and standard curves were generated using the following concentrations for SA 0.01, 0.05, 0.1, 0.3, 0.5 and 1.0 mg/mL. For JA, the concentrations of 0.125, 0.25, 0.5, 1.0 and 2.0 mg/mL were used to generate the standard curve.

Statistical analysis

All experiments were performed in triplicate and statistically evaluated using a paired *t*-test (α =0.05) in Microsoft Excel. For HPLC experiments, each replicate was comprised of three leaves to ensure that enough sample remained after freeze drying.

Results

Figure 4: Flavonoid Content in CAP, Ozone, and UVB treated leafy greens

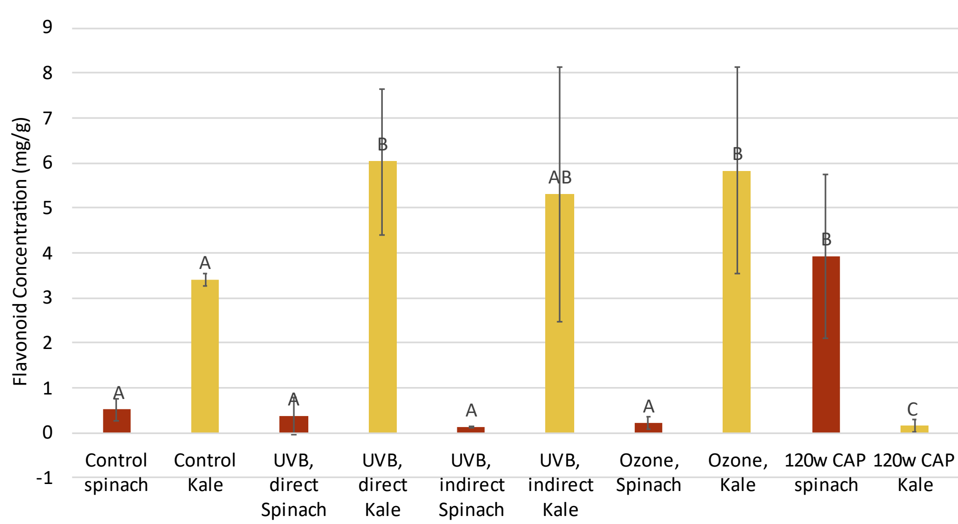


Figure 4: The flavonoid response in spinach and kale resulting from physical sanitization treatment. Flavonoid generation in spinach and kale were evaluated after CAP, UVB or ozone treatment. Ozone and UVB treatments resulted in significant ($p<0.05$) increases in flavonoid content for kale, while only CAP treatment significantly increased flavonoid content in spinach.

Sharply diverging stress responses to the CAP, UVB, and ozone treatments were observed in baby spinach and baby kale (Figure 4). While all three physical treatments elicited a strong stress response that resulted in significant impacts in flavonoid content. In baby kale, CAP treatment resulted in a significant, over 90% reduction in total flavonoids after 60s treatment, however isolated UVB and ozone treatments did not have this effect. Significant increases in flavonoid content were observed in ozone and UVB treated baby kale. With UVB treatment, increases in flavonoids were also observed in kale leaf tissue that had been obscured by aluminum foil and was not directly contacted with UVB radiation. While 120w CAP treatment did result in a significant increase in the flavonoid content of spinach leaf tissue, isolated ozone and UVB treatments did not result in significant changes to the flavonoid content of spinach leaves.

To further understand the role of the stress response to these sanitization treatments their effects on SA and JA concentration were also evaluated (Figure 5). In baby kale, all three treatments resulted in substantial changes to the concentration of salicylic acid, but since all treated samples had SA concentrations less than the limit of detection of the assay, no statistical determinations can be made at 30s or 60s. Both treated and untreated baby kale also had no detectable JA.

In baby spinach, only one sample had detectable JA acid, the 30s ozone treatment appeared to elevate JA levels in one sample above the limit of detection suggesting ozone may increase JA content, but this may be dependent on other factors unique to that sample. CAP, UVB, and ozone treatments resulted in decreases in SA content over time, with SA concentrations observed at 30 seconds higher than at 60s but due to high variability between the three sampling lots, no statistical significance could be determined. However notable results at 30 s show that there was a significantly higher concentration of SA in spinach leaf tissue treated by CAP at 30s, but it was not significant, likely due to the higher variability in the directly treated samples.

Figure 5a: Salicylic acid content of 120w CAP treated leafy greens

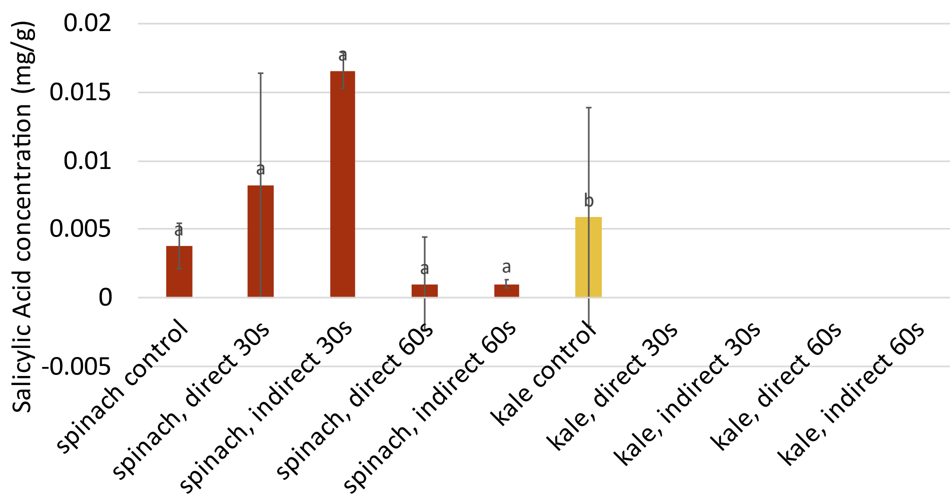
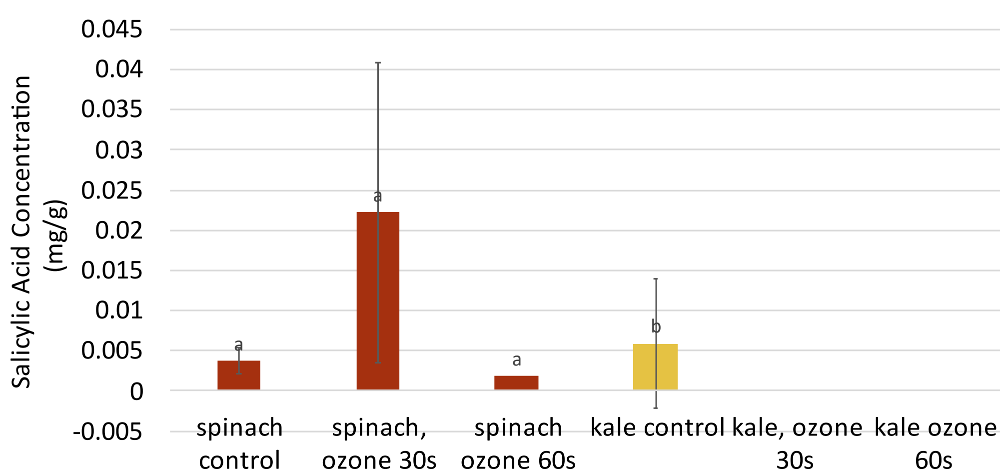
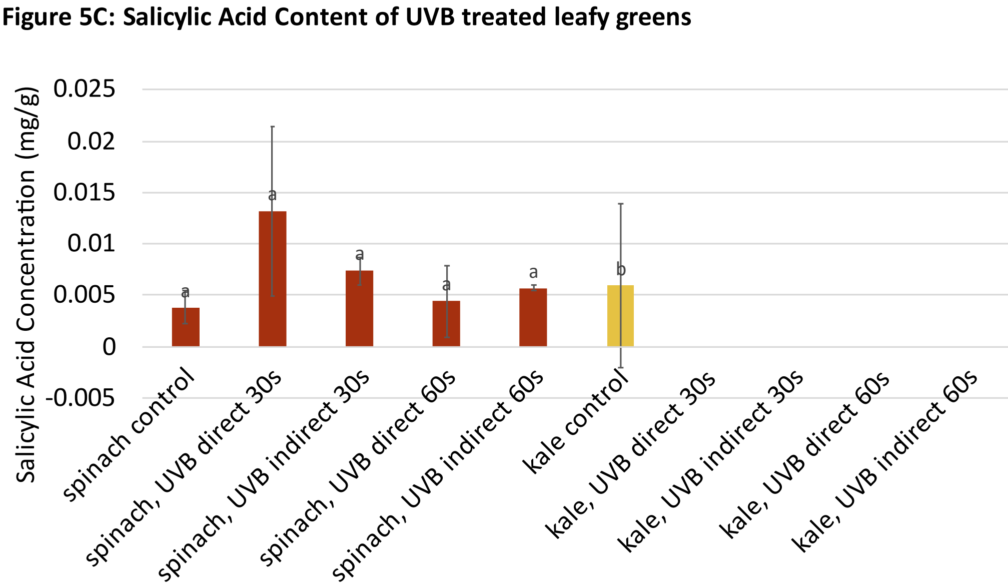


Figure 5B: Salicylic acid content of ozone treated leafy greens





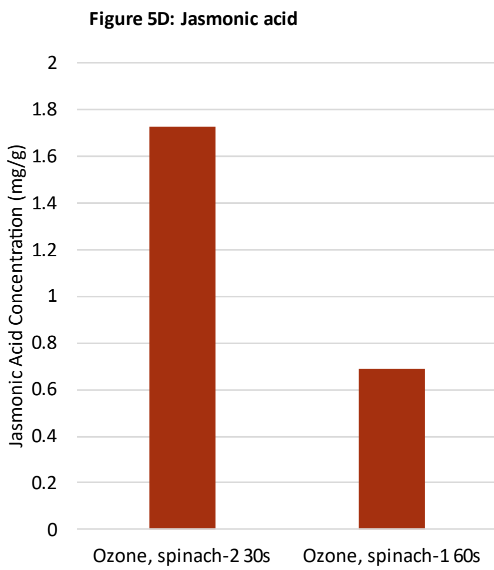


Figure 5: The effect of physical treatments on the SA (A, B, C) and JA (D) content of leafy greens. Physical treatments had very different effects on the SA concentrations on spinach and kale. All physical treatments resulted in no detectable SA in Kale. SA concentrations in spinach were very low with high variability observed, no significant difference was determined compared to control. JA was undetectable in most samples with the compound detected in only one of 3 spinach samples in ozone treatment. This may have been due to the low JA concentration in the more mature leaves.

Discussion

The divergent results observed in flavonoid content for both spinach and kale after ozone and UVB treatments especially when compared to CAP treatment indicates that other products produced by CAP have a substantial influence on the stress response of plants to CAP. While all three treatments were observed to have been impacted by all physical treatments, no significant differences were observed due to high variability and low overall concentrations. MeJA has been shown to trigger stomatal closures via a pathway that involves RONS and SA is involved in controlling stomatal aperture to prevent pathogen colonization (reference). However, SA and JA are not the only hormones involved in the plant response to plasma products and other highly oxidative compounds in Arabidopsis and tomato, Ozone has been shown to affect stomatal openings through a pathway that does not heavily involve these two signaling molecules (Zamora et al., 2021b).

Clues to how plasma products affect plant tissues may be found in the use of plasma activated water and plasma activated liquids where reactive nitrogen species play a critical role in the antimicrobial efficacy of these products and may be critical to understanding the diverging stress responses in spinach. Applying plasma to water will increase its reactive nitrogen species, decrease its pH and increase its oxidation reduction potential (ORP). Prior work indicates that concentrations of certain reactive nitrogen species such as nitric oxide and peroxynitrite and that while ORP and pH remain stable in plasma treated liquids, the reactive nitrogen species generated in the liquids are much more short-lived, and their concentration in these liquids has a strong relationship with their antimicrobial efficacy(Lukes et al., 2014; Neill et al., 2003b; Zhou et al., 2018). Reactive nitrogen species also serve as signaling molecules and their species-specific interactions may be why the flavonoid concentration appears reversed for spinach and kale when the reactive nitrogen species are removed.

One major driver of this difference could be the difference in antioxidant profiles between spinach and kale (Nemzer et al., 2021) . Differences in the antioxidant profile be what is responsible for the increased flavonoid content in spinach and understanding how reactive nitrogen species interact with the plant stress response system is likely key to improving the efficacy of cap treatment while reducing nutrient and quality losses. Reactive nitrogen species are also important signaling molecules in plant tissue and likely have extensive impacts on the plant stress response. Similar to ROS, RNS also display antimicrobial activity and ROS and RNS have been shown to work as antimicrobials simultaneously, typically causing cell death by electrostatic disruption or electroporation (Nasiru et al., 2021) Some RNS species have been observed to further nutritional impacts on plants. NO suppresses photosynthesis by reversibly suppressing electron transport and ATP synthesis in chloroplasts. It is emitted by plants under normal growing conditions, and nitrous oxide is an environmental pollutant (Neill et al., 2003b). NO was thought to be an environmental pollutant with a limited cell signaling role, however it was later found to be generated endogenously in plant cells (Corpas et al., 2019; Corpas & Barroso, 2013; Kohli et al., n.d.) NO plays a role in the control of stomatal apertures, and this also involves reactive oxygen species (Baudouin & Hancock, 2014b)

A major challenge in understanding the role of RNS in CAP treatment in leafy greens will be in the extensive interactions between RNS and ROS in the plant tissue. While evaluating the stress response in a reduced oxygen environment may be useful for characterizing the role of reactive nitrogen species in isolation, prior work indicates that the activity of RNS in plant tissues are influenced by the activity of ROS. Plasma outputs are influenced by charge gas and the waveform frequency and amplitude of the voltage used to produce the plasma, as well as the power level used to generate the plasma. For CAP generated by a DBD electrode in air, the ratio of RNS to ROS is determined by the power level of the plasma device(Paulsen et al., 2022; Shimizu et al., 2012). A possible explanation for the differences in nutritional responses could be examined through a closer investigation of the overall antioxidant profiles of leafy greens (Nemzer et al., 2021). The quenching capacities of antioxidants found in different leafy green species and varietals may be key in determining the best treatment parameters for each crop.

Chapter 4: Objective Three, Evaluating The Systemic Effects of CAP Treatment on Sweet Basil

Introduction

The demand for leafy green vegetables has been steadily increasing and is forecasted to grow as people are eager to reap the health benefits of these foods (*Fresh Vegetables - Worldwide | Statista Market Forecast*, n.d.). However, since these products do not undergo a kill step (*FSMA Final Rule on Produce Safety | FDA*, n.d.) there is an elevated risk of foodborne illness in both leafy greens and fresh herbs. From 1996 to 2015, the FDA reported nine outbreaks linked to the herbs basil, parsley, and cilantro resulting 2699 confirmed illnesses and 84 hospitalizations. Fresh herbs in particular, can be difficult to identify as the etiological agent in an outbreak since they are often incorporated into compound dishes in small amounts. Due to the high contamination risk found throughout the leafy greens production and handling chain leafy greens can be exposed to a variety of organisms. Greenhouse and covered operations may reduce some environmental contamination but may not control human pathogens introduced via water or human handlers (*FDA Releases Final Report on Spinach E Coli Outbreak | CIDRAP*, n.d.; *FSMA Final Rule on Produce Safety | FDA*, n.d.; Gil et al., 2015) Surface water, commonly used in irrigation is a common source for salmonella that appears adapted to a range of different water environments (Bell et al., 2021; Kroupitski et al., 2019) Post harvest washing, while necessary for reducing transient pathogens it can encourage the internalization of pathogens into plant tissue, often due to wash water uptake triggered by a temperature or osmotic difference (Kroupitski et al., 2019; Olaimat & Holley, 2012) Chlorine and other sanitizers are commonly added to wash water to prevent high levels of microbial contamination. Chlorine is commonly used in US fresh produce operations and while highly effective against microorganisms in a solution, the presence of other compounds in the wash water can significantly impact its efficacy (Zhou et al., 2015). Another major challenge in fresh produce safety is the difficulty involved in characterizing the behavior of human pathogens on plants. While *S. enterica* and *E. coli have* been shown to be able to colonize corn, bean, and cilantro plans under humid conditions (Lindow & Brandl, 2003) most enteric bacteria species are not well suited to the harsh, nutrient poor condition found on the surface of leaves (Aung et al., 2018; Carvalho & Castillo, 2018; Vorholt, 2012). As a result, detecting human pathogens in the initial processing stages can be difficult and small populations of human pathogens that manage to attach themselves to fresh produce are often able to successful resist physical washing and sanitization methods (Brandl, 2006; Fletcher et al., 2013; Kim et al., 2020; Van der Linden et al., 2016; Ximenes et al., 2017; Yaron & Römling, 2014)

Cold atmospheric plasma may be uniquely well suited to the food safety challenges presented by leafy greens and fresh and living herbs. The highly oxidative products that are lethal to bacteria affect the plant metabolism in ways that may impact its microflora even if it does not inactivate pathogens to the 5-log CFU/g reduction of pathogens (*Draft Guidance for Industry: Reducing Microbial Food Safety Hazards in the Production of Seed for Sprouting | FDA*, n.d.; *FDA Sampling Fresh Herbs, Guacamole and Processed Avocado | FDA*, n.d.; *FSMA Final Rule on Produce Safety*

| *FDA*, n.d.; *Potential for Infiltration, Survival, and Growth of Human Pathogens within Fruits and Vegetables | FDA*, n.d.; Nwabor et al., 2022) CAP products may modify the phyllosphere organisms as well as the plants own pathogen defenses to make them more resistant to contamination by human pathogens(Arnaud & Hwang, 2015; Yadav et al., 2020). Conversely, the output products may elevate the risk of contamination by clearing commensal existing microflora or bruising the plant in a way that encourages colonization. For this study the systemic effects of CAP treatment were evaluated on the popular living herb sweet basil to provide more insight on how to explore the role of CAP on plant microbe interactions.

Methods

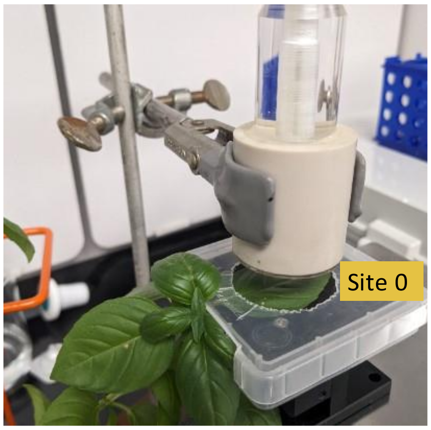
Fresh Produce

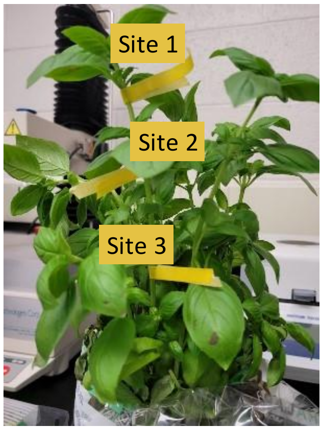
Sweet basil (var. Kiera)plants were also purchased from a local grocery store and stored in a portable growth chamber on a 12-hour light cycle until treatment (ViaGrow, East Point GA, USA).

Plasma Device

A bench top 30kv plasma device was purchased from advanced plasma solutions (Malvern PA, USA) and this power source and dielectric barrier discharge electrodes from the supplier were used for all experiments. All experiments with the plasma device were carried out in a fume hood as a precaution against excess ozone exposure or accidental excessive electrical arcing. Plant samples were treated with the DBD electrode after being placed on an adjustable stage and clamped in place using a magenta box lid (Plantmedia, Dublin OH, USA) that had been cut to allow the charged electrode to interact with the substrate.

When samples were treated, the fine adjustment of the stand was used to ensure that the treated sample was between 5mm and 2.5 mm from the ground electrode, samples were not completely flattened for treatment to prevent physical injury to the plant tissue from contributing to the stress response readings. The samples were treated at three different power levels, settings 1, 3, and 5 which corresponded to 50w treatment, 90w treatment and 120w treatment respectively. Once treatment was completed, the power source was turned off before the sample was removed from under the DBD electrode. For live basil plants treated with CAP, the fine adjustment stand was placed atop a larger adjustable stand to avoid excessive bending of the basil stem to which the treated leaf was attached. Sample sites 1 (adjacent leaf), 2 (neighboring leaf), and site 3 (nearest branched leaf) were also identified before CAP treatment. The directly treated leaf was termed site 0. The electrode setup and sampling sites are shown below.





Rapid Quantification of ROS

The fluorescein assay was used to determine the generation of ROS in basil after CAP treatment. This method was used again since the ROS levels in leaves could be measured rapidly after treatment with little additional handling of leaves after separating the four sampling sites.

After sanitization treatment, each of the leaves from each of the sampling sites were weighed and, then added to a separate whirl-pak bag (Whirl Pak, Pleasant Prairie WI, USA) containing 5mL of 1 μM fluorescein solution and homogenized in a stomacher (Seward Stomacher 80, West Sussex, UK) on the high setting for two minutes. After homogenization, three 100 μL aliquots per sample were placed in an opaque 96 well plate and read for fluorescence at excitation wavelength of 485 nm and an emission wavelength of 525 nm. Fluorescence readings were performed using a Spectramax 5xe plate reader (Molecular Devices, Sunnyvale CA, USA) Fluorescence values were normalized compared to control using the following equation.

$$normalized\ fluoresence = 100 \times [(fluorescence\ sample \times \frac{mass\ control + 5mL}{mass\ control}) - (fluorescence\ sample \times \frac{mass\ sample + 5mL}{mass\ sample})]$$

Statistical analysis

Six biological replicates were used for each experiment and statistically evaluated using a paired *t*-test (α =0.05, α =0.10) in Microsoft Excel.

Results

ROS generation in CAP treated Basil

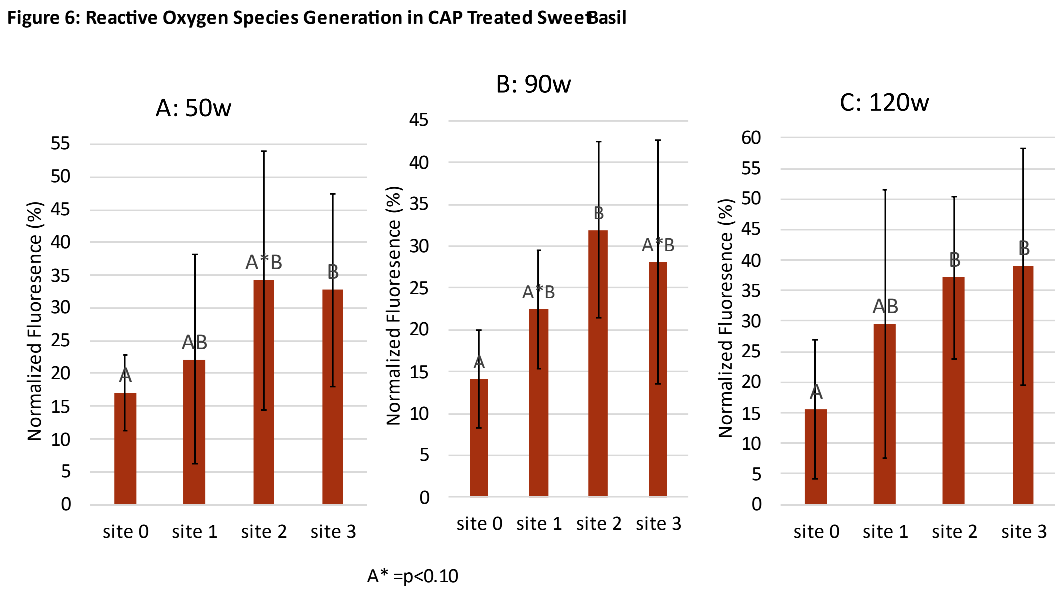


Figure 6: ROS generation in CAP treated sweet basil. All power levels appeared to follow the same general trend, with sample sites 2 and 3 experiencing significantly ($p < 0.10$) lower ROS generation than the directly treated leaf. No significant differences were determined between the same sample site at different power levels.

Reactive oxygen species generation in sweet basil was consistent across power levels, with each treatment resulting in about an 85% reduction in fluorescence at site 0. At 50w and 120w ROS levels at 3 were significant at $p<0.05$ and at 90w it was significantly lower at 90% confidence ($p=0.0516$). A power dependent ROS was observed. At site 2 90w treatment, where the lowest ROS levels were observed for that treatment power. In 120w treated plants both sites 2 and site 3 were significantly ($p<0.05$) lower than site 0 and both sites were not significantly different from each other.

Discussion

Much like the human microbiome, plants microbiota are involved throughout its metabolic processes and contribute positively to the health and growth of the plant. The plants microbiota can be influenced by a number of environmental sources. (Omae & Tsuda, 2022) . Plant age and immune system conditions affect phyllosphere conditions (Sivakumar et al., 2020a) therefore only basil grown in the same facility was used in this experiment. Bacterial species in phyllosphere organisms are often consistent within the same species of plant found in the same field, more diversity within plant species is seen int the fungal phyllosphere organisms (Adi Wicaksono et al., 2023). While CAP treatment parameters will likely change based on species and varietal, field and environmental conditions could influence the effectiveness of CAP treatment which could limit the adoption of the technology.

In addition to the direct effects of CAP treatment on phyllosphere organisms, CAP products may significantly affect the phyllosphere environment. The oxidative products emitted by CAP can modify the leaf surface for example, aliphatic compounds in the cuticle layer can affect the surface wettability of the leaves which can affect attachment or colonization of human pathogens (Sivakumar et al., 2020b). This is potentially more problematic in post-harvest CAP applications where the ability to recover from CAP triggered oxidative damage is more limited. The surface modifications often involve increasing the free sugars available to *Pseudomonas syringae* which in turn facilitates the growth of more opportunistic organisms such as human pathogens potentially shortening shelf life in addition to increasing illness risk (Sivakumar et al., 2020b). When applied pre-harvest, the surface modifications may change the surface environment by modifying the thin layer of moisture that surrounds many leaves, resulting in substantial changes to the microbial environment (Chaudhry et al., 2021; Erickson, 2012). Light may also play a role in influencing microbial communities, as changes in sunlight intensity and day length affect leaf exudates (Mogren et al., 2018). The light emitted by DBD electrodes may significantly impact microbial communities. Additionally, ozone may open stomata which can provide human pathogens access to increased nutrient availability and a shield from environmental stressors on the leaf surface (Erickson, 2012). However, the nutrient scarce highly exposed environment of the leaf surface already selects for organisms with different attributes than enteric pathogens which often grow more successfully in soil (Vorholt, 2012) and the increase in oxidative compounds as well as a potential increase in the availability of nutrients may strengthen the existing population of phyllosphere organisms, increasing its resistance to colonization by human pathogens (Kroupitski et al., 2019; Tsuda et al., 2009). The results in basil indicate that plasma products are rapidly diffused throughout the stem which may minimize the damage that makes it easier for colonization by human pathogens at all power levels. Further exploration of pre-harvest CAP treatment on fresh produce microflora will provide valuable insight on how best to deploy this technology.

Chapter 5: Initial conclusions and Future Work

The electrical output of the DBD electrode combined with the delicate nature of the food products evaluated in this study made evaluating the effect of the treatment on human pathogens very difficult. Applying inoculum to the leafy greens triggered unsafe levels of arcing during CAP treatment, so the plant stress response was evaluated instead to understand how the treatment will affect the plant microflora and human pathogens. The high variations observed in leafy greens samples may have obscured some interesting findings in these experiments, but these high variations are likely to be encountered by processors who may use CAP sanitization on their own products. Additionally, variations in varieties, weather conditions, and the environmental microbial populations in the growing and processing environment likely play a yet undetermined role in the stress response to CAP treatment. The enhancement of flavonoid content in spinach was unexpected and remarkable but is not shared across all species of leafy greens. Understanding the role of RNS and its interactions with the metabolic processes of leafy greens will be useful for understanding the root cause of the flavonoid losses in CAP treated kale. Evaluating the ROS and flavonoid content in altered gas environments with varying levels of nitrogen may be a useful method to evaluate the role of RNS in the plant response to CAP treatment. The experiments of objective two indicate that other sanitization treatments may also enhance the nutrient quality of other species of leafy greens. Studies that evaluate the plant stress response to sanitization treatments as well as other post-harvest handling practices could be valuable for improving best practices for existing technology used in the fresh produce industry. Results indicated that oxidative compounds may enhance the antioxidant content of fresh produce, but understanding the roles these compounds play in the metabolic processes of the model food will be key.

More work will be needed to determine if CAP treatment is suitable for all leafy greens as it is clear different species will likely need different treatment conditions. As new CAP designs are produced, understanding how the electrode layout influences what reactive products are produced will be key to improving the design of CAP for use in foods. Evaluating the stress response of fresh produce to sanitization and other treatments will be a valuable tool for improving the food safety of fresh produce, and it appears that this evaluation may also help improve the nutritional value of these already very healthy foods.

Chapter 6: Objective Four, Identifying to barriers food safety compliance for small and very small processors in the northeastern United States

Background

During my time as a graduate student, I have also had the privilege of working with both the University of Maryland Extension and as an outreach professional the Northeast Center for the Advancement of Food Safety (NECAFS). I have mainly worked on trainings and support for the Food Safety and Preventive Controls Alliance (FSPCA), Preventive Controls for Human Foods (PCHF) training used to help producers become compliant with the new regulations put forth by the Food Safety Modernization Act of 2011. In the summer of 2019, I began work on developing a needs assessment under the guidance of several regional extension professionals who lead the PCHF group within NECAFS. Prior to this work, needs assessments related to food safety have mostly been performed as they relate to farms covered by the produce safety rule and little research had been done for very small businesses that were food processors. For this needs assessment, the NECAFS PCHF team surveyed educators, regulators, buyers, and other professionals that worked directly with food processors, which we termed "Food Safety Communicators" (FSCs). The survey was approved for an exemption by the University of Vermont institutional review board and distributed to the NECAFS mailing list and FSPCA lead instructors in August of 2019.

Results

	Asked of FSPCA lead instructors: *When teaching the PCQI course, which sections do you think are more challenging for small and very small food processors to understand and implement?*					Asked of inspectors: *What were the most common misunderstandings and noncompliance observations or issues you observed in facilities during inspections?*	
	Extremely challenging	**Very challenging**	**Moderately challenging**	**Slightly challenging**	**Not challenging at all**	**Misunderstandings**	**Noncompliance**
Validation Procedures	**30%**	**52%**	**13%**	**4%**	**0%**	**88%**	**63%**
Hazard Analysis	**32%**	**45%**	**23%**	**0%**	**0%**	**88%**	**52%**
Verification Procedures	**9%**	**55%**	**27%**	**9%**	**0%**	**92%**	**56%**
Supply Chain Controls	23%	36%	27%	14%	0%	77%	44%
Developing written operating procedures	9%	45%	36%	9%	0%	65%	44%
Process Controls	9%	41%	41%	9%	0%	58%	56%
Recall Plan Development	5%	45%	36%	9%	5%	54%	41%
Allergen Controls	5%	36%	36%	18%	5%	46%	37%
Sanitation Controls	0%	41%	41%	18%	0%	31%	44%

Table 1: Key food safety challenges for small and very small Northeastern food processors
In the 2019 survey, lead instructors identified Validation procedures, Hazard analysis, and verification procedures as the three components of the preventive controls for human foods rule that were most challenging for small and very small processors to understand and implement. In the 2021 survey, state inspectors were most likely to identify these three areas as sources of misunderstanding and non-compliance during inspections.

Initial Results

The most frequently cited way in which respondents work with small and very small processors was as a consultant, within Extension, Other (incubators, and shared kitchens), and Regulators. Most were located in Pennsylvania, New York, Other States outside of the Northeast, and Massachusetts also mostly serving those in the same states. The most often cited food products were

Ready-to-eat foods, Other, Fruits and Vegetables, Acidified Canned Foods, Confections/Candy/Chocolate, Cereals, Bread and Baked Goods, Condiments, 100% Juice Products, and Beverages other than 100% Juice. The most often cited business sizes were Small and Very Small Business, Farms expanding to sell value added products, and Business not yet selling products. Most respondents said that working with food processors was their main or partial responsibility or they referred processors to another subject matter expert. Over Two thirds (68%) thought that their level of knowledge of regulatory requirements was above average.

Respondents believed that most small and very small food processors (97%) had none to average awareness of PCHF requirements (Table 1). They also thought that most of the processors (79%) had average or less than average knowledge of the basic food safety and sanitation procedures, with nearly one-third (32%) having average knowledge. Respondents also believed that most small and very small food processors (92%) had no to average awareness of risk-based PCHF requirements for writing a food safety plan.

74% of responders felt that “Lack of awareness and understanding of the regulation” was very or extremely challenging. For “Lack of scientific knowledge of potential hazards” and “Lack of knowledge regarding costs of implementation” the percentage of very and extremely challenging responses were 69% and 60% respectively. 81% of responders felt that “Costs for hiring additional workers to manage a food safety program” was very or extremely challenging. “Costs of paying third party consultants” was the second most challenging with 81% of responders stating that this was very or extremely challenging, but fewer responders stating that this was extremely challenging. 65% of responders felt that” Lack of time for training” was extremely or very challenging for producers. For “Training content does not match training needs (materials too advanced)” and “costs for training classes” the percentage of very and extremely challenging responses were 57% and 56%. Respondents most often said they have taken courses in HACCP, Preventive Controls, GMPs, Serve-Safe, and Sanitation. With respect to being an instructor, most often cited were HACCP, Preventive Controls, and GMPs. 83% of responders felt that GMP training was most helpful. The percentages of very helpful and extremely helpful responses for sanitation training and general HACCP were 82% and 69%, respectively.

Upon further analysis, we found that there were substantial differences between regulators and the general survey responder group, particularly on the value of trainings. The results of the survey were concerning as it suggested that current training content and formats may not be useful for improving the food safety practices and outcomes in small businesses. In the spring of 2021, the NECAFS PCHF team developed a follow-up survey sent to state and federal regulators with a special focus on those who perform inspections of very small businesses that may be covered by the PCHF rule. This second survey was created to better identify key areas of non-compliance with the new regulation as well as identify key areas for improvement in the region. On analysis of the second survey we were able to receive responses from 39 regulators in the NECAFS region and the results supported an observation from the first survey. The sections of the PCQI training that instructors reported as the most difficult to teach were also the sections that inspectors reported as common sources of misunderstandings of the regulations as seen in table 1. Selected data from both surveys is included in the appendix. To better serve the needs of small and very small processors in the northeast region NECAFS PCHF portal is currently being redesigned to help direct small businesses in the northeast region find trainings recommended by their extension agents in the area with the hopes of creating an interactive online directory of FSCs to improve food safety outcomes for these valuable businesses in the northeastern United States.

Appendix: Selected Responses from the Needs Assessment sent to Food Safety Communicators

Survey I

Block 2: Background Information on Food Safety Communicators Part 1

Q2.1 Please select what best describes how you work with small and very small food processors

N=71

Other

- Food Testing Lab and Food Safety Consulting
- value added, product development, processing
- Freezing & vak-pak'n plant foods trainer & processor
- Incubator
- Non-profit education
- freezing & vacuum packing trainer
- shared kitchen plus small-batch co-packer
- shared kitchen/small manufacturer
- Kitchen Incubator
- Food Business Incubator, consultant, co-packer
- Shares kitchen and food business incubator
- Small farm Poultry producer and processor
- Rental of kitchen to processors
- Food Recall Insurer

Consulting	16	23%
Extension	16	23%
Other	16	23%
Regulator (State)	13	18%
Retail Food Operations	4	6%
Buyer/Distributor	3	4%
Process Authority	2	3%
Regulator (Federal)	1	1%
Co-Packer	0	0%
Commodity Group	0	0%
Marketing Organization	0	0%
Restaurant	0	0%

Block 3: Background Information on Food Safety Communicators Part 2

Q3.1 What product groups do the small and very small processors you work with process (Check all that apply) N=452

Ready-to-eat foods	38
Other	37
Fruits and Vegetables	37
Acidified Canned Foods	36
Confections/Candy/Chocolate	35
Cereals Bread and Baked Goods	35
Condiments	34
100% Juice Products	33
Beverages other than 100% Juice	31
Seafood	21
Dairy Products	20
Spices	18
Soups	18
Oils and Fats	18
Mushrooms	17
Alcoholic Beverages	14
Sprouts	10
Low Acid Canned Foods	0

Other

- Food Packaging
- coffee
- meat and poultry
- Peanuts and Tree nuts
- cooked whole grains, cooked dry beans, liquids
- wholesale meat; acidified- jars and bottles
- Fermented Foods
- All food types
- variety
- frozen & vak-pak'd vegetables, fruits, cooked whole grains and dry beans and sauces
- Pastured Poultry
- All of the above

Q3.2 What food business size categories best describe all of the food processing businesses you work with? (Check all that apply) N=211

Small Business (500 or fewer employees)	50
Very Small Business (sales less than $1m)	47
Farms expanding to sell value added products	32
Business not yet selling products	31
Larger Businesses (more than 500 employees)	24
Home Based Businesses	24
Prefer not to answer	3
Don't Know	0

Q3.3 Do you conduct one-on-one or small group advice or assistance activities to help small and very small food processors comply with the PCHF regulation?

N=67

Yes, but it is not my main responsibility	24	36%
No, but I refer processors to other people who do	18	27%
Yes, that is my main responsibility	15	22%
No, I do not actively advise or assist food processors	10	15%

Q3.4 Please rate <u>YOUR</u> level of <u>KNOWLEDGE</u> of the requirements for small and very small food processors in the PCHF rule.

N=65

1 No knowledge	2	3%
2	3	5%
3	3	5%
4 Average or somewhat	13	20%
5	13	20%
6	17	26%
7 Expert	14	22%

Block 4: Awareness, Knowledge, & Progress Towards Compliance with PCHF Rule

Q4.1 To what extent do you think the small and very small food processors you work with are <u>AWARE</u> of the requirements in the PCHF rule?

N=64

1 No awareness at all	8	13%
2	18	28%
3	17	27%
4 Average or somewhat aware	19	30%
5	1	2%
6	1	2%
7 Fully aware	0	0%

Q4.2 To what extent do you think the small and very small food processors you work with are KNOWLEDGEABLE of the basic food safety and sanitation procedures necessary to produce safe foods required of all food processors, known as Good Manufacturing Practices (GMP).

N=62

1 No knowledge at all	1	2%
2	8	13%
3	20	32%
4 Average or somewhat knowledgeable	20	32%
5	7	11%
6	4	6%
7 Completely knowledgeable	2	3%

Q4.3 To what extent do you think the small and very small food processors you work with are KNOWLEDGEABLE of risk-based preventive controls approaches for conducting a hazard analysis, implementing preventive controls for potential food safety hazards, and writing a food safety plan (such as HACCP or HARPC).

N=63

1 No knowledge at all	7	11%
2	26	41%
3	13	21%
4 Average or somewhat knowledgeable	12	19%
5	4	6%
6	1	2%
7 Completely knowledgeable	0	0%

Block 5: Perception of Processor Challenges and Barriers

Q5.1 How important do you think the following challenges for achieving PCHF compliance are for small and very small food processors?

Survey answers are sorted below by greatest number of extremely challenging and very challenging responses. The top three responses are bolded.

	Extremely hallenging	Very hallenging	Moderately hallenging	Slightly hallenging	Not hallenging At All	N
Lack of awareness and nderstanding of the regulation	**15 26%**	**27 47%**	**14 25%**	**1 2%**	**0 0%**	**57**
Lack of scientific knowledge of otential hazards	**18 33%**	**20 36%**	**11 20%**	**6 11%**	**0 0%**	**55**

Lack of knowledge garding costs of mplementation	**9 16%**	**24 44%**	**17 31%**	**5 9%**	**0 0%**	**55**
Lack of nderstanding the financial and business risks sociated with non-compliance	10 18%	23 40%	17 30%	5 9%	2 4%	57
Lack of expertise n record keeping nd documentation	16 31%	15 29%	16 31%	5 10%	0 0%	52
Lack of nowledge of basic food safety and processing principles	11 20%	10 18%	25 45%	8 15%	1 2%	55
Lack of ppreciation of the importance of producing safe foods	7 12%	8 14%	14 25%	20 35%	8 14%	57

Q5.2 How important do you think the following <u>MONETARY</u> challenges for achieving PCHF compliance will be for small and very small food processors?

	Extremely challenging	Very hallenging	Moderately challenging	Slightly hallenging	Not hallenging at all	N
Costs for iring additional workers to nanage a food afety program	**25 44%**	**21 37%**	**10 18%**	**1 2%**	**0 0%**	**57**
Costs for ying third party consultants	**22 39%**	**24 42%**	**9 16%**	**2 4%**	**0 0%**	**57**
Costs for grading facilities and equipment	21 37%	17 30%	18 32%	1 2%	0 0%	57
Costs of training and enforcing ompliance with rrent employees	11 19%	25 44%	14 25%	6 11%	1 2%	57

Q5.3 How important do you think the following TRAINING challenges for achieving PCHF compliance will be for small and very small food processors?

Survey answers are sorted below by greatest number of extremely challenging and very challenging responses. The top three responses are bolded.

	Extremely hallenging	Very hallenging	Moderately challenging	Slightly hallenging	Not hallenging at all	N
Lack of time for training	**14 25%**	**23 40%**	**13 23%**	**6 11%**	**1 2%**	**57**
Training ntent does not natch training eds (materials oo advanced)	**15 27%**	**17 30%**	**15 27%**	**7 13%**	**2 4%**	**56**
Costs for aining classes	**10 18%**	**22 39%**	**16 28%**	**9 16%**	**0 0%**	**57**
Difficulty in finding nearby raining classes	12 21%	16 28%	18 32%	8 14%	3 5%	57
Training content loes not match training needs (materials too general)	5 9%	17 30%	14 25%	13 23%	7 13%	56
Training content does not adequately engage adult earning needs and styles	10 18%	11 20%	18 32%	12 21%	5 9%	56

5.4 Have you taken or been an instructor for any of the following food safety courses? (Check all that apply)

	Have taken this course	Have been an instructor for this course
HACCP - General	38	24
Preventive Controls Alliance recognized curriculum	38	22
Good Manufacturing Practices training	35	23

Serve-Safe restaurant training	32	14
Sanitation training	31	14
Allergen Management Training	28	13
Better Process Control School	28	8
Produce Safety Alliance recognized curriculum	27	8
Food defense	23	9
HACCP - Juice	19	7
HACCP - Seafood	17	6
HACCP - Meat and Poultry	16	9
Other food safety courses, Please list:	11	8

Other (Have Taken this course/Have been an instructor for this course, Answers same for both)

- HAACP for retail- NCSU
- Recall
- FDA courses for Produce & Food Inspection credentials
- Employed/owned FDA inspected facility
- HACCP, PrimusGFS Instructor
- FSVP
- Basic Food Microbiology
- SURE Food Safety
- SURE HACCP
- ISVA
- FSVP
- too many to list here
- HACCP - seafood
- FSPCA
- too many
- Dairy HACCP
- Internal Auditing
- Preventive Controls for Regulators
- PIC
- NRFSP
- FSVP

- IA
- Food Recalls
- Allergen awareness

Q5.5 How effective do you think these courses have been, or would be, for helping small and very small food processors comply with the PCHF rule?

Survey answers are sorted below by greatest number of “5, very helpful” and “4” responses. The top three responses are bolded.

	1 not helpful at all	**2**	**3 somewhat helpful**	**4**	**5 very helpful**	**Do not Know**	**N**
Good Manufacturing Practices training	**0 0%**	**2 4%**	**4 8%**	**6 12%**	**37 71%**	**3 6%**	**52**
Sanitation training	**0 0%**	**1 2%**	**6 13%**	**8 18%**	**29 64%**	**1 2%**	**45**
HACCP - General	**0 0%**	**5 9%**	**6 11%**	**11 20%**	**26 48%**	**6 11%**	**54**
Preventive Controls Alliance recognized curriculum	0 0%	3 6%	8 15%	8 15%	25 47%	9 17%	53
Produce Safety Alliance recognized curriculum	2 4%	7 13%	8 15%	9 17%	18 33%	10 19%	54
Better Process Control School	3 6%	6 12%	11 22%	8 16%	15 29%	8 16%	51
Serve-Safe restaurant training	5 9%	9 16%	12 21%	8 14%	15 27%	7 13%	56
Food defense	2 4%	3 6%	14 27%	6 12%	16 31%	11 21%	52
HACCP - Seafood	5 10%	4 8%	6 13%	7 15%	12 25%	14 29%	48
HACCP - Juice	4 8%	7 14%	8 16%	7 14%	11 22%	13 26%	50
HACCP - Meat and Poultry	6 13%	4 9%	5 11%	7 15%	11 23%	14 30%	47
Allergen Management Training	0 0%	3 8%	16 43%	5 14%	5 14%	8 22%	37

Other food safety courses, Please list: (below)	0	0	1	1	8	10

Very Helpful: *UNH extension:" Food Safety Planning NH Commercial Food Processors" "Regulatory quirements", "Proper risk assessment", "Food Safety 1" "Basic Food Microbiology"*

Somewhat Helpful: Recall, arm and Facility Plan velopment"

Other: "FSVP"

Block 6: Processor Resource Needs

Q6.1 When contacted for further assistance in understanding the requirements of the regulation and developing a food safety plan, what <u>types of information</u> are small and small and very small food looking for? (Check all that apply)

N=250

Template or model PCQI food safety plans	41
Where to find general food safety training classes	36
Process authority contact information	33
Where to find a PCQI course	29
Private consultant contact information	26
University extension contact information	25
State regulator contact information	25
Federal regulator contact information	13
Legal advice	13
Other, please list	9

Other

- "Where to find qualified help"
- How to understand what their copacker is doing
- "Writing HACCP or Food Safety Plans, Process Review; Regulatory Guidance Documents"
- "Customization of food safety plans"

- “Want "someone to write 'it' for me and just tell me what I have to do to comply”
- “many do not do well with the learning style that the mandated curriculums are formatted for”
- “additional languages for classes”
- “Help understanding if they are required to comply”
- “They don't really even know to ask for any of these resources specifically. They simply ask "what do I need to know or do to sell this food item?"”

Q6.2 Please list any other types of information you have found to be beneficial for small and very small food processors

- Hazard guide,
- A one-stop facility providing answers to every question raised by a new food entrepreneur
- basic introduction to food safety
- Regulatory Guidance Documents
- Record keeping, internal auditing, employee training,
- Hands on, lots of opportunity for networking and idea sharing; consideration of a vast number of growers/processors part-time engaged
- CGMPs
- They need process specific information and understanding
- Everything from sourcing ingredients and packaging from reputable suppliers to in depth GMP training
- www.vac-smart.com
- More options for different learning styles; more visuals for people who may not have advanced reading skills
- Understanding of the FDA Hazard Guide, how to do a hazard analysis and understanding of critical control measures needed.
- FDA Guidance Documents for Self-Attestation
- We teach very basic 101 food product classes.
- Simply introducing them to FDA regulations and label guidelines is our first step.
- Online templates for developing a food safety plan
- Regional FSMA training centers, Processing Centers, Incubators/Accelerators
- There seems to be some confusion about non-profits needing to be compliant
- Clarity of regulations Federal/State/Local
- One-on-one hand holding is huge!

Q6.3 Based on your experience, what delivery methods are most helpful to small and very small food processors for understanding the requirements of the regulation and for writing a food safety (Check all that apply) N=232

One-on-one advice and consultation	51
Training workshops	46
Printed fact sheets and articles	31

Videos	26
Webinars	26
Web sites	21
Speaking engagements at conferences or meetings	18
Newsletters	5
Social media	4
Other, Please list	4

Other:

- PowerPoint trainings for specific foods
- Hands-on
- Word of mouth
- Anything available at on-demand works best

Q6.4 Please list any other resources you have sound to be beneficial for small and medium food processors

- Engaging workers during food safety training
- Hands on SOP and SSOP writing and other interactive strategies within a workshop
- Industry meetings/ associations
- UMASS extension, Cornell University
- Our incubator staff, our FDA and local health inspectors, university extension programs,
- The library system, and KCSourceLink resources
- Visit smaller processors that have gone through the process of being compliant

Block 7: Value of FSPCA PCQI Course

Q7.1 Are you recognized by the Food Safety and Preventive Controls Alliance as a Lead Instructor?

N=63

Yes	26	41%
No	1	2%
No, but I help teach the course with a lead instructor	36	57%

Questions 7.2 to 7.8 were asked of FSPCA lead instructors only.

Q7.2 When teaching the PCQI course, which sections do you think are more challenging for small and very small food processors to understand and implement?

Survey answers are sorted below by greatest number of extremely challenging and very challenging responses. The top three responses are bolded.

	Extremely :hallenging	Very hallenging	Moderately challenging	Slightly hallenging	Not hallenging at all	N
Validation Procedures	**7 30%**	**12 52%**	**3 13%**	**1 4%**	**0 0%**	**23**
Hazard Analysis	**7 32%**	**10 45%**	**5 23%**	**0 0%**	**0 0%**	**22**
Verification Procedures	**2 9%**	**12 55%**	**6 27%**	**2 9%**	**0 0%**	**22**
Supply Chain Controls	5 23%	8 36%	6 27%	3 14%	0 0%	22
Developing ritten operating procedures	2 9%	10 45%	8 36%	2 9%	0 0%	22
Process Controls	2 9%	9 41%	9 41%	2 9%	0 0%	22
Recall Plan Development	1 5%	10 45%	8 36%	2 9%	1 5%	22
Allergen Controls	1 5%	8 36%	8 36%	4 18%	1 5%	22
Sanitation Controls	0 0%	9 41%	9 41%	4 18%	0 0%	22

Q7.3 Among small and very small food processors who have taken the PCQI course, to what extent do you think they will make progress on developing a food safety system and writing a food safety plan?

N=22

1 Have not started at all	0	0%
2	1	5%
3	6	27%
4 Started to write a plan that is only moderately complete	11	50%
5	2	9%
6	2	9%
7 Completed a plan that is fully operational	0	0%

Q7.4 To what extent do you feel that the small and very small food processors would benefit from taking one or more of the following courses before taking the PCQI course.

Survey answers are sorted below by greatest number of “strong benefit” and “very strong benefit…” responses. The top three responses are bolded.

	No benefit	Slight benefit	Moderate benefit	Strong benefit	Very strong benefit – Definitely needed	N
A course on risk-based identification of food safety hazards	**0 0%**	**1 5%**	**3 14%**	**12 57%**	**5 24%**	**21**
A Good Manufacturing Practices (GMP) course	**0 0%**	**0 0%**	**6 29%**	**11 52%**	**4 19%**	**21**
A general HACCP course	**0 0%**	**4 19%**	**4 19%**	**9 43%**	**4 19%**	**21**
A food science and technology course	1 5%	3 14%	9 43%	7 33%	1 5%	21
A course in food microbiology	3 14%	3 14%	9 43%	4 19%	2 10%	21
Other course: please list *Employee Food Safety Trainings	0	0	0	0	1*	1

Q7.5 Do you use the evaluation tool provided by the PC Alliance at the end of each course you teach?

N=21

Yes, I use only the PC Alliance evaluation form	4	19%
Yes, I use the PC Alliance form and I hand out my own evaluation form	7	33%
No, I do not use the PC Alliance form, but I hand out my own evaluation ms	9	43%
No, I do not use any evaluation methods	1	5%

Q7.6 How useful do you think the evaluation forms provided by the PC Alliance are for assessing knowledge gained during the FSPCA course?

N=18

1 Not at all useful	3	17%
2	0	0%
3	1	6%
4 Somewhat useful	9	50%
5	3	17%
6	2	11%
7 Extremely useful	0	0%

Q7.7 Do you conduct any follow-up surveys to determine if small or very small food processors have taken action to develop and implement a preventive controls food safety plan?

N=21

Yes	1	5%
No	20	95%

Q7.8 Would you be willing to incorporate a standardized evaluation form developed for the region, or to include additional questions to your own evaluation tool, to help NECAFS collect data on small and very small food processors' understanding of the rule and intent to develop food safety plans?

N=21

Yes	13	62%
Maybe	7	33%
No	1	5%

End of Survey I

Survey II

Block 2: Background Information on Food Safety Communicators

Q2.1 "Please select what best describes how you work with small and very small food processors:

N=42

Other

- Retired inspector
- USDA Egg inspector

Regulator (State)	39	93%
Other	2	5%
Consulting	1	2%
Buyer/Distributor	0	0%
Commodity Group	0	0%
Co-Packer	0	0%
Extension	0	0%
Marketing Organization	0	0%
Process Authority	0	0%
Regulator (Federal)	0	0%
Restaurant	0	0%
Retail Food Operations	0	0%

Q2.2 In what zip code are you based?

Results not presented here, match answers for question 2.3

Q2.3 In what state or district are the small and very small processors you work with located?

CT	3
DE	3
ME	4
MD	3
MA	1
NH	1
NJ	3
NY	3
PA	8
RI	2
VT	6
WV	2

Q2.4 What product groups do the small and very small processors you work with process (Check all that apply)? N=270

Other	23
Dairy Products	22
Cereals Bread and Baked Goods	20
100% Juice Products	20
Acidified Canned Foods	19
Confections/Candy/Chocolate	19
Ready-to-eat foods	18
Condiments	17
Beverages other than 100% Juice	17
Fruits and Vegetables	16
Seafood	15
Spices	15
Mushrooms	11
Soups	10
Sprouts	10
Oils and Fats	9
Alcoholic Beverages	9
Low Acid Canned Foods	0

Other

- Meat and poultry (2)
- Products infused with cannabinoids
- Maple syrup
- Frozen meat from farm raised animals
- eggs

Q2.5 Do you conduct one-on-one or small group advice or assistance activities to help small and very small food processors comply with the PCHF regulation?
N=39

Yes, but it is not my main responsibility	15	38%
No, but I refer processors to other people who do	14	26%
No, I do not actively advise or assist food processors	9	23%
Yes, that is my main responsibility	1	3%

Block 3: Awareness, Knowledge, & Progress Towards Compliance with PCHF Rule

Q3.1 To what extent do you think the small and very small food processors you work with are AWARE of the requirements in the PCHF rule?

N=39

1 No awareness at all	5	13%
2	10	26%
3	10	26%
4 Average or somewhat aware	7	18%
5	1	3%
6	2	5%
7 Fully aware	4	10%

Q3.2 To what extent do you think the small and very small food processors you work with are KNOWLEDGEABLE of the basic food safety and sanitation procedures necessary to produce safe foods required of all food processors, known as Good Manufacturing Practices (GMP).

N=39

1 No knowledge at all	0	0%
2	5	13%
3	5	13%
4 Average or somewhat knowledgeable	17	44%
5	7	18%
6	3	8%
7 Completely knowledgeable	2	5%

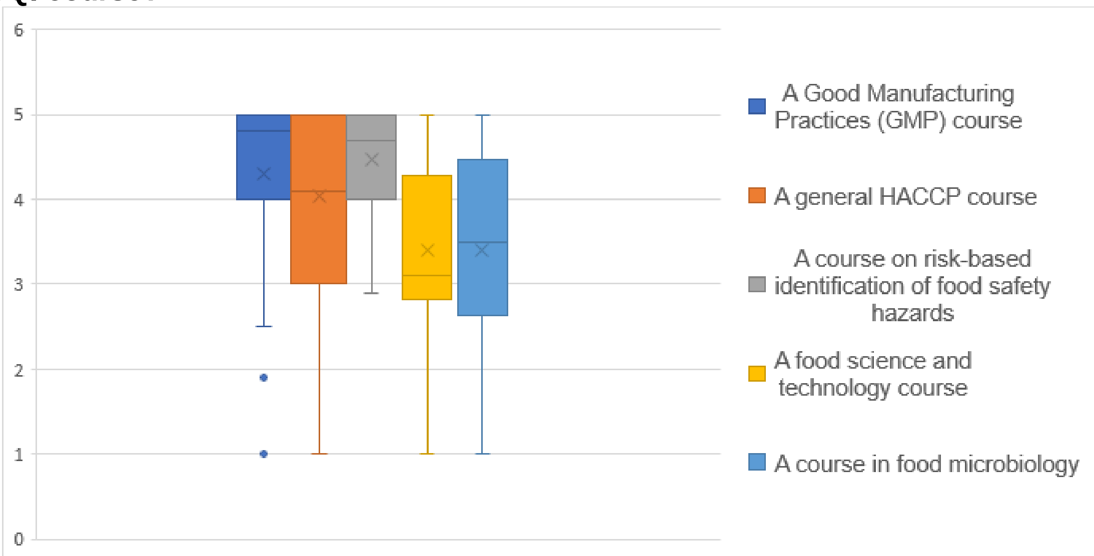
Q4.3 To what extent do you think the small and very small food processors you work with are KNOWLEDGEABLE of risk-based preventive controls approaches for conducting a hazard analysis, implementing preventive controls for potential food safety hazards, and writing a food safety plan (such as HACCP or HARPC).

N=39

1 No knowledge at all	3	8%
2	14	36%
3	10	26%
4 Average or somewhat knowledgeable	4	10%
5	2	5%
6	6	15%
7 Completely knowledgeable	7	18%

Block 4: Processor Training Needs

Q4.1 To what extent do you feel that small and very small food processors would benefit from taking one or more of the following courses before taking the PCQI course?



Q4.2 Have you taken or been an instructor for any of the following food safety courses? (Check all that apply)

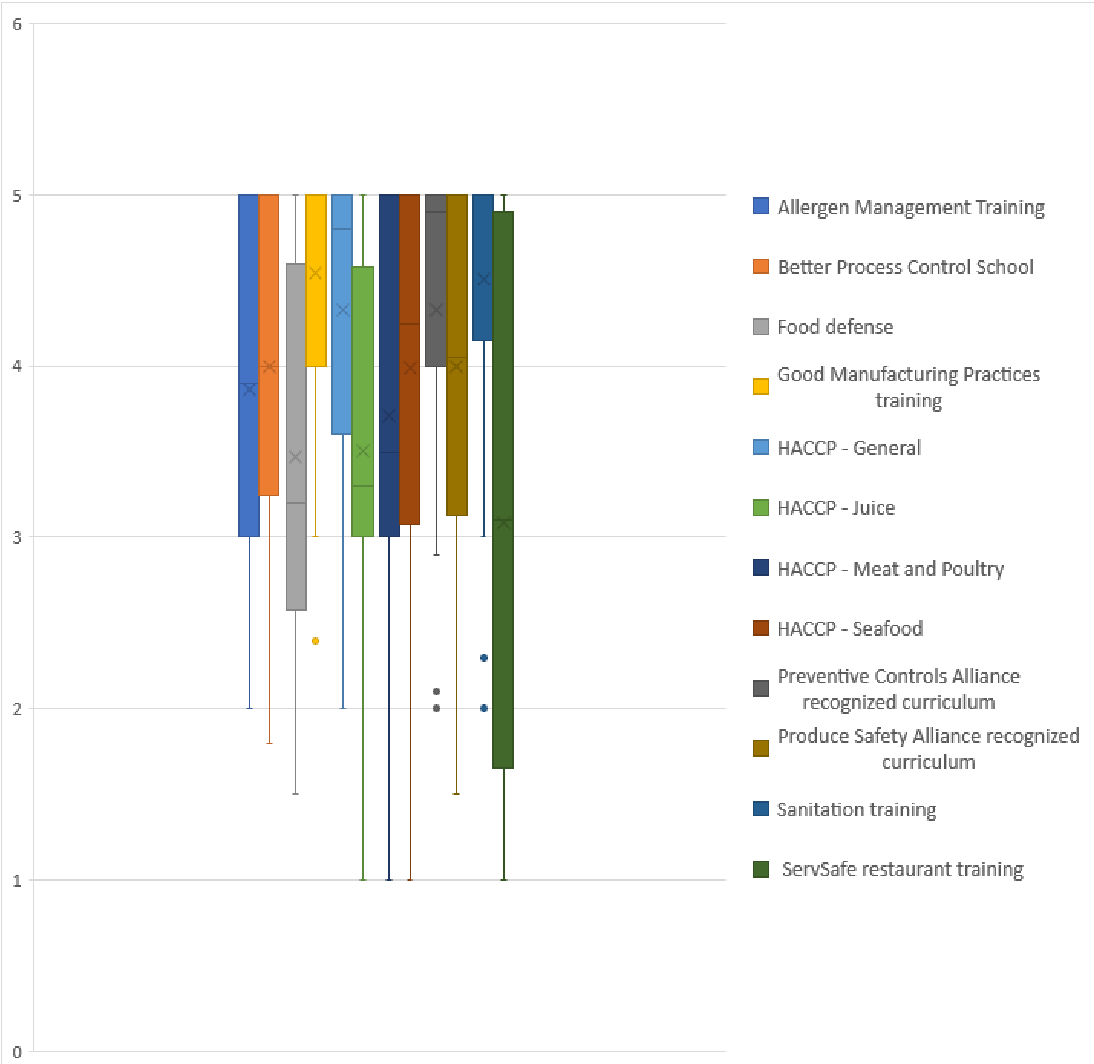
	Have taken this course	Have been an instructor for this course	Have referred processors to this course
Better Process Control School	**15**	**0**	**20**
Serve-Safe restaurant training	**17**	**6**	**19**
Good Manufacturing Practices training	**23**	**3**	**18**
Preventive Controls Alliance recognized curriculum	23	0	17
HACCP - General	27	4	16
Sanitation training	26	5	13
HACCP - Seafood	17	3	10
Produce Safety Alliance recognized curriculum	12	1	9
Food defense	21	2	9

HACCP - Juice	15	2	8
Allergen Management Training	18	1	7
HACCP - Meat and Poultry	9	1	6
Other food safety courses, Please list:	6	2	2

Other courses taken (courses taught indicated by asterisk, courses referred indicated by carat)

- Shellfish HACCP/Seafood Sanitation Control Procedures*^
- ISSC Shellfish* Standardization
- EPI Ready
- FDA courses offered through FDA OTED
- Traceback Investigations
- College level

Q4.3 How effective do you think these courses have been, or would be, for helping small and very small food processors comply with the PCHF rule?



Block 5: Processor Resource Needs

Q5.1 When contacted for further assistance in understanding the requirements of the regulation and developing a food safety plan, what types of information are small and small and very small food looking for? (Check all that apply) N=171

Where to find a PCQI course	30
Template or model PCQI food safety plans	26
State regulator contact information	24

Where to find general food safety training classes	20
Process authority contact information	18
University extension contact information	18
Private consultant contact information	15
Federal regulator contact information	11
Legal advice	5
Other, please list	4

Other

- They usually just want you to give them the answers. Like their consultant.
- labeling expertise
- available local HACCP training courses
- Templates and information on record keeping requirements

Q5.2 Please list any other types of information you have found to be beneficial for small and very small food processors

- Anything they can use without having to think
- Facility Design and Equipment Construction
- Field tickets with Public health statement on them
- How to get the attestation.
- Internet sites
- knowledge of the federal regulations
- Labeling requirements
- On site walk through's and facility specific guidance

Q5.3 Based on your experience, what delivery methods are most helpful to small and very small food processors for understanding the requirements of the regulation and for writing a food safety (Check all that apply) -- N=143

Training workshops	32
One-on-one advice and consultation	31
Printed fact sheets and articles	23
Webinars	23
Web sites	17
Videos	12
Social media	3
Other, Please list	2
Speaking engagements at conferences or meetings	0
Newsletters	0

Other:

- templates for the product they are producing
- Combination with onsite/hands on training with practical application to ecific products manufactured

Q5.4 Please list any other resources you have sound to be beneficial for small and medium food processors

- Virtual Courses
- Training that is built into routine inspections
- State University Extension
- Small group meetings of operators who are manufacturing similar products · interaction as well providing handouts and/or direction to online information has en most valuable. The meetings should be both informative as well directional with pport as needed (problem solving as a group)

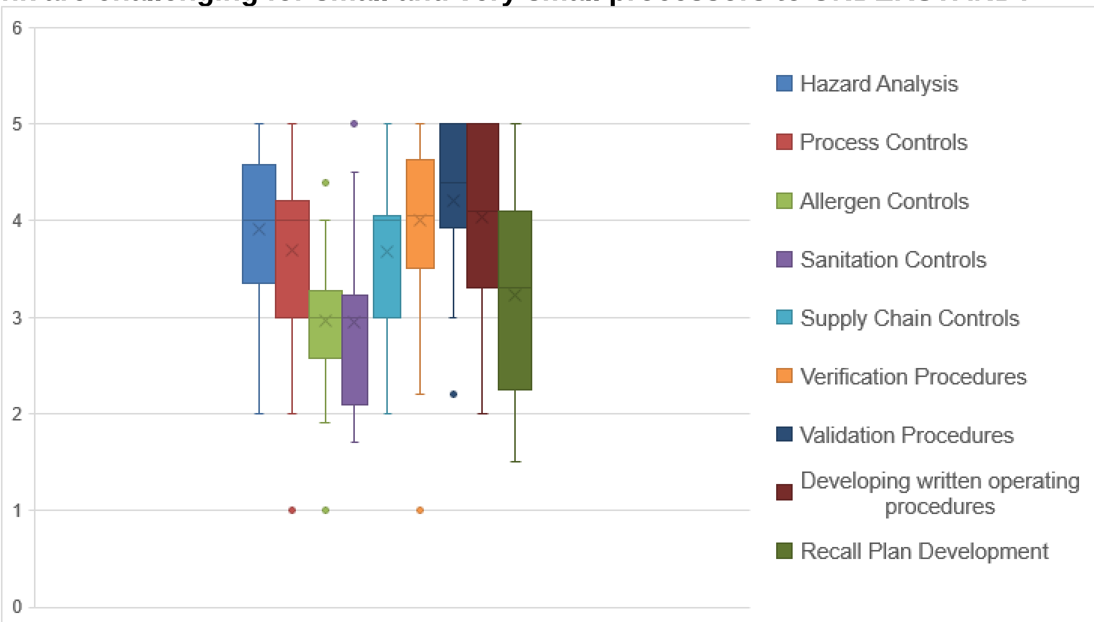
Block 6: Inspection Observations

Q6.1 Do you perform inspections, reviews, or audits of small or very small food processing facilities?

N=39

Yes	35	90%
No	4	10%

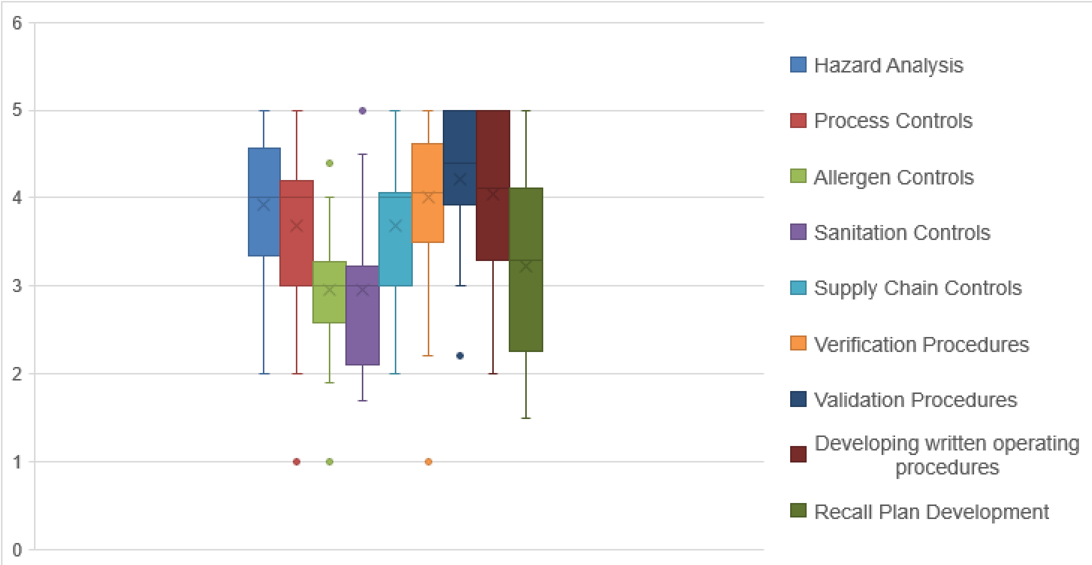
Q6.2 When performing inspections, which sections of the PCHF rule do you think are challenging for small and very small processors to UNDERSTAND?



Q6.2 Please provide any examples you have of processors struggling to understand the PCHF rule.

- Verification vs Validation
- The Amish and some Mennonite communities have low education level and very little to no scientific education.
- Please provide any examples you have of processors struggling to understand the PCHF rule.
- Most of them do. They often barely know GMPs and basic sanitation and hygiene practices. It's like asking someone to learn how to make a space ship before learning how to properly make a canoe.
- Many processors have controls but do not understand the hazards they are controlling, nor how to effectively do verification or validation.
- Lack of understanding the difference between verification and validation for acidified foods/LACF for small operators
- I think the overall process of development of the food safety plan especially accessing and the hazard analysis. There is also a major barrier on the understanding and implementation of environmental monitoring and where or not they need to have it based on their hazards.
- Fresh Juice and Acidified Foods and some small HPP operators
- 21 CFR part 117 is challenging for small processors to understand

Q6.4 When performing inspections, which sections of the PCHF rule do you think are challenging for small and very small processors to IMPLEMENT?



Q6.5 Please provide any examples you have of processors struggling to implement the PCHF rule.

- Understanding the exemptions, and modification sections
- they don't know how to implement and keep records
- some processors have more knowledge than others. responses reflect that. It's not specific to a type of industry, rather an individual.
- Please provide any examples you have of processors struggling to implement the PCHF rule.
- Most have trouble just getting through the Hazard analysis
- Minimal understanding of Microbiology of pathogens associated with particular process and difference between Validation and Verification
- Many operators have no validation of their current processes, and see no need for it.
- Lack of basic microbiology impedes many operators and the PCHF rule seems very complicated and made for larger operations
- I think for most small and very small processors, finding the staff to complete and maintain the requirements particularly the record keeping requirements is challenging.

Q6.6 What common misunderstandings did facilities have about the PCHF requirements during inspections?

Q6.8 What were the most common noncompliance observations or issues you observed in facilities during inspections?

	6.6: Misunderstandings		6.8: Noncompliance	
	n=26	%	n=27	%
Validation Procedures	**23**	**88%**	**17**	**63%**
Hazard Analysis	**23**	**88%**	**14**	**52%**
Verification Procedures	**24**	**92%**	**15**	**56%**

Supply Chain Controls	20	77%	12	44%
Developing written operating procedures	17	65%	12	44%
Process Controls	15	58%	15	56%
Recall Plan Development	14	54%	11	41%
Allergen Controls	12	46%	10	37%
Sanitation Controls	8	31%	12	44%

Q6.7 Please list the top one or two most common misunderstandings so that we can identify any leading commonalities across the region.

- Allergen control/prevention procedures when making multiple bakery/confectionary products and failure to have any written documentation available for reference
- Did not understand process controls.
- do not understand Hazard Analysis development and how to implement process controls
- Hazard Analysis
- hazard analysis and validation procedures
- I think the common misunderstanding is whether or not they have to have a written food safety plan and whether or not they have to have an environmental monitoring program.
- Including all ingredients in the hazard analysis, validation vs verification
- Process controls and Supply chain controls
- Small facilities have SOPs, they just don't have them written down so there's no way to track them.
- supply chain, verification
- Under the educate before you regulate umbrella, we have only conducted two surveys on progress and the items above were noted.
- verification - lack of any
- Verification / Validation procedures
- Ways to control allergens in their products.

End of Survey II

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