ABSTRACT

Title of Dissertation:	ANTIBACTERIAL MECHANISM OF PLANT-DERIVED PHENOLICS AGAINST *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM
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Salmonella enterica serovar Typhimurium (ST) remain one of the main bacterial pathogens responsible for illnesses, hospitalizations, and deaths in the USA. Its ubiquitous prevalence in nature, invasive pattern and increasing antibiotic resistance make it a public health threat, warranting the discovery of novel antimicrobials that can be implemented as either treatments or as forms of control. Plant-derived compounds have been proposed as potential antimicrobials that can be used against gram-negative pathogens, with phenolic acids being of interest for their

prevalence in nature and bioactivity. This research studied the effects of gallic acid (GA), protocatechuic acid (PA) and vanillic acid (VA) against ST. Findings showed these compounds to be able to inhibit bacterial growth *in vitro*, while also showing a reduction in the expression of key virulence genes, without inducing resistance over multiple passages. Further studies using a human epithelial cell line for studying host-pathogen interactions, showed their capability to reduce the number of ST that were able to invade the host cells. Further studies were performed in cecal fluid to test their potency in more complex environments and assess their effects on the microbiome. When in cecal fluid, compounds showed a reduced inhibitory potency compared to *in vitro*, but still exerted antimicrobial pressure against ST. When analyzing relative abundance of other bacteria through 16S-rRNA gene sequencing, there was an overall decrease in the Protobacteria phylum, while no significant negative effect was seen for other phyla like that of the Firmicutes and Actinobacteria. Experiments to determine the mechanism of action against ST showed these phenolic acids to permeabilize the cell plasma membrane, in addition to reducing cell wall synthesis. Scanning electron microscopy showed treated bacteria to have dents at the polar ends of the cell, while others were found in a duplet formation, suggesting further disruption of specific bacterial functions associated to cell division and structure. These findings suggest that despite their similarities, these compounds are capable of exerting different types of antimicrobial pressure against ST that could better inform their future use as control measures against ST, and their potential use case based on the desired outcome.

ANTIBACTERIAL MECHANISM OF PLANT-DERIVED PHENOLICS AGAINST *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

by

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Preface

This dissertation is original, independent work by the author, Zabdiel Alvarado-Martinez under supervision of Dr. Debabrata Biswas.

Dedication

To my parents and grandparents, who's sacrifice throughout the years opened doors for future generations, and who always lovingly supported, instructed, and guided me, teaching me the values of work, education, and patience.

To my wife, who inspires me every day.

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Scholarly articles

Findings from this dissertation have been published in the following peer-reviewed research articles:

1. **Alvarado-Martinez, Z.**, Bravo, P., Kennedy, N.-F., Krishna, M., Hussain, S., Young, A. C., & Biswas, D. (2020). Antimicrobial and Antivirulence Impacts of Phenolics on Salmonella Enterica Serovar Typhimurium. Antibiotics, 9(10), 668. MDPI AG. Retrieved from http://dx.doi.org/10.3390/antibiotics9100668

2. **Alvarado-Martinez, Z**., Tabashsum, Z., Aditya, A., Suh, G., Wall, M., Hshieh, K., & Biswas, D. (2023). Purified Plant-Derived Phenolic Acids Inhibit Salmonella Typhimurium without Alteration of Microbiota in a Simulated Chicken Cecum Condition. Microorganisms, 11(4), 957. https://doi.org/10.3390/microorganisms11040957

Chapter 1: Literature review

Worldwide morbidity and mortality of Salmonella-related disease

Death by enteric infection is comprised of those caused by diarrheal disease, typhoid and paratyphoid fever, invasive non-typhoidal salmonellosis, and other intestinal infectious disease, which together make up the third most common cause of communicable death in the world with approximately 1,766,000 deaths (Roth et al., 2018). Confirmed *Salmonella* related deaths accounted for approximately 330,900 of those deaths, however, when taking cases of non-typhoidal *Salmonella* alone, it reported an approximate toll of 77,500 deaths, when including deaths previously attributed to suffers of HIV that developed invasive salmonellosis (Stanaway et al., 2019). Another study suggests that deaths by non-typhoidal *Salmonella* range around 155,000 per year, when taking into account those caused by gastroenteritis and diarrheal disease (Majowicz et al., 2010a). Studies on foodborne illness attribution at a national level found that though *Salmonella* can be transmitted through many mediums, the most common sources of contamination were eggs, poultry products and produce (fruits and vegetables) (Hoffmann et al., 2017).

Deaths and illness because of *Salmonella* are seen mostly in developing countries, due to issues of sanitation, proximity to reservoir animals, food preservation and other conditions that aggravate the infection like malnutrition, dehydration and

immunological deficiencies (Graham et al., 2000). People in developing countries that lack sufficient potable water are particularly afflicted by typhoidal strains of *Salmonella* which cause typhoid fever, and are also known to be highly invasive, being capable of easily infecting and spreading through the bloodstream, causing bacteremia, which explains its frequent isolation from blood samples from these areas (Andoh et al., 2017). Strains belonging to typhoidal *Salmonella* (TS) account for a great amount of death, but they are mostly transmitted through water, making them uncommon in areas with proper water treatment methods, however, non-typhoidal *Salmonella* (NTS) strains remain widespread across the world, and play a considerable role as major foodborne pathogens (Ferrari et al., 2019). Illness by NTS therefore contributes to a significant portion of hospitalizations and death, making it the most pervasive bacterial foodborne pathogen in developed countries, and being a source of great economic loss (Fletcher et al., 2013).

Though the most numerous accounts of illness can be attributed to *Salmonella enterica* as the etiological agent, this species of bacteria counts with a vast amount of serovars, some more pervasive than others. According to the CDC, more than 2,500 *Salmonella enterica* serovars have been identified, however only around 32 of those serovars are of clinical significance to human health. The importance of specific serovars is mostly based on their capability to infect a human host and cause prolonged disease, as well as its transmission route, animal host reservoir range, survival, environmental prevalence, and pattern of antibiotic resistance (CDC, 2020). Of the two serovar classifications (TS and NTS) the majority of identified *Salmonella* serovars are

found within the NTS group, which are mostly associated with foodborne and zoonotic transmission, since they can be found and isolated from a wide array of animal hosts. Though TS is considered to be better adapted to survive in humans as a host and is known to cause invasive infection, it has a limited host range, making these serovars less widespread than NTS in developing countries (R. A. Cheng et al., 2019). This same study found that based on their contribution to the proportion of salmonellosis that they cause at a worldwide level, the most common, and therefor clinically relevant, NTS strains are Enteritidis and Typhimurium. Though a meta-analysis of worldwide *Salmonella* epidemiology in animal-based foods identified *Salmonella enterica* serovar Typhimurium (Typhimurium) to have the most cosmopolitan distribution when considering all continents and the vehicles of transmission, which in this study were poultry, pork, beef and seafood, though other mediums of contamination have been reported, such as fruits and vegetables (Ferrari et al., 2019). It is important to note that poultry, pork and their respective products meant for consumption have been reported to be two of the most common reservoirs and sources for isolation of NTS around the world, with Typhimurium being one of the most closely associated to these, even in developed countries (R. A. Cheng et al., 2019; Ferrari et al., 2019).

Enteric disease and salmonellosis in the United States of America

When specifically considering the incidence of domestically acquired foodborne illness in the United States of America (USA), it is still a common occurrence that has been estimated to be responsible for at least 9.4 million cases of illness, 55,961

hospitalizations and 1,351 deaths, which have been confirmingly linked to known pathogens (Scallan, 2011). While the concern on the state of public health is important, the economic burden that is attached to the persistence of this pathogen cannot be overlooked. In 2013, the USDA released a report documenting expenses associated to cost of medical care, hospitalization, loss of productivity and cost of death, adding up to a total economic toll of over 3 billion dollars in the United States alone (USDA, 2013). Of these attributable illnesses, bacteria are the second most common group of etiological agents, surpassed only by viruses, which owe their overwhelming bulk of their cases to Norovirus cases (5,461,731). When evaluating illness attribution to individual pathogens, and excluding Norovirus, the positions for top 5 etiological agents are held by bacteria, with *Salmonella* holding the top position. The same study estimates that *Salmonella* is responsible for 1,027,567 cases of illness (94% of which are associated with to be of foodborne origin), 19,336 hospitalizations (27.2% rate), and 378 deaths (0.5% rate).

Taking the compiled outbreak report in 2017 and the National Outbreak Reporting System (NORS) reveals that *Salmonella* was responsible for 202 of outbreaks across water, food, animal contact, environment, person to person and other undetermined sources, which were associated with 4,886 cases of illness, 853 hospitalizations and 13 deaths (CDC, 2018). Food related sources accounted for the majority of those infections, simultaneously making *Salmonella* the top etiological agent responsible for outbreaks in general, and particularly those from food sources (Dewey-Mattia et al., 2018a). This surveillance report found chicken and fruits to be two of the top five food

sources responsible for outbreaks, both of which were contaminated by *Salmonella*. In a similar fashion, pathogen-food category pairing comparisons of outbreak-associated illness, hospitalization and death find *Salmonella* as the top etiological agent per food pairing. Of the *Salmonella* cases that were successfully serotyped, NTS serovars Enteritidis, Typhimurium and Newport were the top agents responsible for outbreaks, in decreasing order based on cases (28, 15 and 13), though other NTS strains were also contributors to the overall numbers, they individually contribute to a lower degree. When looking at the 2016 to 2019 report from the Foodborne Disease Active Surveillance Network, which takes into account the incidence of infections by pathogens commonly transmitted through food from the 10 USA sites that participate in reporting, NTS was responsible for 8,556 cases of infection (second to *Campylobacter*), as well as 2,430 hospitalizations, 46 deaths, making it the top etiological agent in both of those categories (Tack et al., 2020a). In terms of serovars attribution, the report states that the 3 main ones, in order of incidence per 100,000 population, are currently Eteritidis (2.6), Newport (1.4), Typhimurium (1.3).

The high incidence of Enteritidis and Newport NTS strains can be related to their prevalence in eggs and is positively correlated to their consumption in North America (Feasey et al., 2016). Though Typhimurium is third in the independent reports of illness, it is second in outbreaks across a multitude of sources, including foodborne and from direct contact with animals. A previous study done comparing food commodities and associating them to NTS serovar used a Gini coefficient with percent (%) of outbreaks to illustrate and compare diversity of source with incidence (Jackson et al.,

2013). By using the Gini coefficient the distribution and diversity of outbreaks sources among NTS serovars could be compared to each other, which showed found Typhimurium and Newport to have a lower coefficient value (< 0.6), meaning that they had a wider distribution and diversity of sources, as compared to Enteritidis, which had a larger coefficient (≥ 0.8), meaning an attribution to a much narrower source of infection, mostly egg and poultry products. When evaluating the percentage of outbreaks, the study found Enteritidis to be the highest of the three serovars, followed by Typhimurium and Newport, in order of incidence, for that year. However, when taking into account both of these pieces of data together, it can be concluded that out of these serovars, Typhimurium and Newport were the ones with the most diverse range of sources, but of these two, Typhimurium was the one with the highest incidence of outbreaks; and though Enteritidis was highest in outbreaks, it was lower in diversity. More recent studies show that the wide range of contamination-pairing attribution with Typhimurium has to do greatly with this serovar's capability to adapt to various hosts, in addition to being able to survive after harvest of animal food products and in the environment, increasing its ability to spread to other hosts, as well as other food products, such as produce (Fatica & Schneider, 2011). Some of the most common host include animals intended for consumption, such as poultry (both chicken and turkey), cattle (dairy and beef), and swine, but also those intended as pets, particularly household poultry and turtles (Schutze et al., 1999). Produce like seeded vegetables and fruit (particularly cut fruit) have been crucial sources of Typhimurium outbreaks in recent years (CDC, 2020). Studies involving the serotyping of farm isolates found

Typhimurium to be highly prevalent in the soil of such farms, adding to the ubiquitous nature of this serotype (Peng et al., 2016). In part, the prevalence of NTS across so many environments is also perpetuated by animals like wild rodents and birds, because even if these are not intended for human consumption, they serve as additional reservoirs of the bacteria with the ability to transport it across farms (Andrés-Barranco et al., 2014).

With *S.* Typhimurium being one of the top foodborne pathogens in the US, the risk of infection by an antibiotic resistant strain will continue to be on the rise if alternatives to the conventional methods of control are not developed. The wide host rage and ubiquitous nature of this strain is of particular concern from a public health standpoint, as this not only adds to risk of contracting illness from many sources, it also adds to the risk of increasing development of antibiotic resistance within the strain across multiple environments (Fàbrega & Vila, 2013). In addition to the versatility and high adaptability of *S.* Typhimurium to various environmental and biological challenges, it remains one of the most well-studies NTS serovars and has served as an effective model microorganism for studying NTS salmonellosis (Tsolis et al., 2011). (Garai et al., 2012).

Virulence factors in Salmonella: strategies and mechanisms for survival in a host

Over time and with the help of serotyping, *Salmonella* has been found to have an array of hosts, with some serotypes being more common in some hosts than others, but also an efficient invasive capability. Though some serovars are innately more invasive

than others, the same underlying invasive mechanisms can be found through serotypes as they are mostly conserved (Jajere, 2019a). Likewise, when inside the host, two types of cells have been identified as prime targets for *Salmonella*, those being macrophages and epithelial cells of the intestine (Jennings et al., 2017). Invasion of macrophages is more closely associated with serovars belonging to TS, whereas epithelial cell invasion is mostly associated with NTS, though many NTS strains possess the same genes and invasive factors as TS serovars. TS isolates are mostly found as the etiological agent behind invasive cases of salmonellosis, whereas NTS infections are often self-limiting, resolving themselves with minimal medical intervention. However, populations that are at risk, such as children, the elderly and immunocompromised patients, might require further treatment by antibiotics in order to resolve the disease, since these are more prone to development of invasive infections (CDC, 2019). However, healthy individuals are also at risk of dehydration, and, though less common, are still vulnerable to more invasive strains of NTS, which can lead to them entering the bloodstream and spreading to other tissues, causing bacteremia and further health complications, including reactive arthritis (Mayo clinic, 2019). The mechanisms that allow *Salmonella* to invade particularly epithelial tissue are mediated by the *Salmonella* Pathogenicity Islands (SPI)-1 and 2. SPI-1 specifically codes for multiple protein structures that assemble and together form the type III secretion system (T3SS) (Lou et al., 2019a). T3SS is a needle-like structure anchored in the inner plasma membrane that extends across the peptidoglycan cell wall and outer plasma membrane to the exterior of the bacterial cell, facilitating adherence to host cells, and later used to translocate proteins

from the bacteria to the host-cell's cytoplasm which leads to the manipulation of the host-cell cytoskeleton and initiates intracellular survival (Ohl & Miller, 2001).

Invasion and virulence begins with SPI-1 activation and though regulation of T3SS is a complicated process involving multiple regulators and response to many environmental stressors found in the host's intestine, such as ion concentrations (Mg^{2+}), osmolarity, temperature, pH, nutrient availability, bile salts and host receptors, certain genes play major regulatory roles (Puhar & Sansonetti, 2014). One of such genes is *hilA*, which is a master regulator responsible for expression of crucial operons such as the *inv/spa* and *prg/org*, while in turn *hilA* is regulated by three main proteins called HilD, HilC, and RtsA (D. Lin et al., 2008). Crystallographic studies have showed that T3SS begins at the cytoplasmic level, starting with OrgB, which in turn serves as a scaffold-like structure on which SpaO and later OrgA can be held in place for the anchoring of T3SS to the bacterial cell membrane can begin (B. Hu et al., 2017). The structure for the needle and transport complex of the T3SS is composed of many proteins that, through the use of chaperons, assemble structural proteins InvA and SpaP/Q/R/S, forming the base of the needle found in the inner plasma membrane. The inner rings that comprises the transporter proteins on the base of the needle are formed by PrgJ and supported by inner and outer ring proteins found in the inner and outer plasma membranes of the bacteria, specifically PrgH/K and InvG, respectively. Invasion proteins are transported through the neck of T3SS, which extends outwards from the cell and is comprised by PrgI. Contact with the host cell is established through the formation of ring structures composed of SipB/C/D, which are found at the tip of

the T3SS needle, with SipB/C crossing the host membrane to allow for transport and entry of virulence related proteins into the host-cell cytoplasm (Lou et al., 2019a).

Some of the most important proteins that are transported through the T3SS and aid in the later stages of invasion epithelial cell invasion are SpiA, SopE and SptP which are known to affect cytoskeletal arrangement (Ohl & Miller, 2001). SpiA specifically binds directly to the actin in the cytoplasm of the host-cell (C. A. Lee et al., 2000). SopE and SptP are active at different times of invasion through manipulation of GTP binding protein of the Rho family, which regulate gene transcription related to the actin cytoskeleton. This cytoskeletal manipulation will lead to rearrangement of the host-cell structure causing ruffling of the host-cell, which is a crucial step in the invasive process that the bacterial cell uses as it is engulfed into the host-cell, initiating the intracellular life cycle of invasive NTS and activation of the genes of SPI-2, which aid intracellular survival (Waterman & Holden, 2003).

In contrast to the many structural proteins encoded in SPI-1, many of the proteins that have been found encoded in SPI-2 are thought to be enzymes and adaptor proteins that bind to carry out effector functions (Jennings et al., 2017). These are designed to aid in intracellular survival of NTS. Once the bacteria enters the host-cell, it remains inside a *Salmonella*-containing vacuole (SCV), which has been found to be an important resistance structure that protects against cytoplasmic factors that could kill the bacteria, particularly by helping it resist the adverse environment inside the host-cell, such as abrupt pH change, or cellular defense mechanisms, like reactive oxidizing species such as NADPH oxidase, and fusion with lysosomes, which contain lytic

enzymes (Ibarra & Steele-Mortimer, 2009). Some of the most conserved proteins that have been found across multiple serovars are SseF/G/J, SteA/D, SifA and PipB/B2. Preservation of the integrity of the SCV serves important survival purposes, protecting the bacteria, and has been found to be mediated primarily by SifA, though recent studies have also attributed a similar function to two other proteins, SteA/B, which also have been found to have the ability to reduce inflammatory and immune response mediators in the host to reduce defense responses (Bayer-Santos et al., 2016; Geddes et al., 2005). In addition to preserving SCV, SifA and SteA have a role in forming tubulin filaments that extend across host-cell microtubules to allow for vesicle fission and transport of the SCV (Domingues et al., 2014). These proteins also work together with two other proteins, PipB and SseJ, with the former translocating subunits of kinesin-1 motor complexes to the membrane of the SCV (Henry et al., 2006), and the later stimulating host membrane tubulation (Ohlson et al., 2008), which together lead to interactions with microtubules of the host-cell that triggers transport of the SCV across these structures. Other effector proteins like SseF/G are important for establishing and maintaining the SCV positioned close to the endoplasmic reticulum and the Golgi apparatus of the host-cells (Finstad et al., 2012; Salcedo & Holden, 2003). The mechanisms behind this process are similar to those in previously mentioned effector proteins, were host motor proteins are recruited for enabling translocation, which in this case is mediated by dynein. Together, these translocating mechanisms allow the SCV to move to more nutrient-rich areas within the host cell. The strategic positioning of SCV in close proximity to host endoplasmic reticulum and

Golgi apparatus serves a direct metabolic purpose, as it provides the *Salmonella* in the SCV access to nutrients, which will have an impact on rate of replication within this structure. The virulence factors that are associated to T3SS are potential targets for the reduction of invasive potential of NTS and considering the overarching regulatory mechanisms behind T3SS regulation makes them important markers when studying novel antimicrobials against NTS.

Emergence and development of antibiotic resistance

Cases of invasive NTS are more prevalent in countries from sub-Saharan Africa, but similar cases have emerged in North America, particularly involving the serovar Typhimurium, which has shown to be more resistant to killing by macrophages and resembles the pathogenic profile of salmonellosis caused by TS (Balasubramanian et al., 2018; Haselbeck et al., 2017). The public health concern for the prevalence of NTS is aggravated by the alarming increase in emergence of antibiotic resistant strains of the pathogens. The National Antimicrobial Resistance Monitoring System (NARMS) has been tracking the incidence of antibiotic resistant and multi-drug resistant (MDR) bacterial isolates from different kinds of samples, including a vast array of animal hosts (primarily chicken, turkey, cattle and swine), animal products (primarily meats from the aforementioned animals) and humans. The latest NARMS integrated report highlighted the developments in resistance to specific antibiotics in NTS as a whole for antibiotics commonly used for treatment (NARMS, 2019). One of these antibiotics was ceftriaxone, which is commonly used for treating invasive infections (W. C. Yang et

al., 2016), which from human isolates did not show a significant increase (2.8% to 3.4%), but went from 6.3% to 9.3% in chickens and 8% to 12% in turkeys. One change to resistance that is of concern is that to the wide spectrum antibiotic ciprofloxacin. Resistance to this antibiotic reached 8% as of 2017 when previously it was only 1%, this is concerning since it is the recommended treatment alternative to ceftriaxone resistant isolates, as well as being part of the first line antibiotics enlisted for treatment of invasive infections (Raveendran et al., 2008). Antibiotic resistance can also be observed by individual serovars and quantity of antibiotics that it is resistant to. When performing this analysis on the major foodborne NTS serovars, namely Typhimurium, Enteritidis and Newport, at least 26.6%, 29.0% and 11.7% of said isolates were resistant to at least one antibiotic. When increasing the number of resistances to at least two antibiotics the percentage of resistant isolates naturally decreased for all serovars, though Typhimurium experienced the least amount of reduction, as it only went down to 21%, however Enteritidis and Newport both went down to 6.9% and 10.1%, respectively. Lastly when extending the analysis to evaluate multidrug resistance (≥3 antibiotics), percent of isolated belonging to the serovar of Typhimurium were more abundant than all other NTS serovars, especially when increasing the number of antibiotics to 4 and 5 (National Antimicrobial Resistance Monitoring & System (NARMS), 2019). This reporting justifies the growing concern over emerging MDR resistant strains, particularly those from Typhimurium, which when combined with its resistance pattern, multiple hosts/habitats, and as invasive manifestations and pathogenesis, make for a critical pathogen to study.

When evaluating the prevalence of antibiotic resistance in the top three most common NTS serovars between the different hosts and comparing them among each other, there is an increased incidence in animal isolates as compared to human isolates. When running an analysis using the NARMS tools to evaluate antibiotic resistance throughout the past two decades, incidence of isolates from poultry, especially chicken, that are resistant to at least two groups of antibiotics has been on a consistent rise for NTS, reaching a 40.1% out of the total collected isolates, and 45.0% for turkey isolates, which though higher than for chicken, has been decreasing. As of 2018, isolates from other animal sources have been detected, and though there has not been a consistent increase in resistance, the latest reports still show them harboring a substantial percent of antibiotic resistant isolates, such is the case for cattle and swine which have reported 18.3% and 22.2% of isolates as resistant, respectively. When looking at specific serovars of NTS and their hosts, Enteritidis, though prevalent in cases, does not show a significant resistant pattern. On the other hand, Newport, shows a consistently high resistance pattern in isolates form cattle, across multiple years with a 54.5% resistant bacteria isolation, and has been associated with outbreaks form beef products and of being prevalent in cattle (Plumb et al., 2019). However, number of total isolates was not as abundant as with other serovars. When evaluating Typhimurium, chicken and turkey products have been prime sources of resistant isolates, as evidenced by a total of 88.8% and 100% of resistant isolates found from chicken and turkey, respectively as of 2018, though the total isolate number for turkey was significantly lower. Cattle and swine also had a percentage, but low total isolate number, but still yielded a

resistance percent of 50.0% and 80.0%, respectively.

As of 2019 in the 2019 NARMS report, the key antibiotics to which isolates were resistant to were tetracycline, ciprofloxacin, nalidixic acid and ceftriaxone. Of these, resistance to tetracycline was more prevalent, particularly in samples from turkey (48.3%), chicken (38.1%), cattle (27%) and humans (12%). Resistance to ciprofloxacin was more prevalent in chickens (18%) and in humans (8%), nalidixic acid was also more prevalent in chickens (13.6%) and humans (6%), and ceftriaxone being common generally in animals (11%) and humans (3.4%). A similar trend has been seen in other countries with cases of antibiotic resistant NTS isolates, with the addition of resistance to ampicillins, sulfonamides and sulfamethoxazole/trimethoprim (Jajere, 2019b). Though a study that evaluate resistance in farm soil isolates from the Maryland and Washington DC area showed a noticeable prevalence of sulfamethoxazole/trimethoprim (40.9%), cefazolin (34.7%), tetracycline (10.9%), streptomycin (7.0%), ceftriaxone (1.9%), gentamycin (1.3%) and chloramphenicol (1.3%) resistant isolates (Peng et al., 2016). Most of these accounts of antibiotic resistance is to treatments that are often considered first line antibiotics, which are of great importance at the time of treating a more severe infection.

As mentioned before, Typhimurium has a wide host and environment range, which lead to a study evaluating the prevalence of resistance from farm soil and in their respective products. This an important factor to investigate since it directly ties into the patterns that lead to the development of antibiotic resistance pattern that was previously discussed. The study looks at isolates from both conventional and mixed-crop livestock

farms in the Maryland and Washington DC, finding that mixed-crop livestock farms had a higher incidence of *Salmonella* than conventional farms. These findings were justified by the fact that in these farms, the animals and their surroundings, including the soil were they are kept, resources that are used for feeding and watering them, experience more exposure to bacteria-containing feces from wild animals and feces-containing water from runoff sources, which is evidenced in the study as it found increased numbers of *Salmonella* isolates from the farm's water and feed supply, making them key sources of contamination of animals and, consequently, their surrounding environment. When serotyping these isolates, the group found Typhimurium to be the most commonly isolated serovar from both farming systems (Peng et al., 2016). This study was followed up by an analysis of the antibiotic resistance profile of soil isolates from different farming systems with a focus on Typhimurium. What this study found was that though Typhimurium numbers suggest that they are more prevalent in mixed-crop livestock farms, more antibiotic resistance was found among the isolates from conventional farms (Peng, Salaheen, et al., 2018). Samples from the pre-harvest stage of a conventional farm showed to have 85.71% of the isolates be resistant to at least one antibiotic, while 23.81% were resistant to two or more antibiotics at a time (Peng et al., 2016). The same study found that in the post-harvest stage of a conventional farm setting 100% were resistant to at least one antibiotic, while 66.67% were resistant to two or more at a time. These results made sense, since a great part of the cause for the increment in antibiotic resistance throughout recent years has been as a consequence of the historic misuse of antibiotics

within the context of conventional faring. A study evaluating the antibiotic resistance panorama of Typhimurium across the food chain from 1996 to 2016 showed not only an increase in the incidence of resistant isolates, but a diversification of antibiotic resistance (X. Wang et al., 2019). Generation of proximity matrixes based on MIC values to different antibiotics showed a spreading of the points during the time period of 2011 to 2016, signifying the diversification of antibiotic resistance pattern within this time period as compared to previous years. The same study also demonstrated that isolates from animals and meat contain the greatest percentages of resistance to single antibiotics like tetracycline and ampicillin, as well as streptomycin and chloramphenicol to a lower degree. However, those isolates that originate from human samples had more diversity than those from any other source, exceeding that of animals, which in turn exceeded meat. Of the animals, chickens held the most amount of diversity, exceeding that of cattle and swine, which is a trend that has been well documented by antibiotic resistance trackers and could be directly attributed to the historic subtherapeutic use of antibiotics as growth promoters within the poultry industry (Wang et al., 2019).

This trend of increasing antibiotic resistance is mediated by the innate resistance mechanisms that bacteria have developed over time to counteract the inhibitory effects of external stressors, particularly antibiotics (Dcosta et al., 2011). But as mentioned before, the increasing pattern of antibiotic resistance is directly correlated to their pervasive misuse in both agriculture and in the medical field, and it is a phenomenon that has been reported around the world, in both developed and developing countries

(Browne et al., 2020). It has been hypothesized that the incidence of resistance in developing countries is mostly associated with over-prescribing them erroneously for treating diseases that are not relevant, or self-limiting (Ayukekbong et al., 2017). Other sources suggest that the lack of surveillance with regards to the development of resistance, the poor quality of antibiotics, the inaccessibility to newer, more effective antibiotics and their widespread use are also major contributors to this panorama in developing nations. The same report suggests that developed countries have also reported emergent resistance strains in hospitals, which might not be the same case as in developing countries, but still happens as a result of continuous exposure of antibiotics with microbes on these clinical scenarios (Chokshi et al., 2019). The farming sector is another culprit in exacerbating this issue since they have been historically overusing antibiotics. This has created environments in which microbes are constantly exposed to antibiotics, which is an issue since this constant pressure unintentionally selects for more resistant pathogens. Studies that have also delved into the concept of the microbial resistome, which is the collective of genes related to antibiotic resistance that is sustained within an environment and share between bacterial communities, has shown that resistant bacteria can confer resistance to other bacteria via horizontal gene transfer, and this process includes opportunistic and pathogenic bacteria, making even common infections difficult to treat (Escudeiro et al., 2019). Another significant contributor to antibiotic resistance is the environment itself, as soil contains a variety of organic and inorganic compounds, genes and microorganisms that serve as pressures that eventually lead to the emergence of bacteria that are resistant to their particular

environmental challenges (Dcosta et al., 2011; Peng, Salaheen, et al., 2018).

With this in mind, the occurrence of antibiotic resistance within conventional farming can also be linked to the constant exposure to trace amounts of antibiotics that comes about as a result of their excessive use, as in the case of subtherapeutic and growth promoting programs, as well as for regular disease treatment in animals. This environment will also serve as an driving force where there is a selective pressure that selects for increasingly resistant bacteria (Rousham et al., 2018). Agriculture is a particularly strong pressure for antibiotic resistance because of its interaction with many facets of life, as well as the multitude of environments it provides particularly for foodborne pathogens. During the pre-harvest phase, animals are fed with food and water that contains bacteria, in addition to being fed with water that is often times from sources that contain soil and feces, which in turn has trace amounts of antibiotics that will make their way to the bacteria in the animal (Carrasco et al., 2012; Cycoń et al., 2019). Another key source that contains trace amounts of antibiotics that can contribute to antibiotic pressure are animal feces. Most times antibiotics are not properly absorbed or broken down in the animal gut, resulting in their excretion in the fecal matter, which will inevitably make its way to water sources, soil and other animals (Marshall & Levy, 2011). The issue of resistance within the agricultural sector creates a public health crisis when considering its direct connection to products that are intended for human consumption and the prevalence of resistance genes and isolates in products even after they are harvested and processed. Disease-causing foodborne bacteria are an ever-present occurrence in food products, whether it be from plant or animal origin, which

signals the coming of increasingly resistant pathogens that will be in direct contact with the general population via consumer products (Woolhouse & Ward, 2013). Even if no disease is generated in an individual, there is an increased risk of perpetuating antibiotic resistant patterns across different bacterial communities located within the host, while also increasing the risk of expanding the reach of antibiotic resistant patterns across other countries and environments (Rousham et al., 2018).

When evaluating the underlying mechanisms that drive the development of resistance to antibiotics, bacteria have many antibiotic resistance strategies which are conferred through specific clusters of genes that code for proteins and enzymes that directly counteract the effects of antibiotics, but also can experience mutations that make them more resistant (Munita & Arias, 2016). These genes can be found in plasmids that can be transferred horizontally from one bacteria to another, or as part of the resistome which, as mentioned before, contains a myriad gene sequences that can be taken-up and perpetuated throughout a bacterial community, allowing them to synthesize specific proteins that aid in survival (Wright, 2007). These proteins can range from enzymes that can degrade or cleave antibiotics to render them inactive, modifiers that can alter the binding site of the antibiotic to make it less attracted to that site (Wright, 2005), as well as proteins that confer protection by mediating the expulsion of the antibiotic from the bacterial cell through the use of such proteins like efflux pumps (Soto, 2013). Other resistance genes are integrated in the chromosome and can be activated by specific environmental signals, which, over time, may become more sensitive to these signals and therefore become more responsive to counteract

antibiotics (Muthukrishnan et al., 2012). Another common resistance mechanism is mediated through mutations in the target sites of the proteins that a specific antibiotic would bind to, which may reduce affinity for that site and render the antibiotic ineffective (Johnning et al., 2015).

Many examples of these resistance mechanisms can be found in NTS. Though gram negative bacteria such as NTS have thinner peptidoglycan (PG) cell walls, they do have an additional defense barrier in the form of an outer plasma membrane, found outside of the PG wall, which makes them innately resistant to compounds that cannot cross that barrier and confers an additional level of resistance to external pressures (Page, 2012). Resistance to antibiotics know to target this structure, like polymyxin B, has been reported in bacteria that have a mutation that leads to the constitutive over-expression of genes belonging to the operons *pmrCAB* and *arnBCADTEF-pmrE*, which are known to catalyze the modification of the lipid A portion of LPS with PEtN and L-Ara4N, making the membrane less electronegative, hence reducing the antibiotic's affinity (Olaitan et al., 2014). Structural modification is a common mode of defense, as one of the two main ciprofloxacin resistance mechanisms has been linked to mutations in the *gyrA* gene which results in the necessary structural changes in the corresponding protein, DNA-gyrase, to reduce affinity of the antibiotic (Johnning et al., 2015). The other common mechanism of resistance to ciprofloxacin, and other fluoroquinolones, is through direct expulsion of the antibiotic from the cytoplasm towards the outside of the cell. This mechanism also relates to resistance towards tetracycline and is mediated through the use of efflux pumps. These are membrane bound structural proteins that

serve as selective channel that carry the antibiotic from the cytoplasm to the surrounding media, therefore limiting the amount of the antibiotic that will be able to reach its intended target and preventing their accumulation. Many of these pumps have a wide range of substrates, endowing the bacteria with resistance to multiple antibiotics at a time, as is the case with the AcrA pump, which serves as a multi-drug efflux pump (Blair et al., 2009). However, drug-specific efflux pumps also exist, as is the case of TetA and other Tet family proteins, which are coded for by *tet* genes and are specific for extruding tetracycline from the cell (Nguyen et al., 2014). Enzymatic modifications of the antibiotics occurs through the catalyzation of acetylation, phosphorylation and adenylation processes, which lead to ineffective binding of the antibiotic to its intended target. Examples of this kind of enzymatic action over antibiotics has been well documented in NTS, as well as in other enteric gram-negative bacteria, in the form of β-lactamases, which are known to cleave the β-lactam ring in antibiotics like penicillin, rendering its binding site inactive (Michael et al., 2006). A similar mode of has been seen in cephalosporin resistant isolates, which contain the *cmy*-2 gene which codes for an enzyme that similarly cleaves the β-lactam ring of expanded spectrum cephalosporines (Carattoli et al., 2002). Antibiotic resistance conferred by enzymes can also be employed to modify the antibiotic's intended target site, like in the case of aminoglycoside resistance mediated through methyltransferases designed to change the structure of 16S rRNA in the bacteria to reduce the binding strength of antibiotics like gentamycin (Hopkins et al., 2010). Though some of these resistance mechanisms might be rare, or infrequent, the threat of developing multidrug resistant strains of NTS is

ever increasing and is turning towards currently clinically relevant antibiotics. The problem of more antibiotic resistant NTS strains not only has to do with its likeliness to finds its way to a susceptible host that will be harder to treat, but the fact that it can disseminate throughout a number of environments where it will enter into contact with other clinically relevant bacteria and transfer resistance to that community (Gootz, 2006).

Strategies to combat antibiotic resistance

Countermeasures to slow down the increase of antibiotic resistance have been put in place in both the agricultural and medical sectors. National endeavors like the National Strategy for Combating Antibiotic Resistant Bacteria (2015) from the USA, placed an emphasis on wise stewardship of antibiotics, increased surveillance and development of novel antibiotics ("National Action Plan for Combating Antibiotic-Resistant Bacteria," 2015). Examples of this can be seen in farming scenarios, like in the case of organic farming, which eliminates the use of antibiotics in animal production. However, though stewardship is a crucial factor in mitigating the emergence of antibiotic resistant bacteria, it has its limitations. It has been found that though organic farms have a lower incidence of antibiotic resistant bacteria in their products and environment, when compared to conventional farms they carry a higher percentage of bacteria in almost all assets of the farm, including feed, water, environment, products, fecal samples and overall (Peng et al., 2016). This means that additional processes at the pre- and post-harvest levels must be put in place in order to

limit the bacterial load that reaches the consumer. This limitation also makes the animals prone to suffering from diseases that would normally be prevented by sub-therapeutic use of antibiotics (Patel et al., 2020).

Limiting the use of antibiotics has been at the base of this endeavor, however complete elimination is not possible in the current state, which has stemmed a number of novel approaches. The methods that have been proposed for control of NTS can be grouped into four main approaches: vaccination, bacteriophage therapy, pro-commensal modulation and novel antimicrobials. Vaccination has been met with its fair share of challenges when it comes to bacteria with such a numerous account of serotypes and variability, along with the challenges already attributed to vaccine development. There have been attempts at a *Salmonella* vaccine for humans, however, there is still no evidence that this vaccine could lead to prolonged immunity (Galen et al., 2016). On the other hand, bacteriophage therapy, though an approach that is gaining traction within the scientific community that provides a promising solution to bacterial control that does not contribute to the antibiotic crisis, has a limited target range per bacteriophage designed. Effective use of bacteriophages requires them to be strain specific and their effectiveness could be hindered by minor mutations in the bacteria (Principi et al., 2019). There is also the concern of bacteriophages might shift to a lysogenic cycle and might serve as potential carriers of antibiotic resistance genes that could be perpetuated within a bacterial community as they are transported by the phage and later injected into a cell (D. M. Lin et al., 2017). This limits the scope of potential bacteriophages to those that are strictly lytic and must be meticulously designed. Use

of probiotics and prebiotics as pro-commensal strategies for gut microbiome modulation have demonstrated significantly beneficial effects when it comes to prevention and treatment of bacterial infections (Gut et al., 2018). These microbes can produce many metabolic biproducts that aid in the maintenance of the gut structure, preventing the invasion of harmful pathogens, in addition to competitively excluding pathogenic microbes through the colonization of beneficial bacteria (Zuo & Ng, 2018). This method preserves the integrity of the gut microbiome, while eliminating pathogens regardless of the antibiotic resistance panorama. However, there is still much to study, as there is high variability in the outcome of the treatments, especially when considering the person-to-person differences that exist in a population that might lead to differences in response (Zeevi et al., 2019). There is also the matter of immediacy of the effects of the treatment, meaning that there is still the need to evaluate the efficacy of this approach in the case of an acute infection.

Direct elimination of the bacteria is still one of the most feasible methods of control and has a wide range of options when it comes to treatment methods. Antimicrobial compounds can be found naturally from a range of sources, including from animal, algae, fungi, plants and bacteria themselves, giving scientists an incredibly wide selection of potentially antibacterial compounds that could be tested and employed for bacterial control and treatment (Al-Fakih & Almaqtri, 2019; Arias-Rios et al., 2017; Cowan, 1999; Shannon & Abu-Ghannam, 2016). However, there is still the issue of the highly adaptable nature of bacteria and their capability to develop, or re-activate, innate resistance mechanisms that would render novel antimicrobial

compounds ineffective over time (Peterson & Kaur, 2018). Conventional antibiotics for inhibiting bacteria have historically been mostly isolated from fungi and from bacteria alike, with synthetic modification of antibiotics being an effective tool for making stronger drugs that can remain useful for longer before the bacteria develops resistance to it. On the other hand, awareness around the importance of the local gut microbiome and its preservation has brought to light the sever effects that antibiotics can have on gut health, meaning that stronger antibiotic treatments could be a disservice to the host's general wellbeing (Francino, 2016).

Using antibiotic cocktails for treating resistant bacteria has been another proposed method, since it would combine the effects of multiple antibiotics and the extra pressure that is put on the bacteria will reduce the bacteria's ability to respond, while also lowering the chances of developing resistance, since multidrug resistance is less likely to develop than resistance to a single drug (Khalili & Izadpanah, 2015). However, this approach is met with a similar concern for the health of the local gut microbiome and the survival of multiple groups of beneficial bacteria being hindered by antibiotics. Another approach would be to rescue the function of antibiotics that might be no longer effective, though the use of inert compounds that have no bioactive function of their own but can work synergistically to eliminate resistant bacteria (Ejim et al., 2011). This is a promising method, but, similarly to other novel treatment alternatives, requires extensive screening for identifying these compounds as well as developing delivery methods. The innate resistance mechanisms that bacteria have been known to develop against fungi and bacteria-derived antibiotics have been the major driving force for the

search of novel antimicrobial components from other sources that are not as prone to developing resistance in bacteria but can be used to effectively eliminate them. Of the previously mentioned sources, animal-derived antimicrobials are mostly peptides and antibodies (Arias-Rios et al., 2017). Algae-derived compounds with antimicrobial capability, similarly to fungal ones, are mostly metabolites that come about as byproducts (Gyawali & Ibrahim, 2014). However, screening for new potential antimicrobials remains a painstaking and costly progress and there is still the risk of developing resistance. Plants are another source of potential antimicrobials and hold promising advantages.

These other sources are gaining much attention, but in plant-derived compounds there is a vastly diverse source of compounds ranging from peptides and enzymes, to smaller metabolites that are a lower molecular weight but have a range of conformations and functional groups that allow for a wide array of molecular interactions with different structures in the bacterial cell, such as the cell membrane, cell wall, DNA and proteins (Rempe et al., 2017). The reason for plants containing such a wide array of compounds can be associated with their complex signaling pathways, which rely primarily on secondary metabolites that are synthesized within the plant (Marchiosi et al., 2020). Other proposed explanations for this phenomenon are that these compounds provide a form of protection from plant-associated pathogens, since plants do not count with a proper immune system like that of animals. This reasoning would explain why some compounds are more effective than others in the process of eliminating pathogens. However, there is another explanation that helps in accounting

for the diversity of compounds that is found in plants, and that is that as a result of photosynthesis and a highly active metabolism, plants have to manage a large number of highly reactive oxidative species. These reactive oxygen species are known to cause damage to macromolecules in the plant cells, therefore, plants have numerous and diverse small molecules that can neutralize these reactive oxygen species. This function would explain why these compounds have been found to also behave like antioxidant when tested *in vitro* (Hussain et al., 2016). Tied to the discussion of the importance of these compounds for metabolic pathways in plants, some of these compounds serve as the base for pigments that the plant uses to absorb certain wavelengths of the light spectrum and harness that energy to carry out photosynthesis. Though these are the main functions that have been attributed to these compounds, the sheer diversity of structures and functional group modifications that these compounds have leaves open the possibility that they could have interactions with bacteria that result in an antimicrobial effect (Bouarab-Chibane et al., 2019). Of the above-mentioned compounds, phytochemicals that are of polyphenolic nature have gained interest particularly because of their diversity, as well as for their abundance within nearly all parts of the plant and species, though in possibly different quantities, as well as their ease of extraction (Robbins, 2003). Studies have found that the fruits of plants are parts that are particularly rich with amount and diversity of polyphenols (V. Kumar et al., 2019).

Though the mechanism of action behind antimicrobial polyphenolic compounds and their interactions with bacteria, particularly pathogenic ones, has areas that require

further study, the inhibitory effects of polyphenolic-rich plant extracts has been well documented (Santos et al., 2019). Plant-derived polyphenols have been proposed as additives that can extend the shelf-life and quality because of their antioxidant capabilities as they scavenge for reactive oxygen species (ROS), they can chelate free metal ions, which affect bacterial growth, in addition to directly inhibiting bacterial growth (Papuc et al., 2017). The ubiquitous nature of polyphenolic compounds in plants has led to the development of innumerable studies geared towards evaluating the antimicrobial potential of diverse plant extracts of different sources, including from roots, leaves, flowers, fruits and fruit byproducts (Cowan, 1999). The antimicrobial potential of such extracts has been well established to varying degrees of efficiency against multiple bacterial species, both gram positive and negative.

Not only are these compounds capable of eliminating pathogenic bacteria, they could be used as a countermeasure against antibiotic resistant bacteria. Notable evidence of this can be found in previous research that used an extract from blueberry pomace against methicillin-resistant *Staphylococcus aureus* that resulted in the re-sensitization of the bacteria to methicillin, making it susceptible when used in conjunction with the antibiotic (Salaheen, Peng, et al., 2017a). The same extract was used against ST, which exerted clear bactericidal activity (Salaheen et al., 2016a), and when fed to a chicken model it reduced the colonization of *Campylobacter jejuni*, while also serving as a weight promoter (Salaheen et al., 2018). This suggests the possibility of using these compounds as substitutes for antibiotics in the farming industry without compromising food safety and animal health. Polyphenols in these extracts also hold

the potential of serving as a form of multi-target treatment, similar to a multi-antibiotic treatment, or as a source for adjuvants that alone might not be effective antimicrobials, but could enable other previously inactive antibiotics to gain function (Santos et al., 2019). A study done using a grape pomace extract against a multidrug resistant *Escherichia coli* strain found that when used synergistically with the antibiotic that the bacteria was previously resistant to, the minimum inhibitory concentration (MIC) of these antibiotics was brought down to less than half to the concentration of the antibiotic needed when used alone (Sanhueza et al., 2017). This experiment was performed using a raw extract and the specific compounds that are responsible for bringing about this synergistic rescue of the antibiotics are yet to be identified. Though these extracts originate from diverse sources, when evaluated by HPLC it is evident that they contain a similar phytochemical profile and share some of the same compounds that are thought to be crucial for conveying their antimicrobial and possible antibiotic rescuing properties (Altemimi et al., 2017).

One of the challenges associated to the study and subsequent implementation of polyphenol use is the delivery. The relationship between host and polyphenols is largely defined by the host's microbial flora which is known to play a key role in the metabolism and subsequent absorption by host. Though it is known that some polyphenols are readily absorbed by host tissue, others resist the abrupt pH change in the stomach and manage to pass to the small intestine, where they will either be absorbed or further degraded enzymatically (Lewandowska et al., 2013). Though enzymatic degradation by the local microbial flora makes polyphenols a form of

prebiotic that can selectively support the growth of specific groups of beneficial bacteria, it makes the study of the direct effects of polyphenols difficult (L. Zhang et al., 2020). The direct effects of intact polyphenols on a live system are important to consider since *in vitro* they have demonstrated the capacity to inhibit the growth of pathogenic bacteria, exert antioxidant functions and signalers for probiotics, which is why some groups have focused on improving delivery methods for polyphenols, in addition to further studying the chemical properties that facilitates their interactions. Though plants and their extracts contain many of these compounds, the fact that there are thousands of these polyphenolic species, each with their own potential mechanisms of action, function, availability and interaction with probiotics, leads researchers to focus on specific compounds from a particular family. This selection could also be motivated by a previous knowledge about what compounds are predominantly found in a plant extract of interest, and how attainable are those individual compounds for isolated testing.

Polyphenolic compounds are characterized for consisting of one or more 6-carbon phenolic rings that are modified by functional groups. These compounds can be divided into three main groups: phenolic acids, flavonoids and polyphenolic polymers. Phenolic acids are mostly composed of a single benzene (6-carbon) ring with a carboxyl group in the first position, depending on the compound, could contain further functional group modifications across the other five remining carbon atoms. Flavonoids can be further subdivided depending on their functional group arrangements but have the basic structure of a benzene ring fused with a heterocyclic pyran ring that is in turn attached

to a benzene ring. Polyphenolic polymers are a broader term used to describe more complex structures that arise from polymeric arranges that some of these compounds have been known to take, however, these can be often broken down into their constituent parts, releasing monomeric phytochemicals belonging to the groups already discussed (Tsao, 2010). Polyphenols can also be found attached to sugars and other macromolecules, which can alter their bioavailability, absorption and may require enzymatic activity to separate them (Corrêa et al., 2019).

With these basic structural differences in mind, the discussion can be further extended to the functional groups that modify these compounds. The most common forms of modification are with hydroxyl, esters, ethers and methoxy groups that modify the aromatic rings of the phenols (Radulovic et al., 2013). This will yield a wide array of structural diversity and differences in electrochemical properties that are defined by the capacity to accept, donate and reallocate electrons, which will give rise to polar interactions, as well as the disassociation of H+ protons that can give rise to a form of ionic interactions and acidification. These functional groups are at the base of the interactions that these compounds can have with cellular lipids, DNA and proteins. Researchers have made the effort to correlate the number and type of functional groups to antimicrobial and antioxidant capability, and have found that increased lipophilicity increases antibacterial activity, most likely associated to plasma membrane interactions (Sikkema et al., 1995). More functional groups can provide more scavenging potential as it can donate more hydrogen atoms, electrons and can also potentially chelate metal ions, making it a stronger antioxidant (Minatel et al., 2017), but less active

antimicrobials, since they are less lipophilic (Puupponen-Pimiä et al., 2001). However, further studies have suggested that it is not just a matter of number of groups, but the position at which the modification is located, and which groups are being added (Xie et al., 2014). This review suggests that for some polyphenols a methoxy modification could reduce the antibacterial capacity of that compound as compared to one that has a hydroxyl group, but substitution with more lipophilic functional groups like a prenyl, alkylamino, or alkyl significantly increases antibacterial activity surpassing that of the hydroxyl groups.

Chemical structure and functional group modifications will also play a crucial role with respect to environmental interactions. As mentioned before, some of the challenges related to treatment with polyphenols is delivery, particularly because they are prone to degradation, absorption and oxidation/reduction, but solubility and resistance to acidity are going to be important factors as well (Scalbert et al., 2002). These factors are crucial when testing compounds that might demonstrate great potential in an *in vitro* scenario but might not translate as well to an *in vivo* model. There are polyphenols on all groups that are good candidates, however, though phenolic acids have been known to be more absorbable by the host's gut cells, they have shown enough robustness to survive the passage through the stomach and make it far enough to interact with live bacteria in the gut, both probiotic and pathogenic (N. Kumar & Goel, 2019).

Phenolic acids

Because if their molecular structure, phenolic acids behave like weak organic acids. This classification comes from their carboxyl group, which makes these compounds good reducing agents, in addition to being able to free an H+ proton as it disassociates in aqueous solutions, acidifying the medium (Z. Cheng et al., 2002). Their role in plants has been associated with aiding in the synthesis of cell wall components, defense against pathogens and signaling that responds to the surrounding microbes, mycorrhizae and environment, though these roles have not been completely defined (Marchiosi et al., 2020). Phenolic acids can be further divided into two subgroups based on the base structure. These subgroups are benzoic acids and cinnamic acids, with the former containing a simple carboxyl group and the later having an additional alkene group in between the alfa benzoic carbon and the carboxyl group (Marchiosi et al., 2020). It has been well established that this group of compounds accounts for at least one third of polyphenolic compounds in most plant and their derived extracts (Goleniowski et al., 2013). Phenolic acids have particularly gained attention as substitutes for conventional antibiotics in the animal production industry, and have been considered for use in conjunction to other prebiotics and probiotics in order to reduce the colonization of pathogenic bacteria in the gastrointestinal tract of farm animals, particularly poultry (Dittoe et al., 2018).

As mentioned before with regards to general polyphenolic compound structure, phenolic acid antimicrobial activity is highly dependent on the functional groups that modify the fundamental structure. Similarly to polyphenolic compounds, the presence

of functional groups like alkyl chains have been associated with stronger antibacterial activity, but have the cavoite of being more toxic, which is relevant when taking into consideration that these compounds will be interacting with probiotics and host cells (Cueva et al., 2010). On the other hand, phenolic acids derived from benzoic acids have shown to be less toxic, but still be active against bacteria (Merkl et al., 2010). Lipophilicity has shown to play an important role as well, since compounds with more hydroxyl groups had less antibacterial activity than those with fewer hydroxyl groups and those that had methoxy groups (Sánchez-Maldonado et al., 2011a).

One of the phenolic acids with the widest distribution would be protocatechuic acid (PA), also called 3,4-dihydroxybenzoic acid, which has been found in many species of edible fruits, flowers and plant extracts, including from blueberry pomace (Kakkar & Bais, 2014; Salaheen et al., 2016b) .

Response and tolerance to phenolic acids in bacteria

With the current knowledge about the importance of probiotic bacteria and their relationship with the nutrients that their human hosts consume, developing new antimicrobial treatments has to take into consideration the preservation of these microbial communities or improve them when given the opportunity. Research groups have found that a combination of certain fiber-rich and polyphenol rich diets with probiotic bacteria leads to the release of phenolic acids, making them more bioavailable after the breakdown of more complex nutrients (Kling et al., 2016). Though reports are mixed, the current evidence shows that different phenolic acids will have varying

effects on bacteria belonging to both gram-positive and gram-negative bacteria. Phenolic acids against *Staphylococcus aureus* has been well documented, but it is important to note that this bacteria also has a lower acid tolerance when compared to other enteric pathogens (Aldulaimi, 2017). Common probiotics like bacteria belonging to the *Lactobacillus* genus are more acid tolerant but have demonstrated variable resistance to phenolic acids. *L. rhamnosus* and *L. acidophilus* have been demonstrated to be resistant to many phenolic acids, including gallic, protocatechuic and vanillic acid (Pacheco-Ordaz et al., 2018). Further data with regards to the relationship of *Lactobacillus* to phenolic acids suggests that survival is mediated through enzymatic detoxification of these compounds by cleaving the hydroxyl groups (Sánchez-Maldonado et al., 2011a). Other bacteria are capable of further degrading phenolic acids through cleavage of the phenol ring, which fundamentally changes the bioactivity of the molecule (Fritsch et al., 2016). In the case of gram-negative bacteria, reports are more uniform with regards to susceptibility to phenolic acids (Cueva et al., 2010; Pacheco-Ordaz et al., 2018). Though most gram-negative are acid resistant in order to survive the passage through the gut, many groups have demonstrated their susceptibility to phenolic acids. This difference could possibly be related to the main structural difference between gram-positives and gram-negatives, which is the presence of a thick cell wall made of peptidoglycan in the former, and a phospholipid plasma outer-membrane.

There have been many studies geared towards the interaction of enteric gram-negative bacteria to small weak organic acids, namely, phenolic acids like gallic acid,

protocatechuic acid and vanillic acid (Bernal-Mercado et al., 2020; A. Borges et al., 2013; Qian et al., 2020). It is known that these compounds tend to disassociate is aqueous solutions, which leads to a process of oxidizing that acidifies the media in which they are found in (Hirshfield et al., 2003). On the other hand, because of their small molecular size and the slight lipophilicity, these compounds are readily diffused through the bacterial outer membrane, making their way into the periplasmic space and later the cytoplasm. However, it is important to note that the carboxyl group of these compounds give them a polarity that allows them to also interact with other parts of the cell, as well as being responsible for the acidic behavior of these compounds. When inside the cell, the more alkaline environment of the cytoplasm will lead to a dissociation between the phenolic acid and an ionic hydrogen (H+), lowering the intracellular pH of the bacteria as more H+ accumulates.

Mitigating intracellular acidification is important for cell survival because it will inevitably lead to DNA damage, enzyme inactivation, metabolic changes, and can bring about morphological changes in the bacteria, partly linked to changes in osmotic pressure resistance and damage of essential structural proteins (Lund et al., 2014). Cell death will inevitably come about from damage to genetic material and other macromolecules as the function of important catalytic enzymes will also be hindered. DNA damage comes about from a process known as depurination and depyrimidination, in which the nitrogenous bases are protonated and the a base is lost as a result, creating strand breaks (Jeong et al., 2008).

In order to counteract the effects of acidification, bacteria have developed acid

resistance mechanisms that allow them to manage the concentration of protons in the cell, mitigate intracellular damage and counteract the morphological effects that can arise. These mechanisms revolve around the limitation of entry to the periplasmic space and cytoplasm through membrane permeability modification and restrictions on diffusion through proton pump, in addition to active expulsion of protons from the cell, and activation of metabolic pathways that make use of free H+ ions in and out of the cells to generate energy and increase pH (Guan & Liu, 2020a). The acid response process begins with the detection of adverse conditions, and though the process involves the regulation of many genes, a specific group of acid tolerance response genes are crucial for cell survival, beginning with the *rpoS* gene, also known as sigma factor S (σ^S), which is a known stress response gene (Y. Yang et al., 2014). Other acid tolerance genes also have important roles in virulence, since *Salmonella* has to survive drastic pH changes throughout its trajectory through the gastrointestinal tract, as well as survive the acidic intravacuolar environment during its intracellular cycle (Foster, 2001). For this reason, pH acts as a signal that can lead to the activation of important virulence related genes that facilitate invasion of eukaryotic host cells, intracellular survival and resistance to adverse extracellular conditions (Choi & Groisman, 2016). An example of this can be found in the two-component regulatory system of PhoPQ, which has an important role in detecting changes in environmental concentrations of magnesium (Mg^{2+}) and acidic pH. This will lead activation of subsequent virulence related genes, as well as in triggering plasma membrane changes to resist lower pH (Groisman, 2001). On the other hand, the OmpR regulator plays a role in regulating

outer membrane porins OmpF and PmpC, as well as regulating the two-component regulatory system of SsrA-SsrB (A. K. Lee et al., 2000) when responding to osmotic change and pH, respectively, which are often correlated (Chakraborty & Kenney, 2018), but other research groups have reported that it is responsible for allowing a certain degree of cytoplasmic acidification in order for activation of SPI-2 genes to occur (Chakraborty et al., 2015). The controlled acidification of the cytoplasm is a well-studied phenomena in *Salmonella*, as it has been shown to be an important step that leads to the activation of virulence effector proteins of the SPI-2 system, however, it is not a state meant to be maintained in perpetuity as the bacteria needs to initiate pathways dedicated to DNA and protein damage repair from lower pH and needs to maintain a control of H+ accumulation, which is where a diverse array of defensive strategies come into play.

Reducing the entry of protons can be achieved by altering the cell membrane composition to reduce passive diffusion of H+ and other molecules, but that will later be explained in further detail. Other strategies focus on reducing the amount of H+ that can enter the cell by passive diffusion through proton pumps, like in the case of F_1F_0-ATPases, which are capable of pumping out protons from the cell, but can also reduce pore size to control the entry of H+ from the periplasmic space to the intracellular space, in addition to being able to use these protons to generate ATP from ADP, which will aid in the increasing of pH while also contributing to metabolic processes of the cell (Sun et al., 2012). Though the factors that contribute to the activation of one function versus the other is not fully understood, there is a correlation between decrease

in membrane permeability and decreased F_1F_0-ATPase activity in order to restore pH homeostasis (Xu et al., 2020). While controlling the number of protons that enter the cell is important, exporting them out of the cell and using this process in an energetically cost-effective way is also very important for survival. This process is mediated by NADH hydrogenases I and II, which are trans-membranal protein that transfer protons from the cytoplasmic space to the periplasm, generating a proton motive force that reduces pH in the cytoplasm, while also generating energy (Kanjee & Houry, 2013).

As mentioned before, another mechanism of resistance involves the modification of cell membranes with special fatty acids. This has been reported in enteric bacteria, including *Salmonella* were exposure to acidic conditions stimulated the change of unsaturated fatty acids in the membrane to cyclopropane fatty acids (CFAs) (Grogan & Cronan, 1997). The synthesis of CFA is catalyzed by enzymes coded in the *cfa* gene, which is RpoS-dependent, and consists of the replacement of an alkene bond in the unsaturated fatty acid for a cyclopropane (Slonczewski et al., 2009a). It is believed that the advantage of this kind of modification has to do with CFAs being more stable under adverse environmental stressors and reduce the passive influx of weak organic acids and protons. Another significant modification can be seen in the lipid A portion of LPS, with phosphoethanolamine and 4-amino-arabinose as well as with 2-hydroxylation through the activity of LpxO (Sohlenkamp, 2017). These physiological changes in membrane structure confer additional resistance to the acidic environments and help maintain proper cell structure, while reducing permeability and fluidity.

In order to counteract the negative effects of acidification on macromolecules, enteric bacteria synthesize proteins like HdeA, which can be found in the periplasmic space acting as a chaperone that prevents aggregation between proteins (W. Hong et al., 2005), as well as DnaK, which has been found to be involved in protein refolding during stress (Bearson et al., 2006). With regards to DNA damage, the LexA protein controls the expression of the *recA* gene, which triggers a DNA repair mechanism and recombination (Medina-Ruiz et al., 2010).

Once the flow of H+ ions going into and leaving the cell is restored, *Salmonella* has been shown to be able to use the H+ in the cytoplasm as substrates to enzymatically alter amino acids. Amino acid decarboxylases AdiA, CadA and SpeF which correspond to arginine, lysine and ornithine, respectively, catalyze the replacement of the α-carboxyl groups of the amino acids with an H+ from the cytoplasm, therefore reducing the concentration of disassociated H+ protons in the cytoplasm which will lead to an increase in pH (Viala et al., 2011). The polyamines that are generate form this enzymatic reaction will also have the additional function of blocking outer membrane porins, therefore contributing to the prevention of passive influx of H+ protons. A similar process will take place with hydrogenases, which will consume H+ in order to turn them into hydrogen gas (H_2), which will diffuse passively out of the cell (Slonczewski et al., 2009a).

These resistance mechanisms are important to consider when studying the potential application of phenolic acids and other polyphenols as compounds intended for antimicrobial control, since there are intrinsic resistance mechanisms that NTS,

particularly ST, has developed in order to survive and proliferate in adverse environmental conditions. However, these same mechanisms can be exploited in the development of novel antimicrobials and possibly in the repurposing and rescuing of currently inefficient ones. In addition to this, understanding the bacterial response to compounds like polyphenols, particularly phenolic acids like gallic acid, protocatechuic acid and vanillic acid, can facilitate the future discovery of more potent and safe antimicrobials that are commonly found in plant extracts like that of BPE. Understanding the interactions that occur between phenolic acids and *S.* Typhimurium gives valuable information about the potential mechanisms of action of BPE as an antimicrobial and how, in the future, it and the individual compounds that are found in it can be used in a guided manner to elicit specific antimicrobial functions.

Overall Hypothesis and Specific Aims

Individual phenolics known to be in berry byproducts have specific antibacterial mechanisms that disrupt cellular functions in *S.* Typhimurium by which mode of action of natural therapeutics can be defined.

To investigate the hypothesis, the following aims need to be fulfilled:

Aim 1: Investigate prospective candidate phenolics found in berry byproducts and test their individual antimicrobial potential against *S.* Typhimurium.

Aim 2: Assess the effects of phenolic acids on an INT-407 epithelial cell line and their interactions with *S.* Typhimurium.

Aim 3: Evaluate activity of phenolic acids against *S.* Typhimurium in simulated gut conditions, as well as the capability to modulate the surrounding microbiota.

Aim 4: Identify specific phenotypic alterations of *S.* Typhimurium after treatment with individual phenolics to predict mechanism of action.

Chapter 2: Antimicrobial and antivirulence impacts of phenolics on Salmonella enterica serovar Typhimurium

Introduction

Death because of enteric infections was found to be the third highest transmittable cause of death in the world, with diarrheal disease being the main source and infection by the bacterial pathogen *Salmonella* spp. responsible for at least 18.7% of those deaths (Roth et al., 2018). Even though incidence is most pervasive in developing parts of the world, enteric disease caused by foodborne pathogens still accounts for substantial cases of illness, hospitalization, death and economic loss in the USA (Dewey-Mattia et al., 2018b). Of the major 31 pathogens associated to foodborne illness in the USA, *Salmonella enterica* is the leading bacterial etiological agent as it is estimated to be responsible for over 1 million cases of illness, 15,000 hospitalizations, 300 deaths, and an estimated loss of 3.5 billion dollars yearly, associated with the loss of productivity that accounts for cost of care, treatment, recovery and hours of work lost (Scallan et al., 2011; Tack et al., 2020b). Though many of the already identified and sequenced *Salmonella enterica* serovars (>2500) have been linked to disease, *Salmonella enterica* serovar Typhimurium (ST) remains one of the most clinically relevant strains because of its ubiquitous presence in

produce, meat and the environment, as well as being a major source of outbreaks and disease (Carrasco et al., 2012; CDC, 2015; Fatica & Schneider, 2011).

Though self-limiting for healthy individuals, at-risk populations such as children, elders and immunocompromised individuals can experience more invasive forms of the pathogen, requiring more intense treatment with antibiotics (Crump et al., 2015). Invasive *Salmonella enterica* serovar Typhimurium (ST) infections are mediated through the activation of the genes found in the *Salmonella* Pathogenicity Island 1 (SPI-1), which code for an assembly of proteins known as the Type III Secretion System (T3SS) that aid in the attachment and subsequent invasion of host cells (Lou et al., 2019b). The intracellular survival of the bacteria is later achieved through the activation of the genes found in SPI-2, allowing it to avoid the immune system and resist lysis (Jennings et al., 2017). Strains found to be resistant to antibiotics have been corelated with high virulence and a more aggressive invasive capability towards their host, which subsequently results in a harder to treat infection (Angelo et al., 2016; Fluit, 2005; Jajere, 2019a). The continuous and increasing discovery of antibiotic-resistant ST isolates from human samples (U.S. Department of Health and Human Services, 2019), as well as in farm animals meant for consumption, poses a growing public health threat, greatly exacerbated by the need for novel antimicrobial compounds that do not add to the development of resistance.

New antimicrobial agents need to be accessible, easy to synthesize or extract, and effective, but must also be sustainable by not contributing to the rise of antibiotic resistance in ST, or in other bacteria by way of horizontal gene transfer and the

antimicrobial resistome (U.S. Department of Health and Human Services, 2019). Plants and their byproducts are important candidate sources in the discovery of novel antimicrobials, primarily because of their availability, diversity of compounds and their complex chemical makeup (Bythwood et al., 2019; Cowan, 1999). Extracts prepared from various plant sources have been found to contain a vast array of bioactive organic small molecules with antimicrobial properties, particularly polyphenols. These polyphenols are often consumed as a part of ingesting foods that contain them, such as grains, vegetables, herbs and fruits. The range of species and concentration of polyphenolic compounds that can be found in these foods can vary among them and even within the same food group, as these are subject to external factors such as the growth conditions of the plant, environmental parameters, harvesting procedure, and extraction method (Alvarado-Martinez et al., 2019). Studies reporting on phenolic concentration within specific food groups have found cereals to contain a range from 26 to 3300 mg per 100 g (depending on the cereal), cocoa to range from 1204 to 4437 mg per 100 g, tea beverages to range from 29 to 103 mg per 100 mL, and fruits to range from 15 to 2556 mg per 100 g (depending on the fruit) (Goleniowski et al., 2013; W. Li & Beta, 2013).

Previous research on plant extracts with antimicrobial properties found that berry pomace, a byproduct containing the seeds and shell of the berry fruit after juicing, from blueberries (*Vaccinium corymbosum*) and blackberries (*Rubus fruticosus*) contains a high concentration and variety of polyphenols, which can be extracted economically (Gyawali & Ibrahim, 2014). This berry pomace extract (BPE) was

shown to have significant antagonistic effects on ST growth and other cellular functions related to virulence (Salaheen et al., 2014). The two major polyphenolic compounds that make up the majority of the phenolic content in plant extracts, which could be responsible for conferring antimicrobial effects, are flavonoids and phenolic acids. Though the exact mechanism of action is not yet understood, research on the antimicrobial capabilities of phenolic acids has shown potential, particularly against Gram-negative bacteria, since it is believed that they can diffuse passively through the outer membrane, bypassing one of the main defensive barriers against conventional antibiotics and making their way into the cytoplasm, which will lead to acidification and cell death (Cueva et al., 2010; O. H. Lee & Lee, 2010; Salaheen et al., 2016b). Though membrane damage has been cited as one of the main outcomes of phenolic acid treatments, the events that lead to this damage and eventual cell death in ST treated with gallic acid (GA), protocatechuic acid (PA) and vanillic acid (VA), which are some of the most abundant phenolic acids in plants and in BPE, have not been well documented and the mechanism has not been elucidated (Gutiérrez-Larraínzar et al., 2012; Slonczewski et al., 2009b).

This study evaluates the antimicrobial potential of three phenolic acids known to be in BPE, with the aim of comparing their individual effectiveness against ST. This will be determined by measuring patterns of growth inhibition, as well as impairment of cellular functions related to virulence. These findings will serve as a base for discovering the mechanism of action of some of the components that make up BPE, allowing for better guided use of this product in the future, as well as understanding

the antimicrobial potential of the phenolic acid family of compounds against increasingly resistant pathogens such as ST.

Materials and Methods

Bacterial Strain and their Growth Conditions

In this study, *Salmonella enterica* serovar Typhimurium (ATCC 14028) (ST) was used. ST was grown in Luria-Bertani (LB) (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) agar at 37 °C under aerobic conditions (Thermo Fisher Scientific Inc., Marietta, OH, USA). All experiments described were performed in LB broth (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and were incubated under aerobic conditions for 24 h in 37 °C with continuous aeration at 150 rpm through the use of a shaking incubator**.**

Compounds and Stock Solution Preparation

GA (Acros Organics, Geel, Belgium), PA (Sigma-Aldrich, St. Louis, MO, USA) and VA (Alfa Aesar, Ward Hill, MA, USA) were purchased in solid powder form. Stock solutions of gallic acid and protocatechuic acid were prepared by dissolving in sterile deionized water, while vanillic acid was prepared by dissolving in 30% ethanol (Pharmco-Aaper, Brookfield, CT, USA). Stocks for all compounds were prepared to a concentration of 10 mg/mL. Phosphate buffer saline (PBS) was prepared to a pH of 7.2.

Determining MIC and MBC

The MIC and MBC values for individual phenolic compounds were determined using the broth microdilution method as described by Clinical and Laboratory Standards Institute Performance Standards for Antimicrobial Susceptibility Testing M100 (*Clinical and Laboratory Standards Institute*, 2019) and performed previously (Salaheen et al., 2014), with slight modifications. Briefly, an overnight culture of ST cultured on LB agar was used to prepare a bacterial suspension in PBS fixed to an optical density (OD_{600}) of 0.1 (8 log CFU/mL). This bacterial suspension was further diluted and used to inoculate 24-well plates containing LB broth with increasing concentrations of phenolic acids (from 0.5 to 4.5 mg/mL) to achieve a final bacterial load of 4 log CFU/mL in each well. Controls were prepared to have an equivalent media-to-solvent ratio with their respective treatment counterpart. MIC was determined as the lowest concentration at which there was no visible bacterial growth in the well after 24 h. MBC was determined as being the lowest concentration after 24 h at which there was ≥99.9% eradication of bacteria after plating in LB agar. The MBC:MIC ratio was calculated in order to determine if the treatments were bactericidal (<2) or bacteriostatic (>16).

Resistance Development Over Time

Development of resistance to the phenolic acids in ST was evaluated using methods previously described on resistance development to antibiotics with modifications (Ma et al., 2016). Briefly, a 24-well plate was prepared following the

same set up as the microdilution assay. The bacteria from the well with the highest concentration of each of the respective treatments, below MIC, and that allowed growth after 24 h, were selected. An aliquot from the bacteria in this well was taken and again fixed to an OD_{600} of 0.1 to repeat the process of inoculation of a new 24-well plate. This was performed over 12 generations and changes in the MIC were recorded over this time.

RNA Extraction and cDNA Synthesis

Samples were prepared for RNA extraction by inoculating a bacterial suspension of ST to a final concentration of 4 log CFU/mL and incubated overnight. They were individually treated with the sublethal concentrations (below calculated MIC, namely 3.0, 1.5 and 1.0 mg/mL for GA, PA and VA, respectively) of each compound that were subsequently collected. RNA was extracted following the previously described methods with modifications (Peng et al., 2015). Briefly, samples were centrifuged at 14,000 × *g* for 15 min to collect bacterial pellet, and TRI Reagent® (Molecular Research Center Inc., Cincinnati, OH, USA) protocol was used to homogenize pellet and isolate the RNA sample. RNA concentration in the sample was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Marietta, OH, USA) for standardization and to use as the template for cDNA synthesis with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), in accordance with the manufacturer indications.

The cDNA synthesis protocol was set to incubate for 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min.

Quantitative RT-PCR Assay

Changes in the relative expression of genes related to the virulence of ST were measured using qPCR. The qPCR reaction was prepared in accordance with the manufacturer indications of the PerfeCTa SYBR Green Fast Mix protocol (Quanta Bio, Beverly, MA, USA), and performed using the Eco Real-Time PCR system (Illumina, San Diego, CA, USA). Cycle protocol was set to 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, 15 s at 55 °C and 10 s at 72 °C. Custom primer sequences corresponding to the conserved regions of respective virulence genes in ST were used to compared to a reference house-keeping gene belonging to 16S-rRNA (Table 2-1).

Statistical Analysis

Data were analyzed using a Student's t-test to determine the significant difference ($p < 0.05$) between the untreated control and the separate treatment with the individual compound.

Results

Antimicrobial Effect on Bacterial Growth

A microdilution assay was used in order to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each individual compound and to determine phenolic acids with bactericidal/bacteriostatic

antimicrobial potential when used individually against ST. The MIC was taken as the concentration at which there was no visible growth of bacteria in LB broth (Table 2-2). The MICs of GA, PA and VA were 3.5, 2.0 and 1.5 mg/mL, respectively. The MBC was determined as the concentration at which bacteria showed no detectable growth on LB agar after plating from LB broth aliquots. The MBCs of GA, PA and VA were 4.5, 2.0 and 2.0 mg/mL, respectively. (Figure 1). These concentrations exhibited the most acute antimicrobial effect; however, significant reduction as compared to the control was also recorded at lower concentrations. GA exhibited a statistically significant ($p < 0.05$) inhibition of bacteria up to 2.5 mg/mL; PA exhibited a statistically significant inhibition up to 1.5 mg/mL; VA exhibited a statistically significant inhibition up to 1.0 mg/mL. Calculating the ratio of MBC:MIC found the three compounds to be bactericidal, as they fell on or below a ratio of 2.

Evaluation of Resistance Development

Bacteria were transferred from a well containing sub-MICs of each compound that permitted substantial bacterial growth over time. Transfers were performed 12 times (generations) while the MIC from the aliquot was recorded and used as the indicator for changes in susceptibility or resistance (Figure 2). Though no continuous or consistent increase during this period was shown, by the end of the 12 passages, the MIC values for GA stabilized at 3.5 mg/mL, while PA increased to 2.5 mg/mL, showing an increase of 1 mg/mL from the beginning of the experiment, while VA remained at 1 mg/mL from the 4th passage.

Relative Gene Expression of ST

Changes in the expression of ST virulence genes were evaluated through the use of qRT-PCR and gene-specific primers for *fliC, hilA, hilD, invH, prgH, prgK* and *sipA*. When compared to an untreated control, samples from bacteria treated with individual phenolic acids mostly showed a downregulation of examined virulence genes (Figure 6). All virulence genes from bacteria treated with GA showed downregulation when compared to the untreated control, with *hilA, hilD, invH, prgH, prgK* and *sipA* being significant ($p < 0.05$) by 2.51, 3.02, 2.35, 2.24, 2.33 and 2.06 log-folds, respectively. PA-treated samples demonstrated statistically significant ($p < 0.05$) downregulation in *fliC, hilA* and *invH* by 1.87, 2.0 and 0.78 log-folds, respectively, with only a numerical decrease in all but *prgH*. Samples treated with VA showed significant upregulation for *fliC* with an increase of 2.35 log-folds, while the rest of the genes showed a numerical decrease.

Discussion

The use of plant-derived polyphenolic compounds against Gram-negative pathogenic bacteria has gained notice because of the need for novel antimicrobial agents that can counteract the increasing trends of antibiotic resistance. Though polyphenolic compounds belonging to groups such as flavonoids, tannins and catechins have received much attention for their antimicrobial potential, many researchers have focused on compounds belonging to phenolic acids. These comprise at least one-third of the polyphenolic compounds found in plants, are readily

solubilized without need for toxic solvents, and have been proven to have antibacterial potential (A. Borges et al., 2013; Kaurinovic & Vastag, 2019). The low molecular weight of phenolic acids compared to other polyphenols has been cited as a factor that potentially allows for more interactions with bacterial structures, resulting in greater antimicrobial effects (Ravichandran et al., 2011). Though the compounds evaluated in this study share a strong structural similarity, differences in their functional groups could play an important role when it comes to efficiency, half-life and target specificity (de Camargo et al., 2017). Hydroxyl (-OH) and methoxy (-OCH_3) groups have been cited as key attributes that affect molecular interactions, as these create differences in polarity and oxidation rates (Santos et al., 2019).

The phenolic acids evaluated in this study have been previously noted by other researchers, primarily for their roles as antioxidants, documented use as food preservatives, and antimicrobial potential against Gram-negative bacterial pathogens (N. Kumar & Goel, 2019). GA, PA and VA specifically have been shown to inhibit the growth of *Pseudomonas* spp., *Listeria monocytogenes*, *Mannheimia haemolytica*, *Pasteurella multocida*, *Escherichia coli* and some strains of *Salmonella* spp., as well as being able to reduce the pathogenicity of other bacteria such as *Proteus mirabilis* (Aldulaimi, 2017; Bernal-Mercado et al., 2020; López-Romero et al., 2018; Torzewska & Rozalski, 2014). However, the effectiveness against ST, specifically, has not been consistently documented, in addition to the mechanisms of action underlying the molecular interactions that precede cell death remaining poorly understood. The results from this study suggest that GA, PA and VA have

concentration-dependent antimicrobial capability against ST, with all of them being able to inhibit bacterial growth to the point of non-detection at specific concentrations, as exhibited by the MBC values (4.5, 2 and 2 mg/mL, respectively). GA was outperformed by PA and VA in both MIC and MBC tests, with PA and VA requiring lower concentrations of the compound to significantly inhibit growth. However, it is important to note that bacteria treated with VA were further challenged by the ethanol used for preparing the solution. Considering the factors mentioned above, PA could be considered the better of the three compounds as it is more potent and exhibits its effects without interference from additional solvents.

The emergence of antimicrobial resistance poses one of the main concerns and challenges for novel antimicrobial discovery, development, and sustainability (Qian et al., 2020). The use of individual compounds in particular has become increasingly challenging as research has demonstrated that bacteria have intrinsic mechanisms that serve to minimize susceptibility to a variety of known bioactive molecules, in addition to having the ability to adapt to new ones (Dcosta et al., 2011; Wright, 2007). In this study, resistance to individual compounds was measured throughout 12 passages and found ST to still be susceptible to all phenolic compounds at the end of the passages. Even though there was an increase of 1 mg/mL for the MIC value of GA and PA, the concentration did not increase beyond that point. VA's MIC remained stable from early on in the experiment and showed no significant increase at the final passage. The development of resistance could be related to how easily the compound could be oxidized by environmental factors or by the bacteria (Sánchez-

Maldonado et al., 2011b). The methoxy group of VA makes it more stable and less prone to oxidation than GA and PA, which are more susceptible to direct oxidation of their respective hydroxyl groups by bacteria and to the effects of media acidification as a result of bacterial growth (Z. Cheng et al., 2002). With this in mind, the observed increase in resistance to GA and PA could be an indirect result of changes in the metabolic pattern of bacteria and not a direct response to the compounds (N. Kumar & Goel, 2019). Future studies will involve more extensive and numerous passages, as well as experiments that further elucidate the mechanism of action and bacterial response, which will be important for preventing the risk of developing resistance and potentially novel ways in which it can be actively counteracted.

In addition to growth inhibition and cell death, a reduction in virulence as a result of treatment is a desired outcome, especially for ST cases, which are known to be invasive pathogens and use these strategies to prolong survival in the host and create further health complications (Fàbrega & Vila, 2013). Relative expression was measured for genes responsible for synthesis and the assembly of the T3SS, specifically *hilA*, *hilD*, *invH*, *prgH* and *prgK* as well as genes related to other aspects of virulence such as *fliC*, which increases motility and *sipA* which is responsible for intracellular survival. The results of this experiment demonstrated a downregulation after individual treatment with GA, PA and VA in most of the genes that were tested for, with the exception of the notable upregulation of *fliC* in VA and a slight numerical increase in *prgH* in PA. The most significant reduction in all genes was in bacteria treated with GA as all genes related to the structural assembly of the T3SS

that were tested for were downregulated, with the addition of one related to intracellular survival. Downregulation in PA was more evident for *fliC,* which is important for motility, but also in *hilA* and *invH*, the former being a key master regulator for the initiation of the T3SS and the latter being a pilot protein responsible for the co-localization of the base of the needle in the bacterial inner membrane. Though the proposed mechanism of action for these compounds is damage to the membrane and bacterial structure, previous research has reported that damage to the envelope of ST, particularly alterations to the LPS of the outer membrane, leads to the downregulation of genes related to flagellum synthesis and assembly (Spöring et al., 2018). The same study found other virulence-related genes from SPI-1 and SPI-2 to be downregulated. To our knowledge, the specific effects of phenolic acids on changes in virulence have not been examined before for ST, leaving room for further exploration by applying these products for reducing the incidence of invasive salmonellosis, in addition to revealing one of the potential routes by which plant extracts reduce virulence and could have protective qualities towards host cells, as has been reported in the past (Salaheen et al., 2014; van Ampting et al., 2010). In this specific experiment, GA demonstrated the greatest amount of promise as it was able to reduce the expression of most virulence genes, though PA should not be discounted since it also reduced key regulatory genes. The way in which VA impacts virulence is yet unclear, though an increase in *fliC* could be a sign of stress that could warrant deeper investigation into that possible mechanism. These differences in outcome are favorable if in the future there is an attempt to use these compounds synergistically.

Plant extracts are promising candidates because the diversity of bioactive compounds they contain are thought to exert multiple adverse actions over a given bacterium, reducing the risk of developing resistance (Gupta & Birdi, 2017). However, understanding the mechanisms of action of the individual constituents of these extracts will allow for their use in a more guided and efficient manner, even allowing for the possibility of rescuing conventional antibiotics (Ejim et al., 2011; Salaheen, Peng, et al., 2017b). The antimicrobial efficiency of individual phenolic compounds is not as effective as that of leading antibiotics or to that of many raw plant extracts, but in the future may provide alternatives to infections considered as untreatable by antibiotics. This study also presents a way to uncover new targets and mechanisms that could be further exploited when developing and evaluating novel antimicrobials, in addition to exploring the cellular response to weak phenolic acids that lead to the changes in shape and gene expression patterns that were seen in this study, which have not been reported in the past.

Conclusions

In the current study, widely distributed phenolic acids—GA, PA and VA—were tested for their antimicrobial potential against ST. Results show that these compounds have the capacity to inhibit the growth of ST and act as a bactericidal in a concentration-dependent manner. After 12 passages, there was no significant development of resistance in ST to these compounds. In addition to this, GA and PA led to the significant downregulation of important virulence gene regulators—though

VA only showed a numerical decrease, it might have other effects over ST. This knowledge can be used in the future to develop novel therapeutic approaches and techniques that exploit the effects that phenolic acids have over ST, in order to control the pathogen and reduce the severity of infections.

Figures and Tables

Table 2-1. Primers used in determining gene expression of ST strains.

Gene	Protein	Primer Sequence (5'-3')
16S rRNA	16S ribosomal protein	F: GTAGTACGATGGCGAAACTGC
		R: CTTCTCGACCCGAGGGACTT
fliC	flagellum subunit	F: GCAGATGACGGTACATCCAA
		R: CCAGATCAGGCTGTGCTTTA
hilA	SPI-1 transcriptional regulator	F: AATGGTCACAGGCTGAGGTG
		R: ACATCGTCGCGACTTGTGAA
hilD	SPI-1 transcriptional regulator	F: CTCTGTGGGTACCGCCATTT
		R: TGCTTTCGGAGCGGTAAACT
invH	adherence and invasion	F: GGTGCCCCTCCCTTCCT
		R: TGCGTTGGCCAGTTGCT
prgH	T3SS needle support at membrane	F: TGAACGGCTGTGAGTTTCCA
		R: GCGCATCACTCTGACCTACCA
prgK	T3SS needle support at membrane	F: GGGTGGAAATAGCGCAGATG
		R: TCAGCTCGCGGAGACGATA
sipA	actin binding protein for cell invasion	F: CGTCTTCGCCTCAGGAGAAT
		R: TGCCGGGCTCTTTCGTT

Table 2-2. Antibacterial effect of phenolic acids.

Treatment	MIC (mg/mL)	MBC/MIC	Bactericidal/Bacteriostatic
Gallic acid	3.5	1.28	Bactericidal
Protocatechuic acid	2	1	Bactericidal
Vanillic acid	1.5	1.33	Bactericidal

Figure 2-1. Concentration-dependent antimicrobial evaluation for individual phenolic compounds by determining minimum bactericidal concentration (MBC) (log CFU/mL) for *Salmonella enterica* serovar Typhimurium (ST) treated with increasing concentrations (0.25–4.5 mg/mL) of gallic acid (GA) (**A**), protocatechuic acid (PA) (**B**) and vanillic acid (VA) (**C**). Letters denote statistically significant difference ($p < 0.05$) as compared to control and between concentrations (a–e).

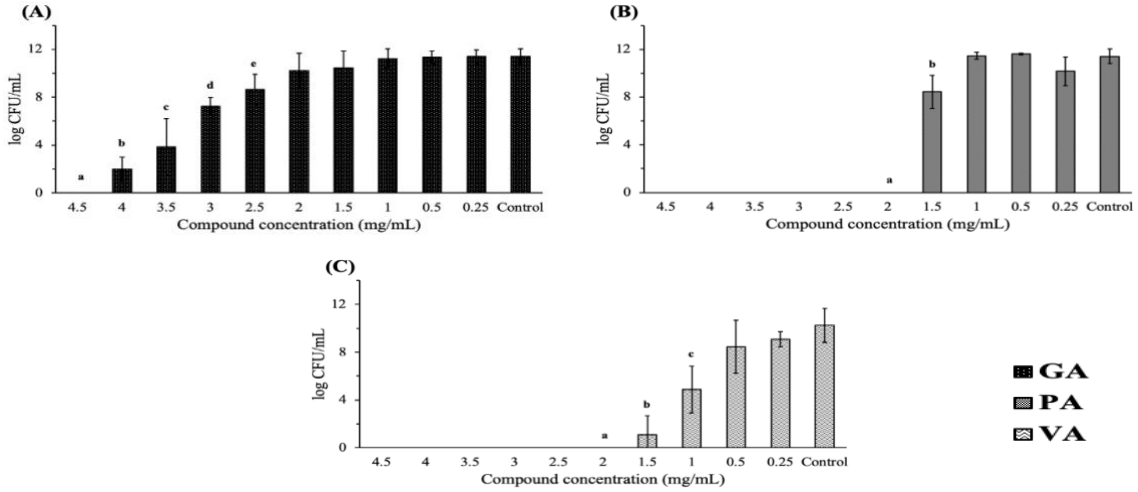


Figure 2-2. Evaluation of changes in susceptibility of ST individually treated with GA, PA and VA over 12 generations.

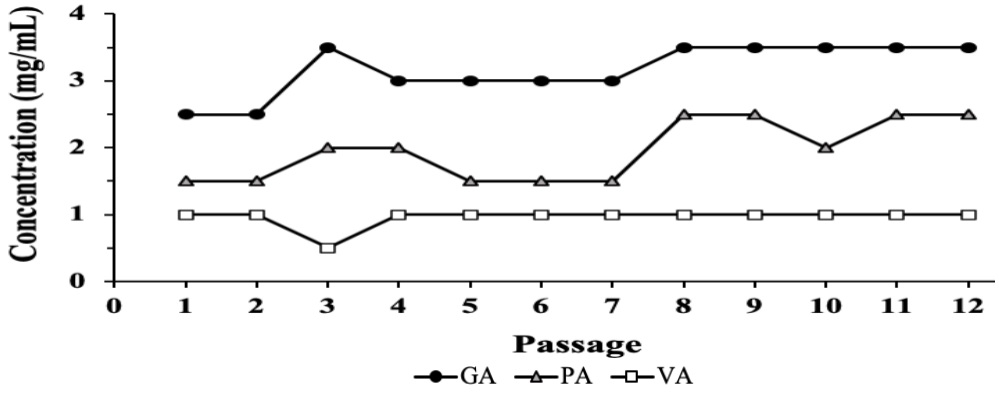
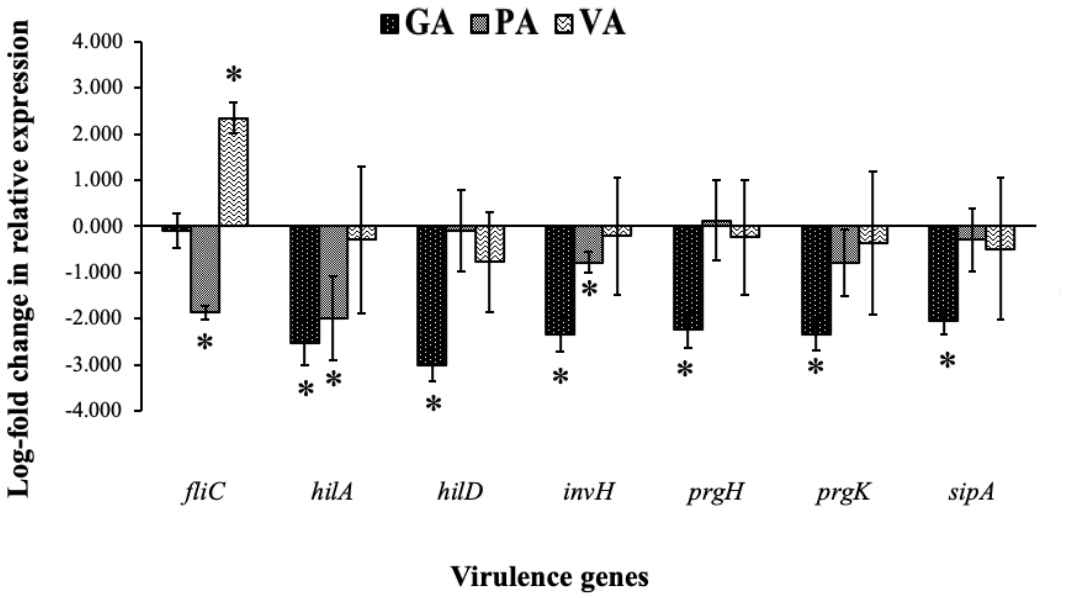


Figure 2-3. Evaluation of log-fold changes of ST virulence genes through measurements of relative gene expression. Significant difference in treatment as compared to control ($p < 0.05$) was denoted with an asterisk (*).



Chapter 3: Plant-derived phenolic acids limit pathogenesis of *Salmonella* Typhimurium and protect intestinal epithelial cells during their interactions

Introduction

Non-typhoidal *Salmonella enterica* is a prominent pathogen that is estimated to be responsible for millions of cases around the world, many of which result in death, making it a major causative agent of gastroenteritis, with one of its main infectious routs being the consumption of contaminated food (Majowicz et al., 2010b; Stanaway et al., 2019). Despite the numerous measures taken to reduce its prevalence, non-typhoidal *S. enterica* also remains one of the major etiological agents of gastrointestinal disease in the USA, as it is the main bacteria responsible for domestically acquired bacterial cases of illness, hospitalizations, deaths, and outbreaks (Interagency Food Safety Analytics Collaboration, 2022; Scallan et al., 2011). *S. enterica* has over 2500 serovars, many of which are common etiological agents of disease and outbreaks in the USA (Andino & Hanning, 2015). However, *S. enterica* serovar Typhimurium (ST) remains one of the most clinically relevant serovars given its ubiquitous prevalence across the environment and various food categories, as well as for its broad antibiotic resistance pattern, and well adapted virulence factors that aid it during infection of its human hosts (Fàbrega and Vila,

2013; Jackson et al., 2013; CDC, 2019; Wang et al., 2019). Though most cases of infection with ST are considered to be self-limiting and mostly manifest as a gastrointestinal disease, infections have been known to persist as they develop into systemic infections that can spread to bones, joints and bloodstream, especially within vulnerable populations, such as children, the elderly, immunocompromised patients and people that cannot clear out the infection in time or are suffering from a secondary infection (Chen et al., 2013). Various control measures have been proposed, including vaccination, and development of new generation antibiotics, however, these have been met with limitations that currently lower their current applicability as proper preventive measures and as treatments, as they also require more research to be implemented safely and effectively (Crump et al., 2023). On the other hand, extracts from plants have also demonstrated promising antimicrobial activity against ST and have the potential to be abundant sources of antimicrobial compounds that do not contribute to the development of antibiotic resistance while also not affect host cells or the host probiotic and commensal bacterial microflora (Patangia et al., 2022; Salaheen et al., 2018; Salaheen, Kim, et al., 2017).

Purified plant-derived compounds commonly found in plant extracts have also been proposed as potential methods of control against ST given their diversity and potential for multiple mechanisms of action that reduce the likelihood of antibiotic resistance, while preserving and sometimes promoting the growth and integrity of pro-commensal bacteria in the gastrointestinal (GI) tract of the human host (Ecevit et al., 2022). Plant extracts are known to contain thousands of bioactive molecules,

many of which are polyphenolic compounds (Y. Zhang et al., 2022). These phenolic compounds can be further categorized as either flavonoids, tannins, stilbenes, lignans or phenolic acids, with the latter accounting for one third of the total phenolic compounds found in some plants (N. Kumar & Goel, 2019). Phenolic acids in particular have been reported to be bioactive, as they display antimicrobial potential against various species of bacteria *in vitro*, have while also being useful as food preservatives and antioxidants (Beya et al., 2021; N. Kumar & Goel, 2019). Previous studies have tested the efficacy of specifically gallic acid (GA), protocatechuic acid (PA) and vanillic acid (VA) against ST *in vitro*, with findings demonstrating these compounds to exert antimicrobial activity against the bacteria, while also showing a downregulation of important virulence genes associated with invasion of epithelial cells (Alvarado-Martinez et al., 2020).

The capability to adhere to and later invade multiple cell types within the gastrointestinal (GI) tract of a human host, especially intestinal epithelial cells, is an important virulence factor of ST that creates further challenges for effective treatments (Ménard et al., 2022). These virulence factors serve as survival mechanism that allows the bacteria to avoid natural shedding by the host, as well as neutralization by the immune system, while also allowing the pathogen to proliferate intracellularly and potentially spread systemically if it reaches deeper layers of tissues (Diacovich et al., 2017). Recent studies have revealed numerous pathways that ST utilizes for invasion of non-phagocytic epithelial cells, providing ST with numerous mechanisms of entry, however one of the best studied and well described mechanisms of action

remains the type 3 secretion system (T3SS), encoded in the *Salmonella* pathogenicity island 1 (SPI-1), which is highly conserved, especially in ST and has also been established as a crucial model for studying the host-pathogens interactions of ST within the context of infection both *in vitro* and *in vivo* (H. Bao et al., 2020; Chong et al., 2019). In addition to the T3SS, ST uses other virulence like flagella-mediated motility to penetrate the layers of mucus and commensal bacteria that line the GI tract in order to reach the epithelial cells and initiate invasion (Furter et al., 2019). For these reasons, in addition to testing compounds for their direct antimicrobial capability, researchers have also suggested testing the anti-virulence potential of novel compounds, as these could serve as additional targets, while also helping in the amelioration of invasion (Birhanu et al., 2018).

Considering the importance of virulence and the previously studies potential of phenolic acids to inhibit ST and reduce relative expression of virulence genes, the purpose of this study is to test the efficacy of GA, PA, and VA for preventing adhesion and invasion of ST during the infection of epithelial cell lines. For these purposes INT-407 cell lines were chosen as a model, considering their susceptibility to ST invasion (Lara-Tejero & Galán, 2009). GA, PA, and VA were tested for cytotoxicity against INT-407, and later used as treatments against ST infection, which was determined directly through calculating colony forming unit (CFU)/mL, in addition to measuring the relative expression of virulence genes associated with regulation of the T3SS system and other virulence factors.

Materials and Methods

Bacterial strain and growth conditions

Salmonella enterica serovar Typhimurium (ATCC 14028) (ST) was used for this study as the infectious agent being studied. Luria-Bertani (LB) agar (Becton, Dickinson and Co., USA) was used as the medium on which ST was grown at 37°C under aerobic conditions (Thermo Fisher Scientific Inc., USA). ST was cultured on LB agar using streaking from a glycerol stock and incubated overnight to obtain individual colonies, which were used later to prepare bacterial suspensions for future experiments.

Cell line and growth conditions

Human intestinal epithelial cells (INT-407) (ATCC CCL-6) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Corning Cellgro, USA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals®, USA) and 50 μg/mL gentamycin (IBI Scientific, USA). All DMEM used in this study was prepared with 10% FBS unless stated otherwise for a specific assay. Cells were incubated at 37°C, in 5% CO_2 and 95% humidity to achieve a 90% confluent monolayer (Thermo Fisher Scientific Inc., USA). Confluent cells were washed with PBS, detached from the cell culture flask using 0.25% trypsin (Gibco, USA), centrifuged, and resuspended for seeding into either a 96- or 24-well plate with fresh DMEM, free of gentamycin, and incubated under the same conditions until 90% of the monolayer is confluent before using for experiments.

Compounds and stock solution preparation

The phenolic acids that were tested in this study were purchased from commercial vendors in powdered form for preparation of working stocks. Gallic acid (GA) (Acros Organics, USA) and protocatechuic acid (PA) (Sigma-Aldrich, USA) by dissolving in sterile deionized water, while vanillic acid (VA) (Alfa Aesar, USA) was prepared by dissolving in 30% ethanol (Pharmco-Aaper, USA). Aqueous solutions of sodium hydroxide (NaOH) and hydrochloric acid (HCl) were both prepared to a molarity of 10 M and used for adjusting pH values of other solutions in this study. GA, PA, and VA were prepared to contain a concentration of 10 mg/mL. Phosphate buffer saline (PBS) was prepared to a pH of 7.2. Working stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) (USA) was prepared by dissolving MTT powder in 1 x PBS to a concentration of 5mg/mL.

Phenolic acid antimicrobial potential within DMEM and at alternate pH ranges

The antimicrobial potential of GA, PA, and VA was tested in DMEM by determining the minimum inhibitory concentration (MIC) of each compound, using the Clinical and Laboratory Standards Institute (CLSI) M100 guidelines (CLSI, 2023). Further analysis to determine minimum bactericidal concentration (MBC) and concentration at which there was no detectable ST growth, were performed through microplate dilution assay, as performed previously, with some modification (Salaheen et al., 2016a). Briefly, an overnight culture of ST from an LB agar plate was used to prepare a bacterial suspension in PBS, which was adjusted to an optical density (OD_{600})

of 0.1, which equals 10^8 colony forming units (CFU)/mL. This bacterial suspension was diluted further for subsequent assays. Increasing concentrations of GA, PA, and VA were prepared, ranging from 0.0625 to 5.0mg/mL in DMEM and inoculated with ST to a final bacterial load of 10^4 CFU/mL, in addition to an untreated control. These were later incubated at 37°C and 5% CO_2 for 24h. After incubation, concentrations exhibiting no visibly detectable growth were determined to be the MIC and confirmed by subculturing and re-inoculating in a new LB agar plate, in addition to having 20µL aliquots collected for further microplate dilution assay.

To determine changes in antimicrobial efficacy of phenolic acid under various pH ranges within DMEM, increasing concentrations of phenolic acids within DMEM were adjusted to a pH between 3 and 10 and tested for inhibition of ST, as has been describe previously with some modifications (Alvarado-Martinez et al., 2023). Briefly, aliquots of DMEM were either independently supplemented with increasing concentrations of GA, PA and VA ranging from 0.0625 to 5.0 mg/mL or left untreated as the control lacking phenolic acids. In addition to testing supplementation with increasing concentrations of phenolic acids, these were tested after adjusting to a pH ranging from 3 to 10 using HCl or NaOH and measured using a pH probe (Metler Toledo, USA). Samples were inoculated with ST to achieve a final volume of 10^4 CFU/mL and incubated for 24h at 37°C with 5% CO_2. Growth of ST was determined visually, and absence of growth was confirmed by subculturing form the sample to a new LB agar plate to allow for re-growth. Growth was interpreted as a decrease or loss of antimicrobial potency.

MTT assay for evaluating cytotoxicity and cell viability

Cytotoxic effects of GA, PA, and VA against INT-407 cells were evaluated using a the MTT reduction assay as has been describes in the past, with some modifications (P. Kumar et al., 2018; Tolosa et al., 2015). Briefly, INT-407 cells were seeded into 96-well plates and grown under 5% CO_2 at 37°C until a 90% confluent monolayer is formed. Confluent INT-407 cells were individually treated and incubated with increasing concentrations of either GA, PA, or VA, while the untreated control contained only DMEM. Additional cytotoxicity tests were performed to determine the optimal pH for INT-407 cell viability and proliferation by adjusting DMEM alone to a pH between of 3 to 10, in addition to testing changes in cytotoxicity of GA, PA, and VA at the pH that supports cell line survivability. After incubation of the designated treatment testing condition, the media and treatment for each well were carefully aspirated, followed by an additional rinse with 100µL of PBS once. Once PBS was removed, 100µL MTT incubation media (1:10 MTT reagent to DMEM without FBS) was added to every well and plate was incubated for 2h under 5% CO_2 at 37°C to allow for the formation of formazan crystals. MTT incubation media was carefully removed leaving undissolved formazan crystals intact, which were later solubilized with 200µL of dimethylsulfoxide (DMSO) and incubating for 15 mins with agitation. After final incubation with DMSO, optical density (OD) read at 550 nm and recorded for calculating cell survival. This assay was performed 3h and 24h after treatment with

phenolic acids with the former being the time later used for adhesion and invasion experiments. INT-407 cell was experienced adverse cytotoxic effects whenever cell viability was reduced by >20%, meaning cell viability must remain at 80% or above for the compound and corresponding concentration to be considered non-cytotoxic (Cannella et al., 2019).

Adhesion and invasion assay.

Impairment of ST adhesion and invasion to INT-407 after treatment with phenolic compounds was evaluated as described before with modifications (Peng et al., 2015). Briefly, a 90% confluent monolayer of INT-407 (~5 Log cell/mL) was used to seed a new 24-well plate. Once the seeded cells reached a 90% confluence, they were ready for infected with ST. A bacterial suspension of ST was prepared by resuspending colonies from an overnight culture in PBS and adjusted to an OD_{600} of 0.1, which was used to inoculate the 24-well plates with INT-407 monolayer in either DMEM, or DMEM supplemented with experimental doses of GA, PA, and VA, as well as GA, PA and VA neutralized to pH 7. A final multiplicity of infection (MOI) of 10 (1:10 ratio of bacteria per cell) was used as the concentration for infection. After inoculation of ST, 24-well plates were incubated for 4 hours, under 37°C and 5% CO_2 to allow time for cell-bacteria interaction. As defined previously for both adhesion and invasion assays, prior to plating, INT-407 cell monolayers were washed three times with PBS, eliminating free unadhered bacteria from the media, with wells for invasion assay being further treated with 250 μg/mL of gentamycin for 1h, eliminating non-invasive

bacteria. For both adhesion and invasion assays, cell monolayers were detached and lysed using 0.1% Triton X-100, with resulting suspension being serially diluted, plated on LB agar and incubated at 37°C to later determine Log CFU/mL of adhered and invasive bacteria.

Assessing relative expression in INT-407 cells and ST

Concentrations of GA, PA, and VA that were previously determined to be effective in reducing adhesion and/or invasion during ST infection, and that did not exert deleterious cytotoxic effects were further used to treat both ST-infected and uninfected monolayers of INT-407 cells to later assess changes in relative gene expression for both bacteria and cell line. After the 4h treatment period, entire contents of the well were collected for RNA extraction, including supernatant with unadhered bacteria and detached cells was collected, as well as the cell monolayer with or without bacteria, which was detached using 0.1% Triton X-100. Total RNA was extracted from the samples utilizing a TRIzol (TRI Reagent®) (Molecular Research Center Inc., USA) extraction protocol, as described previously, with some modification (Rio et al., 2010). Briefly, RNA from infected cell lines and uninfected ones were extracted separately, using the same protocol, which first involved centrifuging samples at 14,000 x *g* for 10 min to collect pellet containing either INT-407 cells, or INT-407 with ST, which was later resuspended in ice-cold 1 X PBS as the first wash step. Centrifuge step was repeated and pellet was resuspended in TRI Reagent® (Molecular Research Center Inc., USA) to homogenize it and release total RNA from sample, which was subsequently

isolated. After extraction, total RNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., USA) and standardized before using them as templates for cDNA synthesis with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) following the instructons outlined by the manufacturer. Synthesis of cDNA was conducted using a thermocycler protocol set to incubate for 25°C for 10m, 37°C for 120m, and 85°C for 5m.

Measuring gene expression through qRT-PCR assay

Changes in the relative expression of genes associated with inflammatory response in INT-407 cell as well as for virulence genes in ST were assessed using qRT-PCR with primers targeting genes associated with these functions. Custom primers were designed to target conserved regions of each respective gene being evaluated, with genes related to inflammatory response in INT-407 being compared to a GAPDH housekeeping gene (Table 3-1) (Y. H. Hong et al., 2006; Rothwell et al., 2004; Smith et al., 2005), while genes related to virulence and invasion regulation in ST were compared to a 16S-rRNA housekeeping gene (Table 3-2) (Peng, Tabashsum, et al., 2018). Reactions for the qRT-PCR were carried out using the PerfeCTa SYBR Green Fast Mix following the guidelines in the manufacture protocol (Quanta Bio, Beverly, USA), with the reads and quantification being performed in the Eco Real-Time PCR system (Illumina, USA). Thermal profile for the qRT-PCR protocol was set to have 30s at 95 °C, followed by 40 cycles of 5s at 95°C, 15s at 55°C and 10s at 72°C. The average Cq value sreported by the qRT-PCR were recorded and later used to

calculate RQ value through normalization with the respective housekeeping gene and untreated control of the assay being performed. Values were reported and visualized as Log functions of the RQ values.

Statistical analysis

Student *t*-test was used to determine statistically significant ($p<0.05$) changes between individually treated groups with the untreated control, as well as evaluated significant differences between treatment groups using ANOVA.

Results

Evaluation of cytotoxicity of phenolic acids in INT-407 cells using MTT assay

Increasing concentrations of DMEM individually supplemented with either GA, PA, or VA were prepared and tested in an INT-407 cell monolayer to determine the potentially cytotoxic concentrations of the phenolic acids at various concentrations (Figure 3-1). MTT assays were performed on the cells treated with increasing concentrations of phenolic acids for 3h (Figure 3-1A) and 24h (Figure 3-1B) with measures being compared to an untreated control at the same timepoint. concentration dependent cytotoxicity at 3h for GA resulted in a 28.15% decrease in cell viability at 0.5mg/mL, followed by a decrease in 41.68% at 2mg/mL and 48.60% at 5mg/mL. PA showed a decrease in cell viability of 62.61% at 4.0mg/mL, while VA lowered cell viability by 21.68%, 22.87% and 87.98% at 1.5mg/mL, 2.5mgmg/mL and 4.0mg/mL. After exposure to phenolic acids for 24h, cells treated with GA had a

decrease in cell viability by 26.68% at 0.25mg/mL and was most cytotoxic at 2mg/mL with a reduction of 70.93%. Treated with PA reduced viability beginning at 2.5mg/mL by 21.34% and had a major decrease at 4.0mg/mL by 66.89%. Cells treated with VA showed an initial decrease in viability of 21.65% at 1.0mg/mL with the most significant decrease being detected at 4.0mg/mL with a 91.50% decrease in viability. Measuring the natural pH of increasing concentrations of each phenolic acid combination in DMEM showed these to be inversely correlated, as increasing concentrations led to a decrease the pH (Figure 3-1C). At a concentration of 0.0625mg/mL, GA, PA and VA had a pH of 7.34, 7.41 and 7.55, respectively, with these decreasing to 4.83, 4.88 and 6.98, respectively at 4mg/mL.

Determining effects of pH on cytotoxicity in INT-407 cell using MTT

Additional MTT assays were performed on INT-407 cells incubated in DMEM adjusted to a pH ranging from 3 to 10 in order to determine the effects of pH alone on cell viability by comparing these to unmodified DMEM (Figure 3-2). MTT assays for cells incubated with the pH adjusted DMEM were performed at 3h (Figure 3-2A) and 24hr (Figure 3-2B). When assessing changes in cell viability at 3h, the most significant decrease in viability was of 93.00% and 69.23% at pH 3 and 4, respectively, however viability improved as pH increased, with pH 6 and 10 showing an increase in proliferation of 0.83%, and 1.87%, while pH 7 only had a reduction in viability of 2.21%. When extending incubation time to 24h, viability at pH 3 and 4 remained the lowest with a decrease in 92.50% and 88.87%, respectively, though it

also improved as pH increased, with pH 6 only showing a 5.61% reduction in viability, while pH 7 had a proliferative effect on INT-407 cells increasing viability by 4.86%. In order to evaluate whether cytotoxic effects of phenolic acids could be neutralized through pH modulation, concentrations of phenolic acids previously determined to be cytotoxic were adjusted to a pH of 7 with MTT assays being performed at 3h (Figure 3-3). After adjusting these to pH 7, GA (Figure 3-3A) saw improvement in cytotoxicity at 1.0mg/mL (54.11%), but remained below the 80% cutoff, however, at 0.5mg/mL, there was an increase to 92.45%, and 86.25% at 0.25mg/mL. Adjusting PA (Figure 3-3B) had a similar effect at 4.0mg/mL increasing viability to 75.08%, however viability decreased to 79.59% and 71.03% for 2.0mg/mL and 1.0mg/mL, respectively. Adjusting VA (Figure 3-3C) at 4.0mg/mL had an improvement in viability exhibited by an increase to 95.83%, while 2mg/mL showed a reduction to 83.65% and 1.0mg/mL to 109.58%, all above the 80% cutoff established initially.

Antimicrobial potency of phenolic acids in DMEM

Growth of ST was quantified by calculating Log CFU/mL in samples inoculated and incubated in only DMEM, or in DMEM individually supplemented with increasing concentrations of either GA, PA, or VA, and growth pattern was compared to that of the untreated control to detect antimicrobial activity of the phenolic acids in DMEM (Figure 3-4). Growth of ST in the untreated control was 10.60 Log CFU/mL. Supplementation with GA reduced ST to undetectable levels at

between the concentrations of 0.125mg/mL and 3.5mg/mL but showed growth at 4.0mg/mL (7.96 Log CFU/mL), 4.5mg/mL (10.87 Log CFU/mL) and 5.0mg/mL (9.86 Log CFU/mL). PA reduced the bacterial load of ST to undetectable levels at 4.5mg/mL and above, with the next significant reduction in ST being observed at 0.5m/mL (7.42 Log CFU/mL). VA was able to reduce ST to undetectable levels at 4.0mg/mL and above, while bacterial load increased as concentration decreased, with 3.5mg/mL showing the next most significant reduction of ST (4.62 Log CFU/mL).

Further tests were performed to determine antimicrobial potential of increasing concentrations of phenolic acids (0.0625-5.0mg/mL) in DMEM after adjusting the solution to a pH value ranging from 3 to 10, which was compared to an un-supplemented DMEM control also adjusted to specific pH values (Table 3-3). Adjusting pH changed the antimicrobial activity of phenolic acids in DMEM compared to their natural pH at their respective concentration, leading to either inactivation in previously determined lethal doses of the compounds or improved potency in previously inactive doses, however, changes in pH did not have an impact on growth when in DMEM alone, as ST growth was detected in all values within the pH range tested. In samples supplemented with GA, there was a loss of antimicrobial function at all pH values for 0.0625mg/mL, while those for 0.125mg/mL lost antimicrobial function between the range of pH of 4 and 7. Antimicrobial potency was regained at pH 7 for concentrations of 0.25mg/mL and above but remained neutralized for all concentrations at pH 4. Similarly, PA showed inactivation at 0.0625mg/mL for all pH ranges but was active at 0.125mg/mL and above when the

pH was 3. The pH range between 6 and 8 rendered PA ineffective regardless of concentration, though it regained antimicrobial activity at 1.0mg/mL for pH 9 and 10, as well as at pH 4 when increasing the dose to 1.5mg/mL. VA retained antimicrobial potency up to the concentration of 3.5mg/mL where it lost activity at pH 6 and above but was effective at 4.0mg/mL regardless of the pH. At 2.5mg/mL, VA lost total activity at every pH tested. When adjusted to pH 7, only GA at 0.25mg/mL and above, as well as VA at 4.5mg/mL were able to completely inhibit ST.

Changes in host-pathogen interactions between ST and INT-407 after treatment

The adhesion capability of ST to an INT-407 cell monolayer as a result of treatment with each phenolic acid was individually evaluated considering the cytotoxicity of each compound, as well as their previously determined effective antimicrobial concentrations (Figure 3-5A). Unadhered bacteria suspended in the media were removed and adhered ST were quantified from a cell monolayer after 0.1% Triton X-100 lysis, which releases the adhered bacteria and allows for calculating Log CFU/mL from a microdilution plating assay. Treatment with GA only showed a slight numerical decrease in adhered bacteria (0.69 Log CFU/mL) at 1mg/mL, while PA showed no to have no adverse effect on adhesion at any concentration. Exposure to VA at 4.0mg/mL was the only treatment to show a statistically significant reduction ($p < 0.05$) of adhesion, shown in a 3.19 Log CFU/mL reduction of ST, as compared to the untreated control. Further assays were performed by adjusting the pH of the phenolic acids and DMEM solution (figure 3-

5B). The change in pH alone had no effect on the adhesive capability of ST. In the case of GA, slight numerical decreases of 0.61, 0.46 and 0.755 Log CFU/mL were observed at 1.0, 0.5 and 0.25mg/mL. PA demonstrated a slightly numerically higher reduction of adhered bacteria, calculated to be 1.28, 1.20 and 0.82 Log CFU/mL for concentrations of 4.0, 2.0 and 0.5mg/mL, while 1mg/mL showed to have a significant ($p < 0.05$) decrease of 1.54 Log CFU/mL. VA showed a statistically significant ($p < 0.05$) reduction of 3.04, 2.67 and 1.94 Log CFU/mL at concentrations of 4, 2 and 1mg/mL, while only showing a slight numerical decrease of 0.87 Log CFU/mL at 0.5mg/mL.

The invasive capability of ST to an INT-407 cells monolayer during treatment was evaluated in a similar way to adherence, with the addition of a gentamycin treatment step to eliminate all external ST, leaving only intracellular ST that was later collected by lysing the cell monolayer with a 0.1% Triton X-100 lysis, and quantified using the microdilution assay (Figure 3-6A). Treatment with GA showed to have statistically significant ($p < 0.05$) reduction on invasion of ST at all the concentrations tested, with 0.5mg/mL having the greatest reduction (2.27 Log CFU/mL), followed by 1.0mg/mL (1.89 Log CFu/mL), 0.25mg/mL (1.70 Log CFU/mL) and 0.125mg/mL (0.99 Log CFU/mL), though no statistical difference was found between the resulting bacterial concentrations of each treatment concentration. PA treatment was only able to significantly ($p < 0.05$) reduce ST by 1.60 Log CFU/mL at 4.0mg/mL, with the remaining concentrations being only numerically lower. VA at 4.0mg/mL had the greatest reduction, exhibited by inhibition of ST to non-detectable levels, though

lower concentrations had the ability to also significantly ($p < 0.05$) reduce ST by 2.74, 1.43 and 0.98 at concentrations of 2.0, 1.0 and 0.5mg/mL, respectively. Further adjusting the pH of the phenolic acid and DMEM solutions lead to improved inhibitory activity for some compounds (Figure 3-6B). GA continued to show significant ($p < 0.05$) inhibitory capability at 0.5mg/mL by reducing intracellular ST by 2.06 Log CFU/mL, followed by 1.0mg/mL (1.93 Log CFU/mL), 0.25 mg/mL (1.47 Log CFU/mL) and 0.125mg/mL (0.41 Log CFU/mL). PA showed numerical decrease in invasion, but bacterial load at 0.5mg/mL was significant ($p < 0.05$), with a 1.89 Log CFU/mL reduction. VA saw an extension in activity range, as in addition to 4.0mg/mL, 2.0mg/mL showed inhibition of ST to non-detectable levels, while lower concentrations like 1.0mg/mL and 0.5mg/mL remained active.

Relative gene expression of virulence genes and inflammatory cytokine genes

The total RNA was collected from INT-407 cell monolayer individually treated with each phenolic acid to evaluate changes in relative expression of genes associated to inflammatory cytokine production using qRT-PCR (Figure 3-7). Average Cq values were recorded and normalized with GAPDH as the housekeeping genes with an untreated sample serving as the control, while results were represented in Log form. Exposure to GA lead to an upregulation of all genes tested, namely, *IFNγ, IL-17*, *IL-1β, IL-22* and *IL-6*, which had values of 0.29, 1.75, 3.87, 2.18 and 0.45 Log though it was not statistically significant as compared to the untreated control. However, when exposed to GA adjusted to pH 7, there was a statistically

significant ($p < 0.05$) 1.99 Log upregulation of *IL-17* when compared to the control though it was not significantly different from its counterpart that did not have pH adjusted to 7. Other notable differences could be observed in the higher upregulation of *IFNγ* for GA at pH 7 (1.02 Log) compared to unadjusted GA, while other genes like *IL-1β* were expressed 2.89 Log less at pH 7, and *IL-6* was the only gene that showed downregulation of 0.20 Log at pH 7. Exposure to PA resulted in numerical downregulation of *IFNγ* and *IL-6* of 0.06 and 0.93 Log, though after adjusting to a pH of 7, all genes were downregulated, with a significant ($p < 0.05$) downregulation of 0.95 Log being reported for *IL-22*. Conversely, exposure to VA lead to an upregulation in every gene with a 1.28 Log increase in *IL-22* being significant ($p < 0.05$), and with the exception of *IL-6*, which was downregulated by 1.35 Log. Adjusting VA to pH 7 had a similar regulatory profile to its unadjusted counterpart, with the exception of *IL-17* upregulation by 0.64 Log being statistically significant.

Changes in the expression of ST virulence genes were also evaluated from total RNA extracted from cells and bacteria collected during the process of infection and later evaluated through the use of qRT-PCR and gene-specific primers for *fliC*, *hilA*, *invH*, *prgK* and *sipA*, with *16S-rRNA* gene being used as the housekeeping gene, and an infected but untreated sample being used as the untreated control (Figure 3-8). Samples treated with GA all showed significant ($p < 0.05$) downregulation of the genes tested, most notably, for *prgK* (1.50 Log) and *sipA* (1.47 Log), though adjusting to pH 7 lead to an even greater downregulation of all genes, especially *hilA* (4.25 Log), though *prgK* (3.81 Log) and *sipA* (3.89 Log) remained low. PA treatment lead

to downregulation of every gene, with the exception of *sipA* (0.23 Log), which showed to be slightly upregulated, though none were statistically significant. Adjusting PA to pH 7 exhibited a greater downregulatory effect as there was significant ($p < 0.05$) reduction of *hilA* (3.76 Log), *prgK* (2.93 Log) and *sipA* (2.93 Log). VA treatment exhibited a significant ($p < 0.05$) upregulation of most genes, especially *fliC* (0.70 Log), followed by *invH* (0.56 Log). Adjusting the pH of VA led to a downregulation of all genes except *invH* (1.00 Log). Though none were statistically significant, *sipA* (1.14 Log) and *invH* (1.02 Log) had the most notable numerical difference.

Discussion

Plants remain significant contributors to the discovery of many bioactive compounds that could be used for their application in controlling many pathogenic bacteria, especially ST. The phenolic compounds within plants have been identified as being notable candidates given their abundance and diversity, which are key factors that contribute to these being able to be used as antimicrobials without contributing to the increasing trends in antibiotic resistance and remain effective for longer periods of time (Ecevit et al., 2022). These compounds have also garnered attention for their antioxidant and anti-inflammatory properties when tested in various types of eukaryotic host cell lines (Abotaleb et al., 2020; Cos et al., 2002; Dai & Mumper, 2010). Phenolic acids specifically have been studied in the past for their antimicrobial activity against multiple pathogenic bacteria, their long use as food

preservatives and their bioactive byproducts (Cueva et al., 2010; Ofosu et al., 2020; Salaheen, Peng, et al., 2017a). Their use against ST specifically have been assessed *in vitro* in the past, as well as their efficacy when used in conjunction to other antimicrobials to enact a synergistic effect (Alvarado-Martinez et al., 2020; Hossain et al., 2020).

Direct inhibition of bacterial proliferation through growth inhibition remains one of the primary methods for control of ST, though recent studies have also been drawing attention to the importance of preventing colonization of host tissues, as well as targeting other virulence factors that the bacteria use to persist in infection (Balta et al., 2021). Using novel compounds that can prevent adhesion and/or invasion of ST to epithelial tissue has been proposed as a potential target for alleviating the disease caused by ST while also preventing further progression and complications of the infection as it will allow the host to shed the bacteria (Birhanu et al., 2021). Despite the GI tract having numerous innate defense mechanisms, including digestive fluids, multiple layers of mucous and commensal/probiotic bacteria, as well as the immune system cells and cytokines, ST is also equipped to either bypass these adverse conditions or even use them to trigger invasion, therefore requiring further intervention to eliminate the pathogen (Hausmann & Hardt, 2019). One proposed invasion model suggests ST can overgrow in the GI tract and even outcompete the local microbial flora in cases where the intestine is inflamed, which leads to the release of reactive oxygen species (ROS), as well as the fermentation of other

products like H_2S, which provides ST with an additional nutrient source (Ribet & Cossart, 2015).

By testing GA, PA, and VA against ST in an INT-407 cell model, this study was able to assess potential applicability of these compounds for the purposes of reducing progression of ST infection in the GI tract. As mentioned before, though the GI tract is a complex ecosystem with many variables that can affect efficacy of an antimicrobial product, cell monolayers have long been used as models for studying host-pathogen interactions between bacteria and eukaryotic cells (Tadala et al., 2022). One of the factors that affects the potency of phenolic acids is pH, which has been shown in the past to either increase their antimicrobial effect or neutralize them (Alvarado-Martinez et al., 2023). The initial antimicrobial assay performed in DMEM showed that inhibitory potential increased with concentration in the case of PA and VA, though they required high doses in order to inhibit ST to undetectable levels. In the case of GA, concentrations between 0.125mg/mL and 3.5mg/mL showed complete inhibition, while concentrations of 4mg/mL and above allowed for bacterial growth. Though this is kind of pattern in uncommon, further tests on the role of pH performed in this study showed that despite the higher concentrations of GA, the corresponding pH at these could be having a neutralizing effect. Regardless of concentration, the pH values of 4 and 5 neutralized GA, which could explain the loss of activity at higher concentrations, as the normal pH for these fell within this pH range. Changes in pH had a similar effect for PA, though it was more noticeable between pH 6 and 7, as bacteria showed capability to grow within this range, though,

lower pH values like 3 and 4 showed an improvement in antimicrobial activity. VA also saw a decrease in activity at pH 6 and 7, while also showing improved activity at 2.5mg/mL in pH between 3 to 5, though at concentrations of 2mg/mL and below there was a loss in the ability to completely inhibit ST growth. This effect of pH could be explained by changes in compound stability, as well as how these conditions can change the protonated state of the compounds, leading to either increased activity or a reduction based on how they will be able to interact with the bacteria (Spiegel et al., 2023).

The efficacy at specific pH was of importance for purposes of optimizing for compound activity but was shown to be important when considering experiments involving the use of INT-407 cells. Epithelial cells were shown to be susceptible to changes in the pH of the media they were incubated in, resulting in a decrease in cell viability. The optimal pH for cell survival was determined to be 7. When testing for cytotoxicity of each compound on INT-407, there was a noticeable decrease in viability as concentrations of each compound increased. The cutoff for cytotoxicity that was chosen was of 80%, meaning a reduction in viability of >20% meant the compound induced cell death and could be potentially cytotoxic at those concentrations (Y. Wang et al., 2021; Y. Y. Zhang et al., 2020). The drop in viability for GA was recorded at lower concentrations than for PA and VA, which led to the use of lower concentrations in general for GA to avoid cytotoxicity, in addition to t previously showing activity at these lower concentrations. PA and VA were overall less cytotoxic, requiring higher concentrations to significantly reduce cell viability.

Adjusting the pH of final phenolic acid and DMEM solution to 7 had a reduction in the cytotoxicity for previously cytotoxic concentrations of some of the compounds. Actively adjusting the pH to 7 in GA showed an improvement for the 0.5mg/mL solution, despite the unadjusted pH for this concentration being similar to 7. This difference could be attributed to the pH adjustment process, which requires the use of NaOH and HCl, depending on the case. In the case of PA, even at higher concentrations, even though it still fell below the 80% cutoff. In the case of VA, there was also an improvement in previously cytotoxic concentrations.

Adhesion and invasion were used as the markers for assessing the host-pathogen interactions between ST and INT-407 during an infection period, and treatment with each phenolic acid. The MOI that was chosen for these assays have been reported to represent more closely which was found in an *in vivo* condition (Lathrop et al., 2018; Lhocine et al., 2015). Adhesion is an important step for initiating infection that will progress to invasion of the host cells, which in turn is mediated by the T3SS (Dostal et al., 2014). Treatment with GA or PA did not show significant reduction in the adhesive capability, while VA did have activity at the higher dose of 4.0mg/mL, though this could also be the result of changes in the host cell given this concentration was found to have cytotoxic effects. Adjusting the pH to 7 did not improve GA activity, though it did enhance that of PA at 1mg/mL and expand the activity of VA to also be effective at lower concentrations. Though adjusting to pH 7 in previous experiments had a neutralizing effect on the antimicrobial potential against ST growth, other researchers have reported similar

scenarios in which specific natural compounds do not affect ST growth, but still display activity for inhibiting specific functions within the bacteria such as inhibiting the T3SS (Y. Zhang et al., 2018). When measuring invasion, the amount of intracellular ST detected was found to be the lowest when treated with 0.5mg/mL of GA, which was further improved after adjusting to pH 7. Though the other compounds showed an ability to inhibit invasion, GA achieved the greatest reduction with the lowest concentration. VA had the most significant reduction at higher concentrations, exhibited by a reduction in ST to undetectable levels when treated with 4mg/mL, which was later expanded to 2mg/mL as well after adjusting to pH 7. Previous research has shown the importance of pH as a key signal for ST to initiate the invasion of a host cell (Allam et al., 2012), but in the current study, a similar adhesion and invasion pattern for both regular DMEM and pH 7 DMEM suggests no adverse effect exerted by pH alone, however, when paired with a specific concentration of a phenolic acid, there could be an improvement in activity, whether mediated by the corresponding state of the compound, or by the response of either ST or INT-407.

Quantifying changes in relative expression for INT-407 cell genes associated with production of inflammatory cytokines gave information pertaining the potential anti-inflammatory activity of each phenolic acid, which could have significant effects on the progression of ST infections. In an infection model, production of cytokines like INFγ have been found to lead to better outcomes in terms of infection progression (S. Bao et al., 2000). Though not statistically significant, GA treatment

showed an upregulation of the *INFγ* gene, with pH 7 adjustment leading to a higher value. Similarly, IL-17 has been found to have a critical role in the early stages of infection, with studies showing its suppression to lead to greater epithelial damage (Mayuzumi et al., 2010). The current study showed both GA and VA to upregulate *IL-17* genes, with adjusting to pH 7 leading to a more significant expression in GA. In the case of IL-22, this cytokine has been associated with also having a protective function during ST infection but has recently been shown to aggravate the infection in some cases (Lo et al., 2019). This variation in outcomes makes it uncertain as to what would be the optimal outcome when using phenolic acids to modulate cytokine expression. However, it is important to note that PA adjusted to a pH of 7 was the only treatment to downregulate the expression of the *IL-22* gene. GA also had a notable upregulation of *IL-1β* gene, despite not being statistically significant. This was ameliorated when adjusted to pH 7, and similarly to *IL-22*, the use case for using these compounds to regulate this gene could be dependent on the context, as previous studies have noted its role in sensing and responding to bacterial lipopolysaccharides but can also have a pyroptotic effect on the host cells (Bent et al., 2018; Diamond et al., 2017). On the other hand, the *IL-6* gene, which is associated with acute inflammation was downregulated for PA and VA, as well as their pH adjusted counterparts, in addition to adjusted GA, which could have a use for reducing prolonged bouts of acute inflammation (Fuster & Walsh, 2014).

Relative expression of ST virulence genes during infection gave insight into the mechanism that might be behind the inhibition of invasion. Among the genes

tested that are directly associated with invasion, *hilA* is a major transcriptional regulator, *invH* is mainly responsible for protein translocation, *prgK* is a structure protein that serves as the base of the T3SS, while *sipA* has been shown to be critical in the manipulation of actin filaments, mediating bacterial entry (Jepson et al., 2001; Kimbrough & Miller, 2000; Lou et al., 2019a; Pati et al., 2013). On the other hand, though *fliC* is not directly associated to invasion, it is still considered an important virulence factor since it mediates motility that can help bypass host defenses (Cummings et al., 2006). GA had a significant downregulatory effect, which was further reduced by adjusting to pH 7. In the case of VA, there was upregulation of the virulence genes tested, suggesting a difference in mechanism of action considering that VA was able to reduce invasion.

Conclusions

The use case of plant-derived antimicrobial compounds has been expanding considering their activity. The current study has evaluated the applicability of GA, PA and VA for inhibit key virulence factors in ST like adhesion and invasion, while also considering the effects that these treatments can have on the host cells. Results showed the potential that some of these compounds have for reducing invasion particularly and provides insight into future optimization steps that can be taken in order to enhance the efficacy of these compounds, while also reducing detriment to the host cells.

Figures and Tables

Table 3-1: Primers used in quantifying gene expression of INT-407 cells.

Gene	Cytokine	Primer Sequence (5'-3')
GAPDH	dehydrogenase	F: GGTGGTGCTAAGCGTGTTAT
		R: ACCTCTGTCATCTCTCCACA
IFNγ	interferon gamma	F: GTGAAGAAGGTGAAAGATATCATGGA
		R: GCTTTGCGCTGGATTCTCA
IL-17	neutrophil activation	F: GCAGATGACGGTACATCCAA
		R: CCAGATCAGGCTGTGCTTTA
IL-1β	pro-inflammatory cytokine	F: GCCATGGACAAGCTGAGGAAG
		R: GTGCTGATGTACCAGTTGGG
IL-22	host defense at mucosa	F: CTCCGATCCCTTATTCTCCTC
		R: AAGCGGTTGTGGTCCTCAT
IL-6	acute inflammation	F: GAACTCCTTCTCCACAAGCG
		R: TTTTCTGCCAGTGCCTCTTT

Table 3-2: Primers used in quantifying gene expression of ST.

Gene	Protein	Primer Sequence (5'-3')
16S-rRNA	16S ribosomal RNA protein	F: GTAGTACGATGGCGAAACTGC
		R: CTTCTCGACCCGAGGGACTT
hilA	SPI-1 transcriptional regulator	F: AATGGTCACAGGCTGAGGTG
		R: ACATCGTCGCGACTTGTGAA
fliC	flagellum subunit	F: GCAGATGACGGTACATCCAA
		R: CCAGATCAGGCTGTGCTTTA
invH	adherence and invasion	F: GGTGCCCCTCCCTTCCT
		R: TGCGTTGGCCAGTTGCT
sipA	actin binding protein	F: CGTCTTCGCCTCAGGAGAAT
		R: TGCCGGGCTCTTTCGTT
prgK	base structure formation of T3SS	F: GGGTGGAAATAGCGCAGATG
		R: TCAGCTCGCGGAGACGATA

Table 3-3: Antimicrobial potential of increasing concentrations of phenolic acids in DMEM with adjusted pH.

pH	Concentration (mg/mL)																																												
	DMEM	0.0312			0.0625			0.125			0.25			0.5			1			1.5			2			2.5			3			3.5			4			4.5			5		
	N/A	G	P	V	G	P	V	G	P	V	G	P	V	G	P	V	G	P	V	G	P	V	G	P	V	G	P	V	G	P	V	G	P	V	G	P	V	G	P	V	G	P	V
3	+	+	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-	-	+	-	-	+	-
7	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-	-	+	+	+	+	+
8	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	-	+	-	-	+	-	+	+	-	+	+	-	+	+	-	+	-	-	+	-	-	+	-
9	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-	-	+	-	-	+	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
10	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-	-	+	-	-	+	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-

* In addition to visible detection, ST growth (+) or complete inhibition (-) was confirmed by spot platting aliquots from the gallic acid (G), protocatechuic acid (P), vanillic acid (V) and DMEM samples in fresh LB agar, incubated overnight and re-assessed for confirming growth.

FIGURE 3-1: MTT assay assessing changes in cell viability as a result of cytotoxicity for INT-407 cell monolayer treated with increasing concentrations of GA, PA and VA for 3h (A) and 24h (B), as well as the corresponding pH values for each phenolic acid concentration (C).

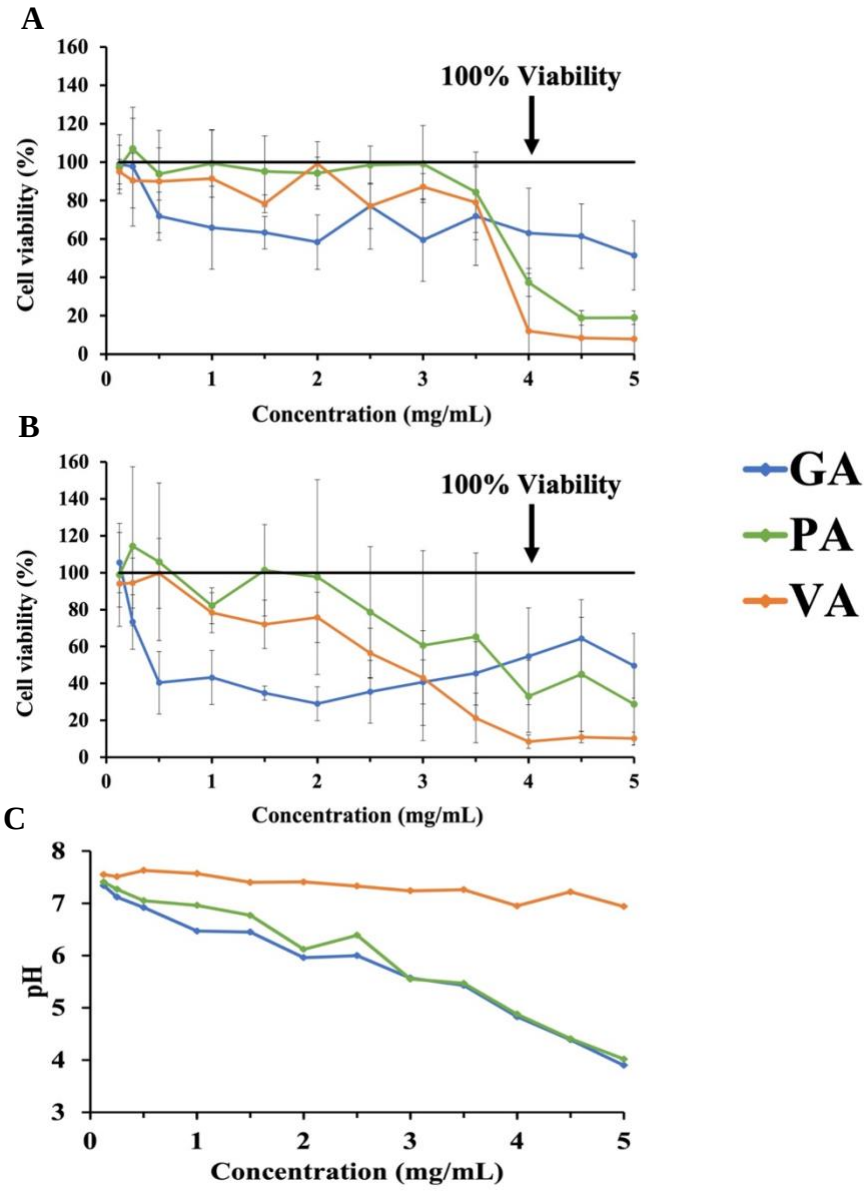


FIGURE 3-2: Effects of pH on cell viability evaluated through MTT assay after 3h (A) and 24h (B).

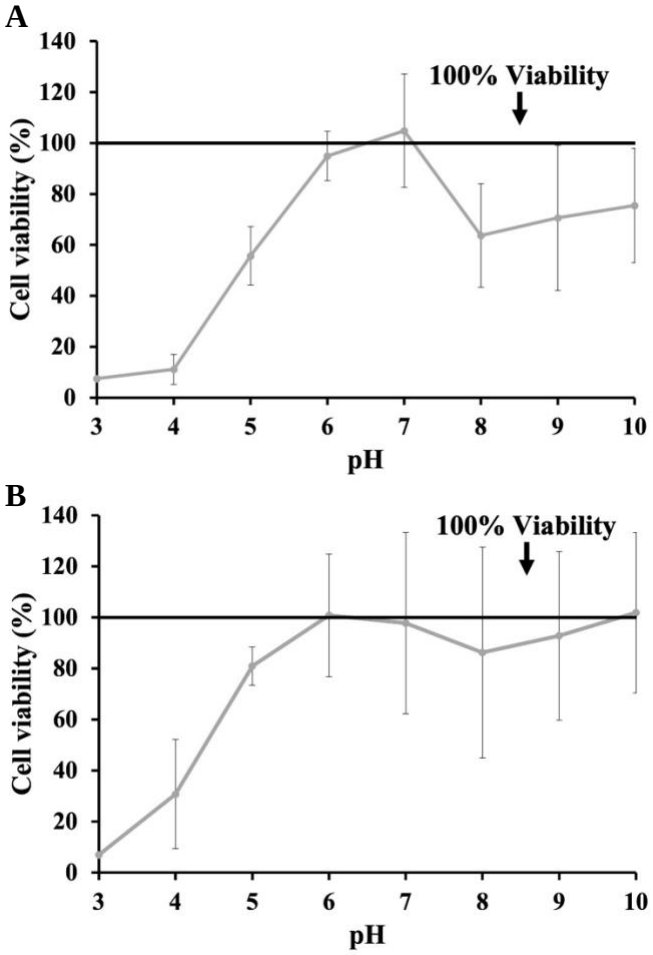


FIGURE 3-3: MTT assay assessing changes in viability as a result of cytotoxicity after adjusting incubation solution to a pH of 7 for GA (A), PA (B) and VA (C).

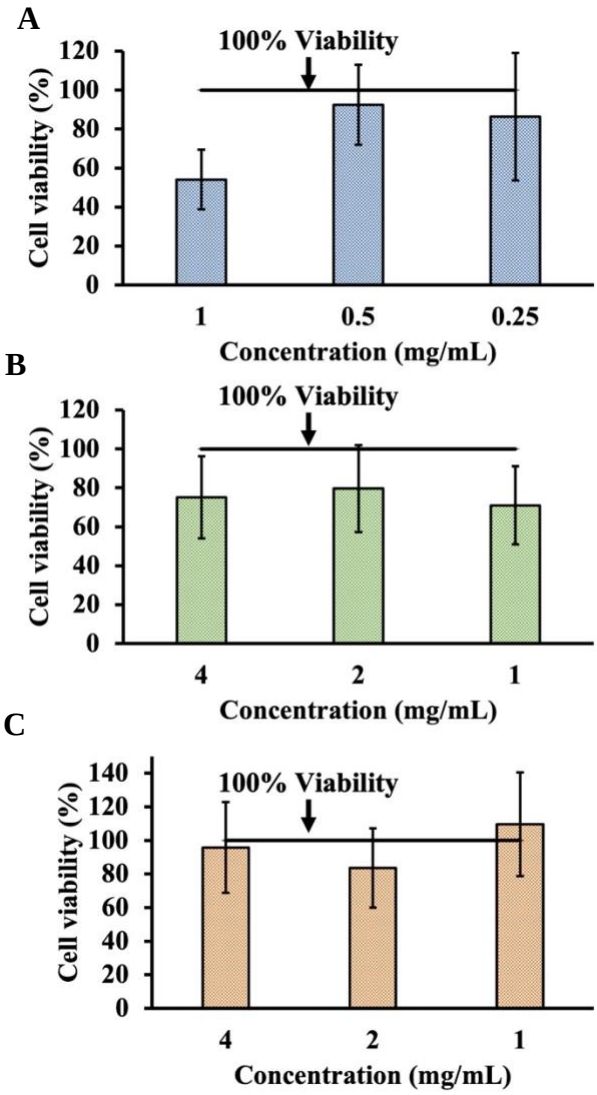


FIGURE 3-4: Testing increasing concentrations of GA, PA and VA to determine antimicrobial profile of phenolic acids when tested in DMEM, along with the corresponding pH at given concentrations. Statistically significant differences ($p < 0.05$) between treatments when compared to the untreated control at their respective timepoint was denoted with letters *a-b*.

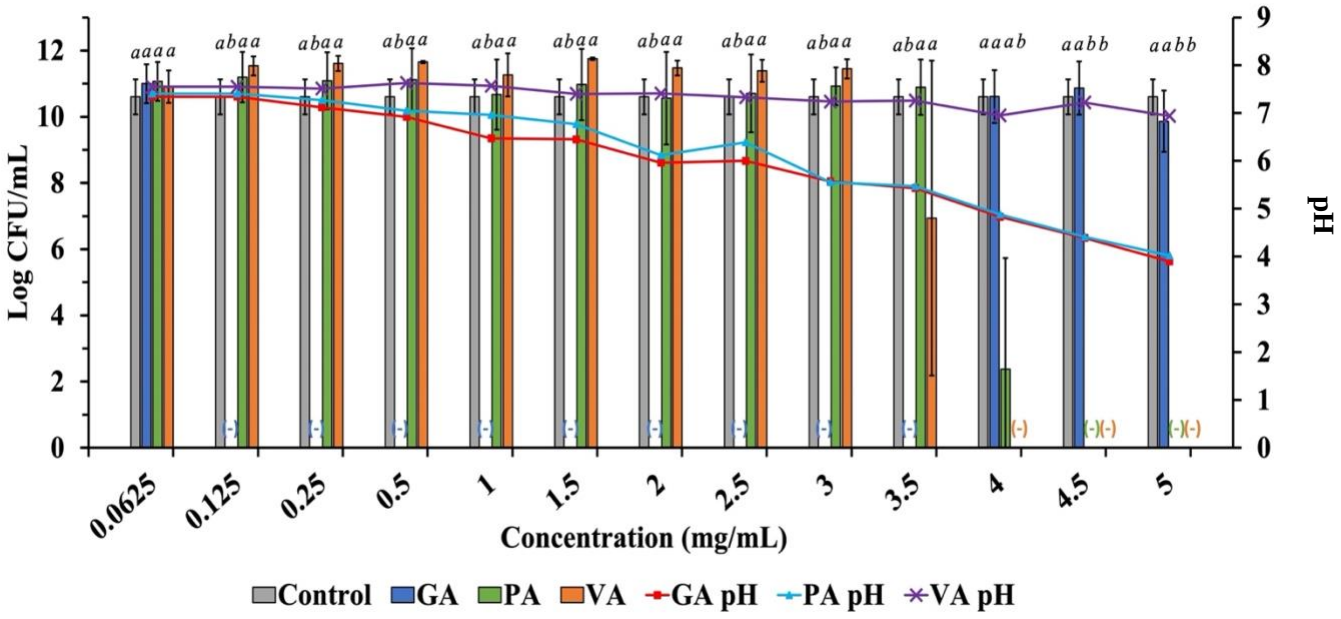


FIGURE 3-5: Bacterial quantification of ST adhered to INT-407 cell monolayer after 3h of incubation with a treatment dose of either GA, PA or VA at their normal pH (A) or adjusted to a pH of 7 (B). Statistically significant differences ($p < 0.05$) between treatments when compared to the untreated control at their respective timepoint was denoted with letters *a*-*b*.

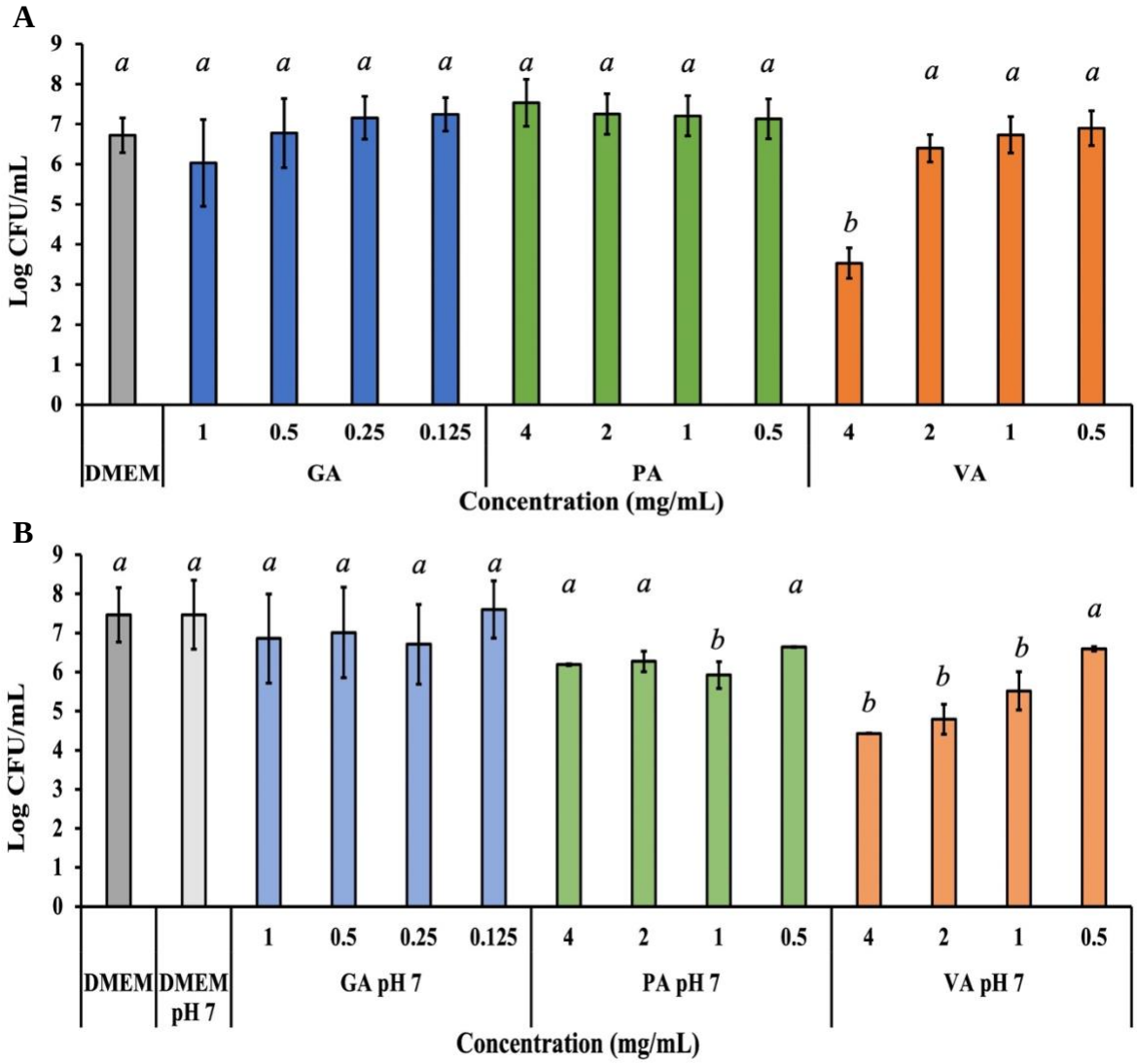


FIGURE 3-6: Bacterial quantification of intracellular ST that invaded the INT-407 cell monolayer after 3h of incubation with a treatment dose of either GA, PA or VA at their normal pH (A) or adjusted to a pH of 7 (B). Statistically significant differences ($p < 0.05$) between treatments when compared to the untreated control at their respective timepoint was denoted with letters *a*-*b*.

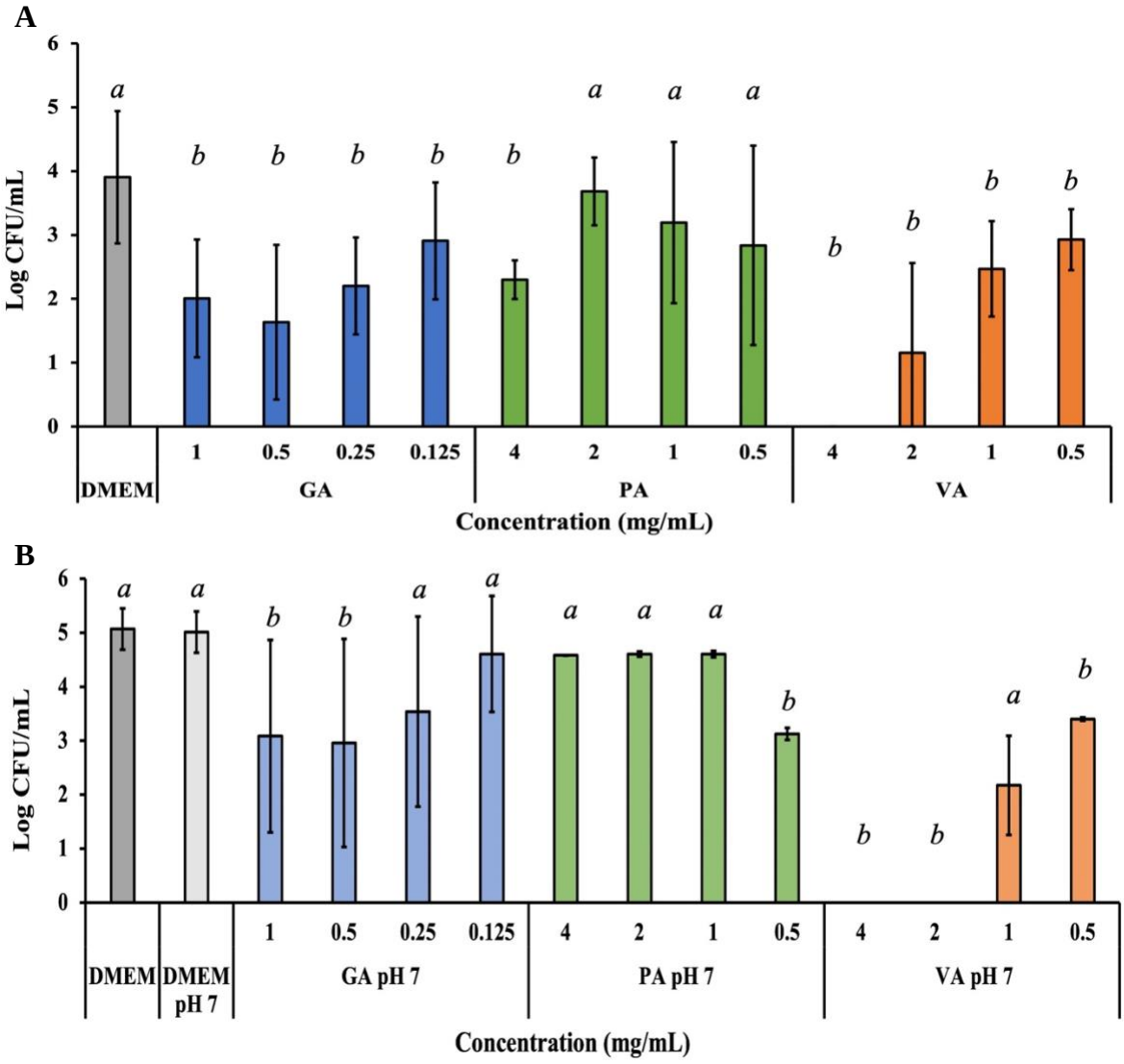


FIGURE 3-7: Relative log-fold expression of host genes associated with production of inflammatory cytokines, using *GAPDH* as the housekeeping gene and an untreated control for normalization. Statistically significant difference of treatment as compared to the control was denoted by a star (*), while statistically significant between normal and pH adjusted treatments were denoted by two stars (**).

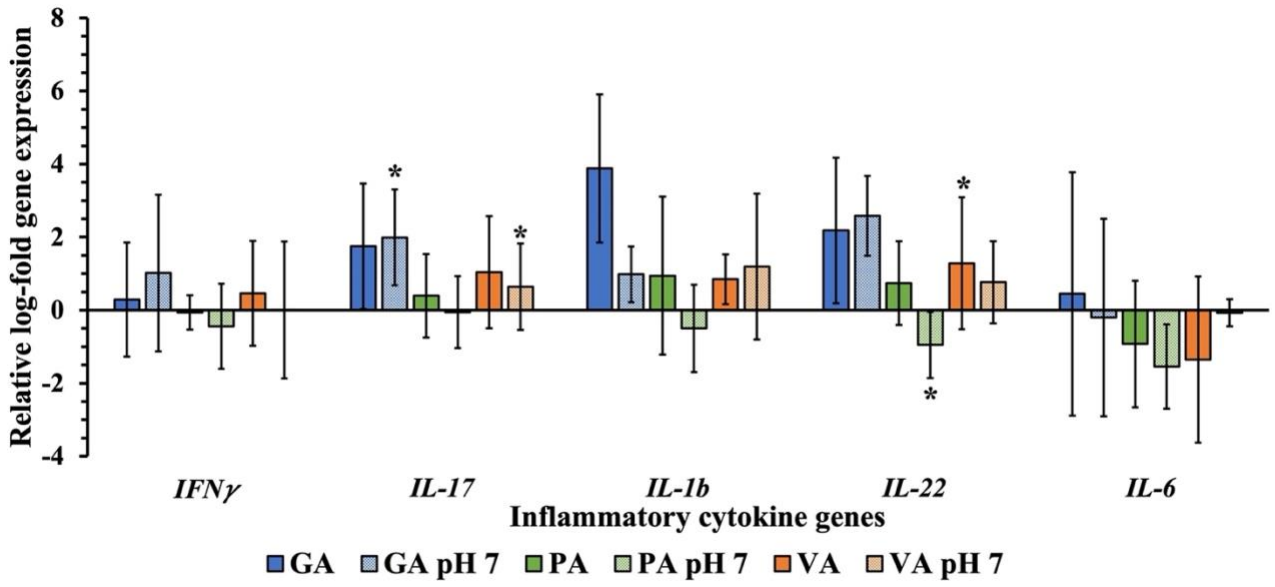
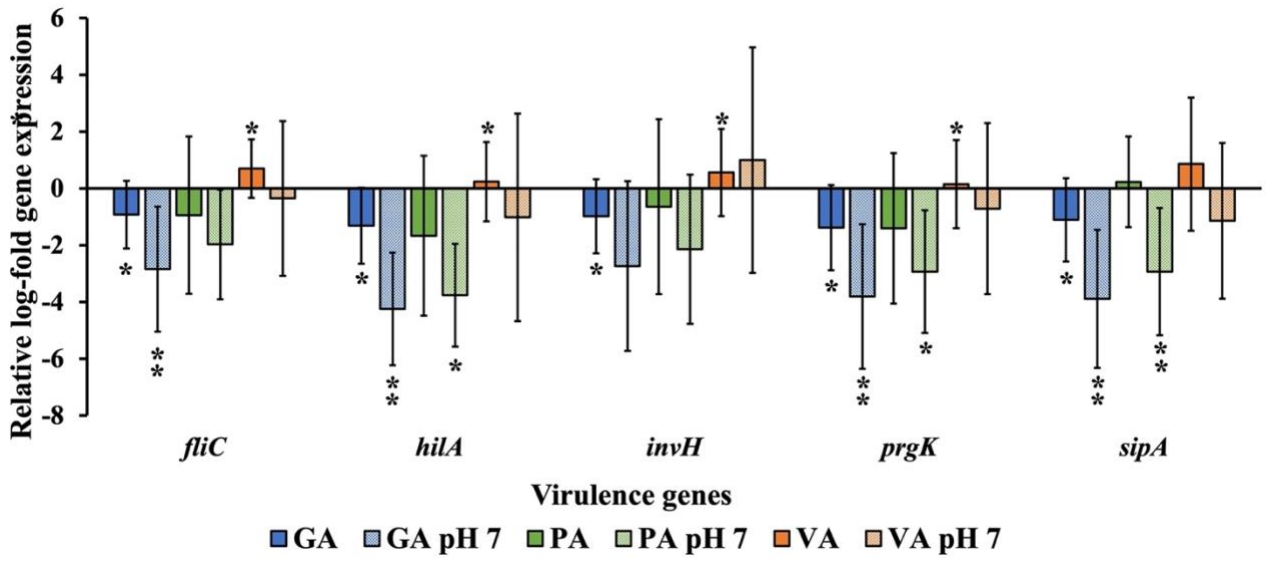


FIGURE 3-8: Relative log-fold expression of ST genes responsible for regulating virulence, using *16S-rRNA* gene as the housekeeping gene and an untreated control for normalization. Statistically significant difference of treatment as compared to the control was denoted by a star (*), while statistically significant between normal and pH adjusted treatments were denoted by two stars (**).



Chapter 4: Purified plant-derived phenolic acids inhibit *Salmonella* Typhimurium without alteration of microbiota in a simulated chicken cecum condition

Introduction

The current demands for novel antimicrobial compounds, that can target and eliminate pathogenic bacteria without negative consequences, is critical as multiple antibiotic resistant bacterial pathogens continue to be a major cause of disease, hospitalization, and death (Scallan et al., 2011). This emerging issue represents an overwhelming economic burden on the Unites States (USA) (Hoffmann et al., 2015). Farm animals have been attributed as one of the major reservoirs associated with transmission of various pathogens, with poultry being the main animal source for several enteric bacterial pathogens, particularly various serovars of *Salmonella enterica* (SE) (Interagency Food Safety Analytics Collaboration, 2021). Restrictions on the sub-therapeutic use of antibiotics has also contributed an increase in bacterial load found in animal food products (K. L. Tang et al., 2019). In addition to this, there is a growing trend of multiple antibiotic resistant SE, with serovars like *Salmonella enterica* serovar Typhimurium (ST) being of specific concern because of its sharply growing multi-antibiotic resistant trend, as well as its invasive capabilities to human intestinal epithelial cells, and ubiquitous presence in multiple animal hosts, foods, and

environments (Puhar & Sansonetti, 2014; X. Wang et al., 2019). Though antibiotic stewardship has reduced some of the presence of antibiotics in animal products and the environment, these measures potentially expose consumers to food products that contain higher loads of pathogenic foodborne bacteria naturally found as part of the animal's normal microbial flora (Patel et al., 2020). Despite these efforts, antibiotic resistant bacteria have not been eliminated from these scenarios since antibiotic resistance genes endure in the environment and within animal hosts, which serve as reservoirs for genes and other bacteria that can horizontally transfer and acquire these genes, even in the absence of previous antibiotic use (Bythwood et al., 2019). With this scenario, despite the abundance of antibiotics that are available for preventing and treating bacterial diseases like salmonellosis, the increasing panorama of bacterial resistance to clinically relevant antibiotics has greatly limited the use case for these compounds (U.S. Department of Health and Human Services, 2019). Previous research has suggested that reducing the bacterial load of an animal at the pre-harvest level can lead to a decrease in pathogens present in the subsequent food product, which translate to a lower incidence of foodborne infection, in addition to a lower incidence of zoonosis, meriting the search for novel antimicrobial compounds that do not contribute to bacterial resistance pattern (Alali & Hofacre, 2016).

As ST is commonly found in the gastrointestinal (GI) system of poultry as part of its normal microbial flora, and colonizes and thrives in the jejunum, ileum, and cecum without causing disease, while the chicks routinely shed the bacteria, releasing it in the feces (Harvey et al., 2011). The cecum is one of the organs with most abundance

of bacteria, both in terms of numerical quantities and taxonomic diversity, consisting of a complex microbiome that includes bacteria that are commensal to the animal and non-pathogenic to humans, but also contains many pathogens including ST (Clavijo & Flórez, 2018). Therefore, cecum is an important organ of chicken as a source of poultry-borne enteric pathogens, and it can be targeted to control colonization of poultry-borne human pathogens. In addition, the bacterial dynamics that take place in the cecum must also be taken into account as these also have important implications for the host's overall health (Wickramasuriya et al., 2022).

Further, recent studies have revealed the importance of maintaining a diverse and stable gut microbiota for promoting and maintaining host health, and in the case of poultry, the cecal microbiome has a big role in this process (Clavijo & Flórez, 2018). Other studies have proposed influencing the gut microbiome through the introduction of prebiotics and probiotics as a strategy for improving poultry health, welfare, and production, as well as reducing the prevalence and colonization of pathogenic bacteria in their GI tracts, which would result in safer food products, as well as less cross-contamination (Salaheen et al., 2018; Salaheen, Kim, et al., 2017; Tabashsum et al., 2020). These specifically demonstrated the efficacy of plant extracts as both antimicrobial agents that could reduce the prevalence of common foodborne pathogens in an *in vivo* chicken model, while also being capable of stimulating animal growth and promoting the microbial diversity within the gut of the animals that were given these products. Though these methods are promising and could be used to replace the use of conventional antibiotics in animal production, they require more

study directed at understanding their mechanisms of action and how they can be implemented in a more targeted manner.

Recent findings indicate that plant extracts are promising candidates for discovering novel antimicrobials which contain a variety of diverse polyphenolic compounds (Takó et al., 2020). The plant derived flavonoids, tannins and phenolic acids can be easily extracted and administered orally with little or no detriment to the animal and have proven benefits to them and their microflora (Aldulaimi, 2017; Alvarado-Martinez et al., 2019). Though there are many types of phenolic acids, each with their own unique chemical makeup and bioactive potential, many of them have been previously reported to have antimicrobial and anti-virulence properties against ST *in vitro* (Alvarado-Martinez et al., 2020). However, the effectiveness of pure phenolic acids on targeted pathogens in complex biological system such as cecum have not been studied well yet.

In this study, we aim to evaluate the effectiveness of gallic acid (GA), protocatechuic acid (PA) and vanillic acid (VA) in a simulated chicken gut environment, specifically one containing cecal fluids collected from chickens, which contain the initial local bacterial communities commonly found in this animal, as well as the surrounding matrix containing the nutrients, macromolecules and compounds that form part of the contents in this part of the gut. This provides an environment that accounts for crucial interactions that could affect the efficacy and potential of pure, individua phenolic acids as future methods of microbial control, while also serving as a model that can help elucidate more information with regards to the mechanism of

action behind plant extracts. This study also seeks to evaluate gaps in compound potency associated to changes in medium composition used for testing antimicrobial compounds and environments in which these compounds are meant to be used. To address this, compound stability and potency was first evaluated within a wide pH range in nutrient-rich media. Effects on the growth pattern of ST in cecal fluid was also evaluated during treatment with phenolic acids. In addition to evaluating the effects of phenolic acids on ST, 16S-rRNA gene sequencing was used to evaluate changes in the microbial composition of the pre-exiting microbiota of the cecal fluid samples, and later analyzed to determine changes in species diversity.

Materials and Methods

Bacterial strain and growth conditions

Salmonella enterica serovar Typhimurium (ATCC 14028) (ST) was used for this study. Luria-Bertani (LB) (Becton, Dickinson and Co., USA) agar and broth was used as the medium on which ST was grown, and it was incubated at 37°C under aerobic conditions (Thermo Fisher Scientific Inc., USA). XLD (Hardy diagnostics, USA) and SS (Becton, Dickinson and Co., USA) agar were used as differential media that allowed for the identification and differentiation of ST from other gram-negative coliforms.

Compounds and stock solution preparation

The phenolic acid compounds that were assessed in this study were purchased in powder form from commercial vendors. Stock solutions were prepared for gallic acid (GA) (Acros Organics, USA) and protocatechuic acid (PA) (Sigma-Aldrich, USA) by dissolving in sterile deionized water, while vanillic acid (VA) (Alfa Aesar, USA) was prepared by dissolving in 30% ethanol (Pharmco-Aaper, USA). Aqueous solutions of sodium hydroxide (NaOH) and hydrochloric acid (HCl) were both prepared to a molarity of 10 M and used for adjusting pH values of other solutions in this study. All compounds were prepared to contain a concentration of 10 mg/mL. Phosphate buffer saline (PBS) was prepared to a pH of 7.2.

Phenolic acid antimicrobial potential at alternate pH range

Antimicrobial potential of phenolic acids against ST were evaluated at increasing concentrations of each compound within a pH ranging from 3 to 10 following previously described methods with certain modifications (Burel et al., 2021). Briefly, LB broth samples were independently supplemented with increasing concentrations of GA, PA and VA ranging from 0. 07813 to 4.5 mg/mL, while aliquots of each concentration were concurrently adjusted to a specific pH value ranging from 3 to 10 using either HCl or NaOH, depending on the desired pH value of the solution being prepared. These solutions were used to inoculate with ST fixed to an optical density (OD_{600}) of 0.1 in PBS (10^8 CFU/mL) that was further diluted to achieve a final bacterial load of 10^4 CFU/mL in the final volume. These samples were incubated

aerobically for 24hr at 37°C and screened for visible growth, which was confirmed by plating in LB agar. Growth in LB agar was interpreted as a sign of inactivation of the phenolic acid either by changes in pH and/or changes in minimum inhibitory concentration (MIC).

Simulated chicken cecum model design

Previous research on the antimicrobial potential of these phenolic acids against ST (ATCC 14028) determined the lethal concentration (elimination of bacteria to the point of no detectable growth) to be 4.5mg/mL for GA, 2mg/mL for PA and 1.5mg/mL for VA, with minimal change in susceptibility over various passages (Alvarado-Martinez et al., 2020). A simulated chicken cecal environment was designed in order to study the effects that the phenolic acids GA, PA and VA have on complex environment with a diverse a bacterial community like the chicken gut, while also evaluating differences in the effectiveness of these compounds when it comes to inhibiting the growth of ST. This model was designed as has been described in the past by previous research groups, with some modifications (Paul Tetteh Asare et al., 2021). Briefly, to attain the complex microbial composition of the gut, as well as the environmental matrix that they inhabit, cecal fluid samples were collected from two groups of previously sacrificed chickens (*Gallus gallus domesticus*), one being comprised of 3 21-day-old birds and the other of 4 28-day-old birds (IACUC, protocol number R-FEB-20-04). The two groups with their respective sets of birds served a duplicates, while cecal contents from each bird within a group were combined to

account for variance between birds. These were dissected to remove the cecum, which was later emptied of the cecal fluid and suspended in PBS immediately after the initial collection. Samples belonging to their respective collection group were pooled together and homogenized through vortexing to ensure equal distribution of cecal contents across multiple samples, as well as initial microbial composition. Groups consisted of an untreated control group, and three treatment groups individually supplemented with previous experimentally determined lethal doses of each corresponding compound, namely GA (4.5 mg/mL), PA (2 mg/mL) and VA (2 mg/mL) (Alvarado-Martinez et al., 2020). All samples were inoculated with a bacterial suspension of ST previously prepared by using ST grown overnight in LB agar at 37°C, and later fixed to an OD_{600} of 0.1 in PBS. The final concentration of bacteria in each sample solution was 10^4 CFU/mL. After inoculation, samples were incubated for 24hr and 48hr at 37°C under aerobic conditions and sample aliquots of 1mL were taken at 0hr, 24hr and 48hr, part of which was used for determining ST growth and the rest was stored for later total DNA extraction. The pH was measured across all timepoints for all treatment types and controls using a pH probe to identify any deviations from the initial pH.

Quantification of ST growth after treatment with phenolic acids

The growth of ST was quantified in all samples (Control, GA, PA and VA) from both duplicate collection groups, at the three predetermined timepoints (0hr, 24hr and 48hr). This was performed through a microdilution assay as has been described before (Salaheen et al., 2016a), with some modifications. Aliquot from each sample

were taken at the pre-determined timepoints and diluted using PBS before plating in XLD and SS. The media selected allowed for the differentiation of coliforms in samples containing a mixed culture of bacteria with only bacteria that developed black colonies being considered for quantification and counted as positive for ST (Yue et al., 2014). CFU/mL values were calculated for each sample and later presented in Logs CFU/mL.

16 S-rRNA microbiome analysis

The microbial composition of each sample was evaluated through the use of 16S-rRNA gene sequencing as has been described before (Salaheen, Kim, et al., 2017). Briefly, total DNA was extracted from all samples using the QIAamp Fast DNA Stool Kit (QIAGEN, USA) using the manufacturer guidelines. The extracted DNA was quantified using a NanoDrop (ThermoFisher, USA) to validate the extraction yield and later normalize samples to a fixed concentration. Taxonomic analysis was performed by amplification of the variable v3 and v4 regions of 16S-rRNA, allowing for phylogenetic classification of the bacteria in the sample through next-generation sequencing and further diversity analysis. Briefly, Nexter DNA Library Prearation Kit (Illumina, USA) was used to prepare samples for sequencing. KAPA HiFi HotStart ReadyMix, primer amplicons and DNA samples were combined, and a PCR reaction was performed with the following protocol: 95°C for 3min; 25 cycles of 95°C for 30sec, 55°C for 30sec and 72°C for 30sec; 72°C for 5min. Product cleanup for eliminating free primers and primer dimer species was performed using the AMPure XP beads. Index PCR was later performed to generate library using the Nextera Index

Kit (Illumina, USA), following the manufacturer guidelines. The PCR reaction was carried out as follows: 95°C for 3 min; 8 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; 72°C for 5 min. Library products were cleaned up for a second tie using the AMPure XP beads. Library concentrations were quantified and normalized, in order to prepare an equimolar pool of DNA amplicons, which is later combined with the PhiX reference control provided by the manufacturer. After heat denaturation (96°C for 2min), the sample pool was loaded and pair-end: 2 x 300 bp sequencing was performed using the MiSeq v3 600Cycle Kit (Illumina, USA) in the Illumina MiSeq System (Illumina, USA).

Analysis of taxonomic abundance

A total of 11,903,602 quality-filtered reads were used for taxonomic analysis and profiling of each sample. Sequence analysis was performed using the MiSeq Reporter-BaseSpace workflow tool for FASTQ, which uses the Greengenes Database. Further analysis involved the taxonomic quantification and classification of the bacterial profile of each sample at the phylum and genus level (Peng & Biswas, 2020). The mean richness and diversity at the species level were analyzed in each sample by determining alpha (α) diversity, which was determined by using the Shannon Index as has been established before (Jovel et al., 2016). Evenness at the species level for each sample was also determined by calculating the Inverse Simpson Index (Nagendra, 2002). Similarity at the species level and species overlap between treated samples when compared to control, were also analyzed by determining beta (β) diversity, which was

calculated using Sorenson's Coefficient (Finotello et al., 2018). Number of operational taxonomic units (OUT) in each sample were calculated for each sample and compared between their respective timepoints using a Venn diagram for further comparison and visualization, which was generated using the web-based InteractiVenn tool (Heberle et al., 2015).

Shannon Index (H):

$$Proportion\ (p) = \frac{Individuals\ of\ one\ species\ (n)}{Total\ number\ of\ individuals\ (N)}$$

$$H = -\sum_{i=1}^{s} p_i \ln p_i$$

Inverse Simpson Index (D):

$$D = 1/(\sum_{i=1}^{s} p_i^2)$$

Sorenson's Coefficient (CC):

$$Number\ of\ species\ in\ common = C$$

$$Total\ number\ of\ species\ found\ in\ community = S_n$$

$$CC = \frac{2C}{S1 + S2}$$

Statistical analysis

Student *t*-test and ANOVA were used to determine statistically significant ($p<0.05$) changes between individually treated groups with the untreated control.

Results

Antimicrobial potency of GA, PA, and VA at various pH ranges

The antimicrobial potency of increasing concentrations of the three phenolic acids in LB broth were evaluated by adjusting the pH of each individual solution to a value ranging from pH 4 to pH 9 (Table 4-1). ST was used to inoculate each solution containing increasing concentrations of either GA, PA, or VA (0.0156 – 4.5 mg/mL), in addition to a control containing only LB broth, all of which were fixed to a specific pH value. After incubation of ST with each unique mixture for a 24hr period, the samples were screened for signs of visible growth and confirmed by plating in LB agar. Changes of pH altered the inhibitory efficacy of each compound significantly, leading to either their inactivation, or increasing their potency, requiring less concentration of each of the compound to inhibit ST growth to undetectable levels. As a control, an initial testing of tolerance to a wide range of pH was assessed in only LB broth, finding ST to be unable to grow below a pH of 4, and demonstrated limited growth above pH 9 (data not shown). Considering the antimicrobial pressure these levels exert on the bacteria without the addition of any of the compounds being evaluated in this study, lead to the selection of the current pH ranges. Further changes in antimicrobial activity were observed in GA, which retained inhibitory potential against ST across the widest

range of pH, only showing loss of function at pH 4, up until the concentrations of the compound fell below 1 mg/mL, at which point there was loss of function at pH 5 as well. Potency of GA saw an improvement between the ranges of pH from 6 to 9, lowering the lethal dose of the compound to 0.125 mg/mL and to 0.0625mg/mL at pH 8 and above. Efficacy of GA was lost at concentrations below 0.0313mg/mL in all pH levels. PA showed inactivation between pH of 5 and 7 at 2 mg/mL, but also demonstrated an improvement in potency at pH 8 similar to GA, as the lethal dose of PA required for inhibition of ST growth was lowered to 0.125 mg/mL, while retaining efficacy up to 0.0313mg/mL at pH 9. VA showed the lowest tolerance in changes of pH with inactivation between pH 5 and pH 6, even at higher concentrations. At 2 mg/mL of VA, there was inactivation of between the ranges from pH 5 to pH 9, evident in the loss of antimicrobial activity. At pH 4, VA showed an improvement in antimicrobial effect, inhibiting all detectable growth of ST at a lower concentration of 0.5 mg/mL (Table 4-1).

Quantification of ST growth in chicken cecum fluids in the presence or absence of GA, PA, or VA

Growth of ST in the simulated cecal environment was evaluated by collecting cecum fluids from each time-points and quantifying the bacterial load (CFU/mL) using plate microdilution assay. The bacterial count for each sample were calculated and converted to Log CFU/mL (Figure 4-1). The moment of inoculation with ST in the extracted cecal fluid was used as the starting point for the time scale, as 0 hr. At the

initial time-point (0hr), there was no significant difference between the control and the treatments (Figure 4-1A). After 24hr, ST population grew to 9.65 Log CFU/mL in the untreated control, with CFU/mL values being significantly (p<0.05) lower in treated cecum fluids at subsequent timepoints. After 24hr, ST in samples treated with GA and PA increased slightly to 6.37 and 8.38 Log CFU/mL, increasing form the initial inocula at 0hr, but remaining lower than the untreated control. In the same assay and timepoint, VA decreased ST from its initial concentration to 4.84 Log CFU/mL. After 48hr, ST concentration in the untreated control showed a slightly decreased to 9.41 Log CFU/mL, while values for the treated sample remained lower. Despite remaining lower than the untreated control, growth of ST was increased slightly in cecum fluids to 6.63 Log CFU/mL and 7.52 Log CFU/mL in GA and PA treated samples at 48hr time point, respectively, whereas in same assay, the cecum fluids treated with VA, growth of ST was reduced to 4.21 Log CFU/mL. The pH was measured at each timepoint of non-treated control and treated samples (Figure 4-1B), finding the initial pH of the untreated control to be pH 7.53, which decreased to pH 7.17 after 24hr and later increased to 7.96 after 48hr. Supplementation with phenolic acids provoked a significant change in initial pH, as the GA treatment group started at pH 4.52, but gradually increased to pH 4.53 and pH 6.82 after 24hr and 48hr, respectively. Initial pH in the PA group was slightly higher than GA initially, with a pH of 5.76, but also increased gradually to pH 6.3 and pH 7.32 after 24hr and 48hr, respectively. VA was initially the same pH as PA, as it also started at a pH of 5.76, however, fluctuations over time were less drastic, as it

decreased to pH 5.66 at 24hr and later to pH 5.84 at 48hr, showing less variation than the other treatment groups.

Changes in microbial makeup at major phyla and genus levels due to treatments

Changes to bacterial population distribution as a result of treatment with phenolic acids were studied using 16S-rRNA sequencing, which was later analyzed to uncover the taxonomical composition in each sample at both the phylum and genus level (Figure 4-2 and Figure 4-3, respectively). The experiments were performed with a technical duplicate for each biological replicate, and the results shown are the averages of the independent values obtained from each run. Relative bacterial abundance was determined from the percentage (%) of total reads classified to the respective taxonomic level, which at the phylum level was an average rate of 97.80% and at the genus level was an average rate of 86.50%. Changes in the bacterial populations in the samples treated with the respective phenolic acids were compared to the untreated control of the same timepoint. Reads from the control at 0hr revealed the four major phyla to be Firmicutes (87.76%), Bacteroidetes (4.14%), Actinobacteria (3.44%) and Proteobacteria (1.38%). At the 24hr of the untreated control, the top four major phyla remained the same as 0hr, however, the relative abundance of Firmicutes decreased (63.56%), while it increased for Bacteroidetes (8.98%), Actinobacteria (5.91%) and Proteobacteria (19.51%). Samples treated with GA at the same timepoint had statistically significant ($p<0.05$) increase of Firmicutes (71.86%), Actinobacteria (10.72%) and in the less abundant Cyanobacteria (0.05%), while there was a significant

decrease of Proteobacteria (6.65%). Samples treated with PA showed a slight numerical increase in Firmicutes, significant change ($p<0.05$) was seen in a decrease of Bacteroidetes (6.76%) and the less abundant Synergistetes (0.02%). Samples treated with VA showed a significant ($p<0.05$) increase in abundance of Firmicutes (84.45%) and Cyanobacteria (0.03%), while there was a significant reduction in Proteobacteria (1.08%) and Bacteroidetes (5.70%).

At the 48hr of the untreated control, the top four major phyla remained the same as in 24hr, with a numerical decrease in relative abundance of Firmicutes (57.50%) and Actinobacteria (5.13%), while there was an increase of Proteobacteria (28.59%) and Bacteroidetes (7.02%), with the latter being significant ($p<0.05$). Samples treated with GA only saw statistically significant ($p<0.05$) change in the increase of Actinobacteria (14.40%) and the lesser Cyanobacteria (0.04%), however there was a numerical decrease in Proteobacteria (17.38%) and increase in Bacteroidetes (8.21%). Samples treated with PA had only numerical variations when comparing the top phyla with the untreated control, with a decrease in the less abundant Tenericutes (0.32%) being significant ($p<0.05$). Samples treated with VA showed statistically significant ($p<0.05$) increase in the abundance of Firmicutes (79.69%), while there was a decrease of Proteobacteria (1.12%) and Tenericutes (0.19%).

Taxonomic classification at the genus level was performed by calculating the percentage of relative abundance for the top 45 bacterial genera discovered in each sample, while genera that fell below these values were added and placed under *Other*. The ten most abundant genera at timepoint 0hr were *Lactobacillus* (18.22%),

Clostridium (11.54%), *Ruminococcus* (9.50%), *Faecalibacter* (6.07%), *Alistipes* (4.08%), *Eubacterium* (3.74%), *Bifidobacterium* (3.17%), *Blautia* (3.07%), *Flavonifractor* (2.69%) and *Intestinimonas* (1.82%), while *Salmonella* (0.004%) was below these values. At the 24hr timepoint, the ten most abundant genus were *Escherichia/Shigella* (13.91%), *Lactobacillus* (12.19%), *Clostridium* (10.45%), *Alistipes* (8.91%), *Bifidobacterium* (5.45%), *Ruminococcus* (4.10%), *Faecalibacterium* (3.66%), *Acinetobacter* (3.46%), *Blautia* (2.05%) and *Flavonifractor* (1.98%), with *Salmonella* (0.059%) increasing over this time. Out of these, when comparing to the initial 0hr timepoint control, the increase of *Escherichia/Shigella* and *Alistipes*, as well as the decrease in *Eubacterium* were statistically significant ($p<0.05$). Samples treated with GA showed a significant increase in *Bifidobacterium* (8.90%) and *Faecalibacter* (4.64%), though there was a slight numerical decrease in *Lactobacillus* (11.50%), there was also a decrease in *Escherichia/Shigella* (5.03%), *Clostridium* (9.64%) and *Ruminococcus* (3.77%), as well as a slight increase in *Salmonella* (0.079%). Samples treated with PA had a similar abundance of the top genus as compared to the untreated control, with *Alistipes* (6.71%) being significantly ($p<0.05$) decreased, while *Salmonella* (0.043%) was lower. Samples treated with VA had a similar abundance in the top ten genera, demonstrating a numerical increase in *Lactobacillus* (13.90%) and *Clostridium* (12.05%) with a significant ($p<0.05$) change being observed in the abundance of *Escherichia/Shigella* (0.37%), *Alistipes* (5.66%), and an increase in the abundance of *Ruminococcus* (6.49%), while *Salmonella* (0.0025%) was experienced numerical reduction.

At the 48hr of the untreated control, the top ten bacterial genera were *Escherichia/Shigella* (13.57%), *Acinetobacter* (12.19%), *Clostridium* (9.77%), *Lactobacillus* (9.38%), *Alistipes* (6.96%), *Bifidobacterium* (4.68%), *Faecalibacterium* (3.85%), *Ruminococcus* (3.09%), *Flavonifractor* (1.79%) and *Pseudoflavonifractor* (1.66%), with *Salmonella* (0.051%). When compared to the 24hr untreated control, statistically significant ($p<0.05$) change was seen in increased abundance of *Acinetobacter* and a decrease in *Alistipes*. Samples from the same timepoints treated with GA demonstrated a change in the distribution of the previously reported top ten genera, as there was a numerical increase of *Lactobacillus* (12.82%), *Alistipes* (8.13%), *Klebsiella* (7.28%) and *Salmonella* (0.12%), with statistical significance ($p<0.05$) being found in *Bifidobacterium* (12.62%), *Enterococcus* (4.29%) and *Eubacterium* (3.01%), on other hand, a numerical reduction of *Escherichia/Shigella* (8.44%), *Clostridium* (9.77%), and *Ruminococcus* (1.73%), with significance being found in *Clostridium* (4.74%), *Acinetobacter* (0.02%) and *Pseudoflavonifractor* (0.81%) . Samples treated with PA showed a numerical increase in *Escherichia/Shigella* (15.29%), *Lactobacillus* (10.53%) and *Bifidobacterium* (6.45%), with *Klebsiella* (0.75%) being statistically significant ($p<0.05$), while a decrease was observed in *Faecalibacterium* (2.71%) and *Salmonella* (0.038%), with *Acinetobacter* (6.70%) being statistically significant ($p<0.05$). Samples treated with VA demonstrated a numerical increase in *Clostridium* (12.32%), *Ruminococcus* (6.01%) and *Eubacterium* (2.61%), with *Flavonifractor* (3.40%) being statistically significant ($p<0.05$), while

there was a numerical decrease in *Salmonella* (0.002%), with *Escherichia/Shigella* (0.21%), *Acinetobacter* (0.055%) and Klebsiella (0.018%) being significant ($p<0.05$).

Changes of bacterial composition assessed through alpha and beta diversity indexes at species level due to treatments

The taxonomic distribution of bacteria at the species level was analyzed based on the percentage (%) of total reads classified at this taxonomic level, which was on average 50.96% from the total classified reads. Species number was quantified (Figure 4-4), while further analyses on species distributions and diversity were performed using calculations for alpha distributions (Shannon Index and Inverse Simpson Index), in addition to comparing distribution between classified bacterial communities within samples through beta distribution (Sorrenson's coefficient) (Figure 4-5). At the 0hr timepoint there was an average of 593 species identified (Figure 4-4A), while at the 24hr (Figure 4-4B) timepoint there was a statistically significant ($p<0.05$) increase in the average of species identified (1053). When comparing the number of species in the samples treated with phenolic acids to that of the untreated control at the same timepoint, there was a slight numerical decrease in GA and VA (991 and 932, respectively), while in PA there was an increase (1249). At 48hr (Figure 4-4C) there was a slight increase of species number (1073) but not statistically different from 24hr. When comparing species among samples at 48hr , samples treated with phenolic acids demonstrated lower numbers of species in the cases of GA (988), PA (914) and VA (611), as compared to the untreated control.

Shannon Index was calculated as an indicator of alpha diversity for each sample in order to determine microbial diversity within each (Figure 4-5). The untreated control at the 0hr (Figure 4-5A) timepoint had a value of 3.91, with a slight decrease to 3.85 and later 3.62 at 24hr and 48hr, respectively. Comparatively, at 24hr (Figure 4-5B), samples that were treated showed a slight numerical decrease as compared to the untreated control, as seen in the slightly lower values for GA (3.90), PA (3.84) and VA (3.82).. At 48hr (Figure 4-5C), values were lower than their 24hr counterparts, as seen in values for GA (3.54), PA (3.79) and VA (3.81), despite the later two being higher than the untreated control at the same timepoint. The Inverse Simpson Index (Figure 4-6) was used as another indicator of alpha diversity in terms of evenness, which showed the untreated control at 0 hr to be 27.30 (Figure 4-6A) and to also decrease slightly to 21.80 and then significantly to 14.92 ($p<0.05$) over 24hr and 48hr periods, respectively. Further, at 24hr (Figure 4-6B), values for the samples treated with GA, PA and VA were 23.70, 23.24 and 23.73, respectively, which was slightly higher than the untreated control, but was still lower than the 0hr sample. At 48hr (Figure 4-6C), values for the samples treated with GA and PA decreased further to 16.54 and 21.08, respectively, while VA significant increased to 24.29 ($p<0.05$).

Further analysis of the species number required finding the common bacteria among the replicates of each group, which allowed for the comparison of OUT between the control and treatment groups at a given timepoint using a Venn diagram for each timepoint (Figure 4-7). When considering only the number of common species found across all replicates of each group, there was a total of 312 common species identified

in the control at the 0hr timepoint (Figure 4-7A), while these numbers increased to 628 and 677 at the 24hr (Figure 4-7B) and 48hr (Figure 4-7C) timepoints. After 24hr of treatment, number of common species within the replicates of each group were 342, 871 and 603 for GA, PA and VA, respectively. After 48hr of treatment the common species within replicates of each treatment group increased to 580 for GA but were reduced to 565 and 175 for PA and VA, respectively. Using the three-way Venn diagram tool allowed to evaluate common species amount control and treatment groups at each timepoint. There were 293 core species detected between all three control timepoints, with 24hr and 48hr sharing 223 common species, which was higher than when compared to the 0hr timepoint. When comparing common species between groups within the 24hr timepoint using a four-way Venn diagram there were a total of 291 core species detected between all treatment groups and control. Control and PA had the most similarity as they shared an additional 102 species in common.. At the 48hr timepoint, there was a total of 163 core species across all groups, with Control and PA having the most in common as they share an additional 72 species in common.. The microbial distribution at the species level between bacterial communities in each sample were further analyzed by calculating and comparing the beta distribution between respective treated samples and their corresponding untreated control. Beta distribution between communities was determined by calculating the Sorenson's Coefficient (Figure 4-8). The coefficients calculated from the comparison between untreated samples at 0hr with those from 24hr and 48hr timepoints (Figure 4-8A), were 0.59 and 0.61, showing these to share similarity, however, when comparing the

bacterial communities between the 24hr and 48hr timepoints, the coefficient value increases to 0.71, suggesting these to have more species overlap. When comparing the similarities between untreated and treated bacterial communities at the 24hr timepoint (Figure 4-8B), the values for GA, PA and VA were 0.59, 0.711 and 0.68. When comparing the similarities between untreated and treated bacterial communities at the 48hr timepoint (Figure 4-8C), the values for GA, PA and VA were 0.69, 0.70 and 0.54.

Discussion

The search for novel antimicrobials, which can be used in farm animal production, remains an important endeavor for the future of food production and safety of products, however, recent uptrends in antibiotic resistance, particularly in enteric bacterial pathogens, and current knowledge regarding the importance of maintaining balanced microbiomes requires careful consideration. Additionally, novel antimicrobials should not have negative effects on the communities of beneficial and commensal bacteria in or on the host, in addition with inhibiting targeted pathogens. Further, these compounds should also aid in making food products safer while also meeting demand, in addition to maintaining animals healthy and preventing cases of zoonosis. Direct elimination of bacterial pathogens through the implementation of antimicrobials remains one of the main strategies for improving food safety but influencing the microbiome through bioactive compounds has been demonstrated to also be a promising approach to achieve the aforementioned goals in the industry (Téllez et al., 2015). Maintaining a diverse gut microbiome has been established as a

hallmark of good health as it contributes to better bioavailability of nutrients and weight gain in chicken models (Salaheen, Kim, et al., 2017). A balanced gut microbiome also helps in the prevention of pathogenic bacterial colonization of the GI tract through competitive exclusion and growth inhibition by producing other bioactive antimicrobial metabolites (Kamada et al., 2013). The compounds used in this study (GA, PA, and VA) had been previously tested *in vitro* against ST, in which they demonstrated antimicrobial potential (Alvarado-Martinez et al., 2020), however, the current study expands on the potential for these compounds to be used in scenarios closer to those that would be encountered inside of the chicken GI tract and evaluates their effect on the normal microbial flora that is commonly found there. Controlling pH with weak acid has been used as a method for increasing their antimicrobial potency in food products as they are often used as preservatives (Lambert & Stratford, 1999). In the case of GA, PA and VA, pH was found to have a significant effect over the antimicrobial potential of each compound. GA not only retained its antimicrobial potential across a wider range of pH and concentrations, but also saw an improvement in activity at certain values, whereas PA lost activity closer to neutrality, while retaining and improving at the ends of the range tested, while VA lost much of its antimicrobial potential at most of the pH values tested, once tested at concentrations below the previously determined MIC. A similar antimicrobial pattern has been previously reported in other organic weak acids where researchers have reported an optimization in inhibition of bacterial growth based on the pH of a given compound at the time of the assay (Burel et al., 2021). Explanations for this have

been proposed to be related to the associated and/or disassociation state of organic acids at specific pH values, which influences their capability to freely diffuse through the bacterial envelope. This has been reported in both Gram-positive and Gram-negative bacteria but is especially relevant in the later since their outer membrane is often viewed as a resistance structure that is to be overcome in order for antimicrobial compounds to be effective in inhibiting their growth (Cunningham et al., 2009; Kundukad et al., 2020). Lower pH values have been associated with more associated states in weak acids, which potentially makes some of them more effective at inhibiting bacterial growth, however this correlation has been found to also be dependent on the ratio between the pH and pKa values of each individual compound, as it has been found to play a significant role in the interactions that will be possible with specific bacteria (Cunningham et al., 2009). In this study, even when at lower concentrations, higher pH values passed neutrality (pH 7), consistently sustained higher antimicrobial activity, especially in GA and PA, though the former being effective up to a pH of 8 and concentration of 0.0625mg/mL, and the latter being effective up to a pH of 9 and a concentration of 0.0313mg/mL. Though the effects of phenolic acids could also be amplified by certain bacterial stressors associated to alkaline stress and enzyme inactivation at these higher ranges of pH, they require further investigation into the mechanics involved in weak acid activity when fixed to higher pH levels, since these still fall within the commonly understood range that is permissible for ST growth (Saito & Kobayashi, 2003).

When evaluating the antimicrobial potential of these phenolic acids against ST in the simulated cecal environment, there was a reduction in potency as compared to previous reports. GA had the ability to limit the growth of ST over time, as compared to the untreated control, however there was no complete elimination of the bacteria despite the use of the previously determined lethal dose of the compound. VA demonstrated the most significant reduction of ST by reducing more ST with each increasing timepoint, while also being the only treatment to reduce the bacterial count to a value below the initial inoculation size. PA had the least effective outcome of the phenolic acids evaluated, as it had no significant reduction of the bacterial growth over any of the timepoints, allowing for the proliferation of ST to reach levels similar to that of the untreated control. Though previous research demonstrated GA to be the least potent of the three compounds *in vitro*, in this study it proved to be more effective than PA, which resembles it in structure. GA has an additional hydroxyl group (-OH) compared to PA, while VA has a methoxy group ($-OCH_3$). The additional functional hydroxyl group could make GA more resistant to oxidation, especially in environments with varying pH ranges (Siquet et al., 2006). The difference in functional groups also contributes to the polarity of each compound, which influences the compound's ability to freely diffuse across the bacterial membrane (N. Kumar & Goel, 2019). In addition to oxidation, commensal gram-positive microbes have been found to break down phenolic compounds and use them as carbon sources, which is a process that has been reported to either confer antimicrobial potential to the resulting products of these reactions or can lead their

inactivation as the microbes further break them down (Álvarez-Rodríguez et al., 2003; Bottery et al., 2021; Kawabata et al., 2019). The pH at each timepoint could also have played a role in the potency of each compound at a given timepoint. While GA was able to remain effective at a wider range of pH values when altered manually, when in the cecal fluid setting it was initially at pH of 4.52, while *in vitro*, pH 4 showed to lower the potency of the compound. At a pH of 5, GA regained efficacy, therefore this could serve as an explanation as of how GA was able to reduce some of the bacteria load at 24hr and maintain ST at similar levels over 48hr. On the other hand, PA, though requiring lower concentrations for antimicrobial potency *in vitro*, at a range between pH 5 and 6 there was a loss of potency, which coincides with the results seen in the cecal fluid, where pH between 0hr and 24hr fell within this range. This could have been a factor that contributed to the lower efficacy of PA in the cecal samples. The effects on pH for VA when in the cecal fluid were not as clear, since despite it loosing efficacy *in vitro* at a pH between 5 and 9, it was the most effective in reducing ST even though the pH of the simulated cecal environment did not go lower than pH 5 during the treatment. This could be related to the different compound structure of VA, which makes it more resistant to oxidation and disassociation in natural environments but could also be related to the bacteria being more favored *in vitro* by a specific compound in LB broth that makes them more resistant to VA in these altered pH ranges (P. L. Tang et al., 2015). These results highlight the importance of understanding the final pH at which a compound is being intended to use, since it might influence the potency of the compound,

especially in those as dynamic as phenolic acids, which might also favor those that are more resistant to these changes, or more electrochemically stable.

Results from the 16S-rRNA gene sequencing and subsequent microbiome analysis revealed that Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria were the most abundant phyla, which is consistent with the pre-established literature regarding composition of chicken microbiome in the cecum (Cardenas et al., 2021; Di Marcantonio et al., 2022). These phyla persisted as the most abundant across timepoints and treatments, though they did experience changes in the percentages of the abundance ratios within each group that were time and treatment dependent. Firmicutes, which mostly consist of Gram-positive probiotic and commensal bacteria, were the most abundant in all sample groups, though they were gradually reduced over time in the untreated control, which had no antimicrobial pressure. Though Bacteroidetes and Actinobacteria increased in abundance over time in the untreated control, Proteobacteria, which mostly consist of Gram-negative pathogenic bacteria, had the most significant increase under the same conditions. When treated with GA and VA, Proteobacteria were less abundant than in untreated samples of the same timepoint, while also preserving the number of Firmicutes, as seen in a Firmicute abundance more similar to that of the one found at the initial conditions of the cecal fluid before incubation. Of the three compounds tested, the one with the most significant reduction of Proteobacteria and promotion of Firmicutes was VA, followed by GA, with PA having a slight numerical difference with the untreated control at the same timepoint.

Analysis at the genus level also showed that some of the most notable genera that were greatly represented within each phylum belonged to the either Firmicutes, Proteobacteria, Bacteroidetes or Actinobacteria. Within the phylum Firmicutes, the most common genera found were common probiotics like *Lactobacillus* and *Ruminococcus*, except for *Clostridia*, which, depending on the species can be either considered part of the normal flora, or could be an opportunistic pathogen (Y. Lin et al., 2017). Within the phylum of Bacteroidetes, which normally mostly consists of gram-negative bacteria, many of which are known to colonize the GI tract of animals (Rychlik, 2020), the genus *Alistipes* was found to be the most abundant. This bacterium genus has been discovered recently, but recent data suggests that it is commonly found within the cecum of chickens and has been found to have performance and growth promoting activity associated to broiler chickens (Parker et al., 2020; Torok et al., 2011). Within the Actinobacteria phylum there are mostly gram-positive soil bacteria like *Bifidobacterium*, a known probiotic bacteria that has been studied and implemented in chicken models, in which they exhibited an improvement in health parameters, as well as demonstrated the potential to reduce the colonization of commonly known gram-negative foodborne pathogenic bacteria (Baffoni et al., 2012; Dev et al., 2021). The Proteobacteria phylum is most associated with harboring a vast array of common gram-negative pathogenic bacteria, including those belonging to the genus of *Salmonella*, however in this study, the most predominant genus within this phylum was *Escherichia*. This is a genus of common enteric bacteria that can be commensal, but also have highly pathogenic and virulent

serotypes that are common foodborne pathogen known for contaminating meat and produce that can lead to infection, causing diarrheal disease and other complications (C. A. Borges et al., 2019; Ewers et al., 2009).

Changes in the relative abundance of the main genera within each group varied across time and treatment. Most notable changes were seen in *Lactobacillus*, which naturally deceased in every group over time, but when treated with GA, PA and VA, their relative abundance remained numerically similar or higher than the untreated control at a given timepoint, showing no detrimental effect of the compounds to this genus. Other gram-positive Firmicutes like *Ruminococcus* naturally decreased over time in the untreated control, but VA promoted them over time. This effect over the Firmicutes could be associated with the VA providing them with an additional nutrient source, while also being further metabolized into other potentially bioactive molecules that could be modulating other genera (R. Hu et al., 2022). Other probiotic gram-positive genus, *Bifidobacterium* saw an increase in abundance over time if left without supplementation, but in all samples treated with phenolic acids, there was an increase in abundance compared to the control of its respective timepoint, with GA having the most notable improvement. On the other hand, GA treatment saw reduction in the prevalence of the *Clostridium* genus after 24hr and 48hr, with the largest decrease being seen at the later timepoint. Previous studies have evaluated the effects of GA in inhibiting the growth of specific species of *Clostridium*, while reporting either no effect or support of specific species of *Bifidobacterium* (Gwiazdowska et al., 2015). VA treatment has been reported in the

past as a potential method for inhibition of *Clostridium*, especially within the context of fermentation (He et al., 2021), however other studies have detected phenolic acid resistance genes within other specific *Clostridium* species, which could have played a role that explains how this genus was promoted by VA in the current study (Luo et al., 2019, 2021). Samples treated with PA saw a genus level relative abundance similar to that of the untreated control at each respective timepoint, while only a slight numerical increase was seen in *Bifidobacterium* and *Lactobacillus*.

Regarding the abundance of the *Escherichia* genus within the Proteobacteria phylum, there was a noticeable difference between the groups treated with GA and VA, when compared to the untreated control and group treated with PA. Phenolic acids have long been known for being especially bioactive against gram-negative bacteria for their capability to diffuse freely through the outer membrane of gram-negative bacteria (Krygowski & Szatyłowicz, 2006). This outcome is desirable considering that most poultry-borne pathogens are gram-negative Proteobacteria. In addition to this, the abundance of Proteobacteria is considered a marker of dysbiosis and can be induced by common pathogenic Gram-negative bacteria, while also being able to displace Firmicutes (Bindari & Gerber, 2022). The balance between the natural antimicrobial potency of each compound and their tolerance to changes in the environment could account for why PA had little effect over the Proteobacteria, whereas GA and VA were more active in modulating the microbiome in general, since the presence and number of specific functional groups has been reported to influence the interaction of specific phenolic acids with Gram-negative bacteria

(Sánchez-Maldonado et al., 2011b). However, the PA treated samples had a noticeable reduction in *Acinetobacter*, which is a Gram-negative bacteria that has been associated with causing nosocomial infections, as well as for having an antibiotic resistance pattern and negatively affecting chicken growth, though GA and VA remained more effective against this genus (Jochum et al., 2021; Xiaolong Zhang et al., 2022). The negative correlation between *Escherichia* growth and probiotic genera of bacteria like *Lactobacillus* and *Bifidobacterium*, shows an inverse relationship between the abundance of these bacteria, which supports the results found in the current study (K. Yang et al., 2022). Previously published research has also proposed treatment with GA as a potential method for promoting probiotic bacteria and reducing enteric pathogens by way of promoting the inverse relationship that exists between their growth patterns (Y. Li et al., 2019).

Analysis of the microbiome at the species level was performed by quantifying the number of species, but also by calculating the alpha diversity using Shannon Index and Inverse Simpson Index for identifying diversity and evenness within the bacterial species found in each sample, while beta diversity was calculated using Sorenson's Coefficient, which analyses similarity of microbial populations between samples (Finotello et al., 2018). These indices have been used as markers for gaining a better understanding on the composition of complex microbial communities and are especially useful when analyzing and interpreting 16S-rRNA gene sequencing data (Tikhonov et al., 2015). Microbial diversity in the host GI tract has gained importance in animal production for its potential in improving animal health and yield, as well as

making them more resilient to disease, increasing their weight, and reducing the load of foodborne and zoonotic pathogens, though some of the correlations associating specific genera of bacteria to improvements in animal performance remain unclear (Peixoto et al., 2021). With the importance of microbial diversity in mind, there has been increasing concern over the negative effects that interventions involving synthetic antimicrobials and conventional antibiotics have on the microbial composition of the GI tract, as these have been shown to cause dysbiosis that makes the host more susceptible to disease and inflammation, but has also been found to alter the gut microbiome in a way that allows for other opportunistic pathogens to colonize the GI tract and remain there long-term, posing an additional threat to food safety (Francino, 2016). With the importance of microbial diversity in mind the number of species and their similarities between groups were quantified and compared by using a three- and four-way Venn diagram that identified similar OTUs of the core microbiome. Further, beta diversity was evaluated through the Sorenson's Coefficient, in which two microbial populations are compared to each other, yielding a quotient between 0 and 1, with values closer to 1 indicating more similarities in the species found between the microbial population being compared, and values closer to 0 indicating populations that are more dissimilar to each other (Diane Kiernan, 2014; Fazlutdinova et al., 2020). Alpha diversity was evaluated through the Shannon Index, which determines species diversity within a given group, while Inverse Simpsons was used for determining evenness, which is a parameter known to be inversely correlated to diversity, meaning that a higher Inverse Simpsons Index denotes less evenness,

which is commonly associated with more diverse communities (Doan et al., 2017; Willmann et al., 2019).

When analyzing the average number of species found in each group and narrowing them down to a list containing the common OTUs found among replicates, there was a reduction in the number of total OTUs that could be attributed to a given group, which could be explained by the natural variability that exists between reads and collection times (Neu et al., 2021). Furthermore, this analysis showed the number of unique OUT species in the control to increase over time along with the number of total species identified. At 24hr control and treatment groups all shared a similar core microbiome, on the other hand at the 48hr timepoint, control, GA and PA become more similar, suggesting that at this stage both compounds were exerting a similar antimicrobial pressure over the same microbes, possibly related to the compounds reaching a similar association state, while VA displayed a different pattern. Further comparison between groups using the Sorenson's Coefficient corroborated that over time, microbial communities became more dissimilar from those found at 0hr, but more similar between more advanced timepoints. When comparing treatments to the control at their respective timepoint, PA showed more similarity to the control at 24hr as it had a value closer to 1, than in the GA and VA treatments, while at 48hr, GA and PA were more similar to their respective control, which is in agreement with the core and unique OTUs detected in the Venn diagrams.

Differences in the Shannon Index for the control at 0hr, 24hr and 48hr timepoints were not statistically significant but did show a slightly decrease as time

progressed. A similar pattern was followed by the Inverse Simpson Index, throughout the three timepoints, with the values for 48hr being statistically significant, meaning that the microbiome at this stage was becoming more even, or less diverse. These indexes reveal that despite an initial increase in the number of species detected from 0hr to 24hr, only accounting for species number does not represent the actual composition of the microbial community at that time, since indexes also consider the amount of hits for each species detected. These results are not unexpected, since if left uninterrupted with a fixed amount of nutrients, over time, specific bacteria will out-compete and out-grow others, displacing them and reducing their overall abundance, even if they can still be detected (Estrela et al., 2021). When analyzing samples within the same timepoint, there was also no statistically significant difference between treatment groups, as compared to the control, but there was a slight numerical increase in the Shannon index of GA treated group at 24hr, in addition to a reduction in evenness, as exhibited by a higher Inverse Simpson Index than the control. On the other hand, a slight reduction in diversity was seen in GA treated group at the 48hr timepoint, but evenness remained lower than the control at the same timepoint. Samples treated with the other compounds, PA and VA at the 48hr timepoint, saw a slight increase in diversity and a reduction in evenness, especially in those treated with VA having the most significant effect in reducing evenness. These results suggest that the compounds tested do not have a significant effect on the alpha indexes for microbial diversity across time, but when using another alpha index, it did reveal an improvement as related to evenness, especially at

the 48hr timepoint as all treated groups were above the control at the same timepoint, most notably VA.

Conclusions

The use of organic weak acids like phenolic compounds has emerged as a viable alternative to conventional antibiotics and synthetic antimicrobials as forms of pathogen control, especially for ST in poultry products. This study evaluated their efficacy in simulated environment using cecal fluid as the medium. Though high doses of these compounds are needed to elicit antimicrobial effect against ST, they can still reduce the overall load the bacteria and other similar Gram-negative pathogens, with minimal disruption to the overall integrity of the microbiome in the GI tract. The effects of these compounds can be attributed to their molecular structure, which allows them to have a specific effect over important Gram-negative pathogens but is a feature that requires further study in order to understand how to implement these products efficiently and effectively in an *in vivo* scenario.

Figures and Tables

Table 4-1. Antimicrobial potential of phenolic acids in LB with adjusted pH.

	Concentration (mg/mL)																					
pH		**0.016**			**0.031**			**0.063**			**0.125**			**0.25**			**0.5**			**1**		
	LB	**G**	**P**	**V**	**G**	**P**	**V**	**G**	**P**	**V**	**G**	**P**	**V**	**G**	**P**	**V**	**G**	**P**	**V**	**G**	**P**	**V**
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
6	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-	+	+
7	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-	+	+
8	+	+	+	+	+	+	+	-	+	+	-	-	+	-	-	+	-	-	+	-	-	+
9	+	+	+	+	+	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+

pH		**1.5**			**2**			**2.5**			**3**			**3.5**			**4**			**4.5**		
	LB	**G**	**P**	**V**	**G**	**P**	**V**	**G**	**P**	**V**	**G**	**P**	**V**	**G**	**P**	**V**	**G**	**P**	**V**	**G**	**P**	**V**
4	+	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-
5	+	-	+	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
6	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-	-	+	-	-	-	-
7	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-	-	+	-	-	-	-
8	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*Plating samples in LB agar was used to confirm growth (+) or complete inhibition (-) of ST after pH-adjusted treatments with gallic acid (G), protocatechuic acid (P) and vanillic acid (V).

Figure 4-1. Antimicrobial effect of GA, PA, and VA on ST in cecum fluid conditions, measured in Log CFU/mL at 0hr, 24hr and 48hr timepoints (**A**) while pH was measured at the same timepoints at which sample aliquots were taken (**B**). Statistically significant ($p<0.05$) between treatments when compared to the untreated control at the respective timepoint is denoted with letters *a-d*.

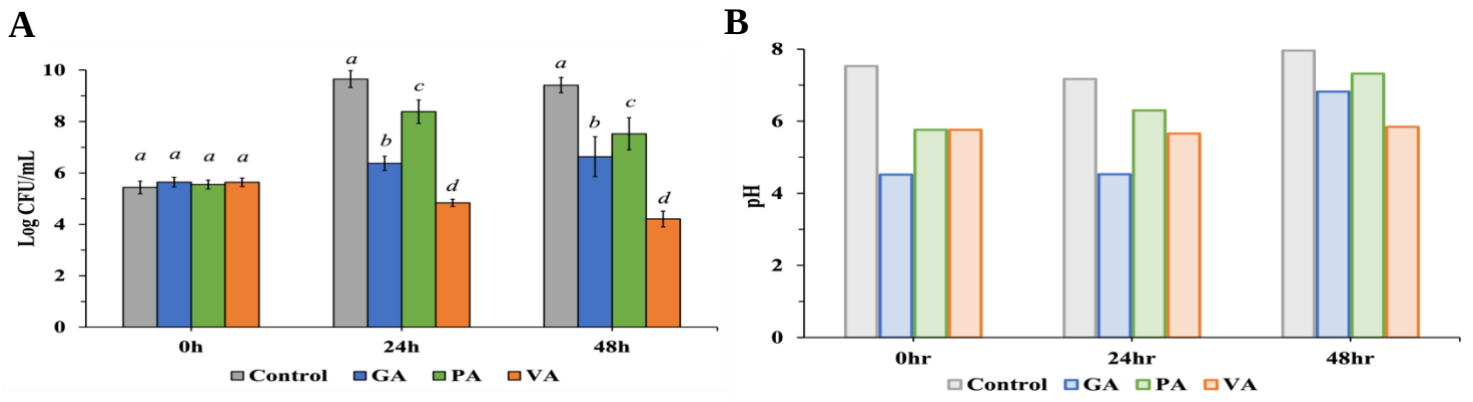


Figure 4-2. Relative percentage of abundance of microbes at phylum level for top 12 phyla in 16S-rRNA gene sequencing data at 0hr (**A**), 24hr (**B**) and 48hr (**C**).

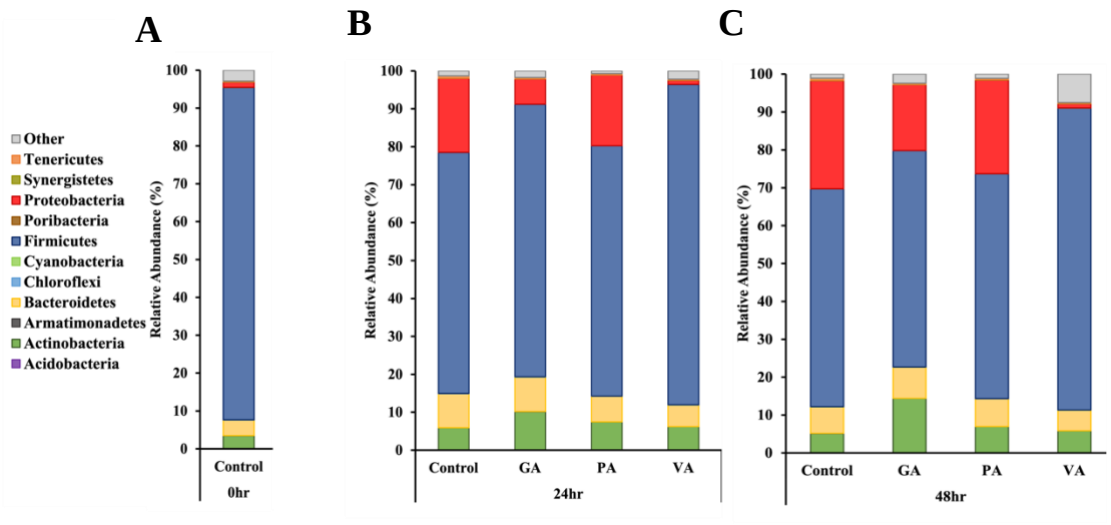


Figure 4-3. Relative percentage of abundance of microbes at genus level for top 25 genera in 16S-rRNA gene sequencing data at 0hr (**A**), 24hr (**B**) and 48hr (**C**).

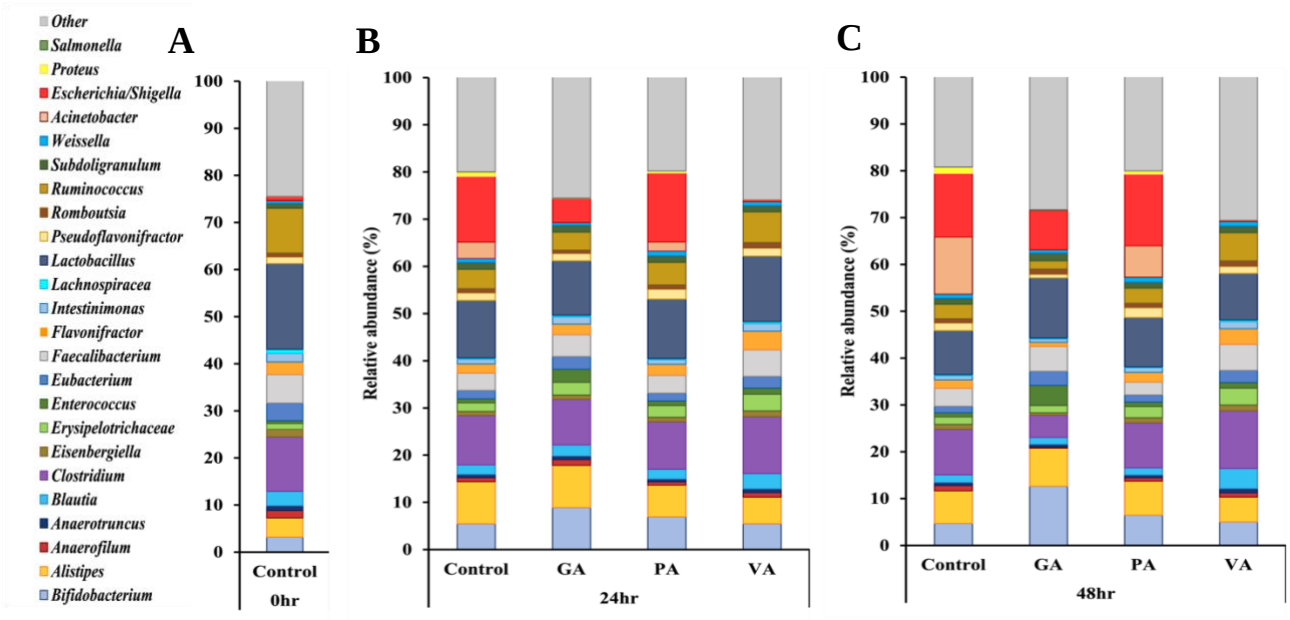


Figure 4-4. Number of identified species in 16S-rRNA gene sequencing data at 0hr (**A**), 24hr (**B**) and 48hr (**C**).

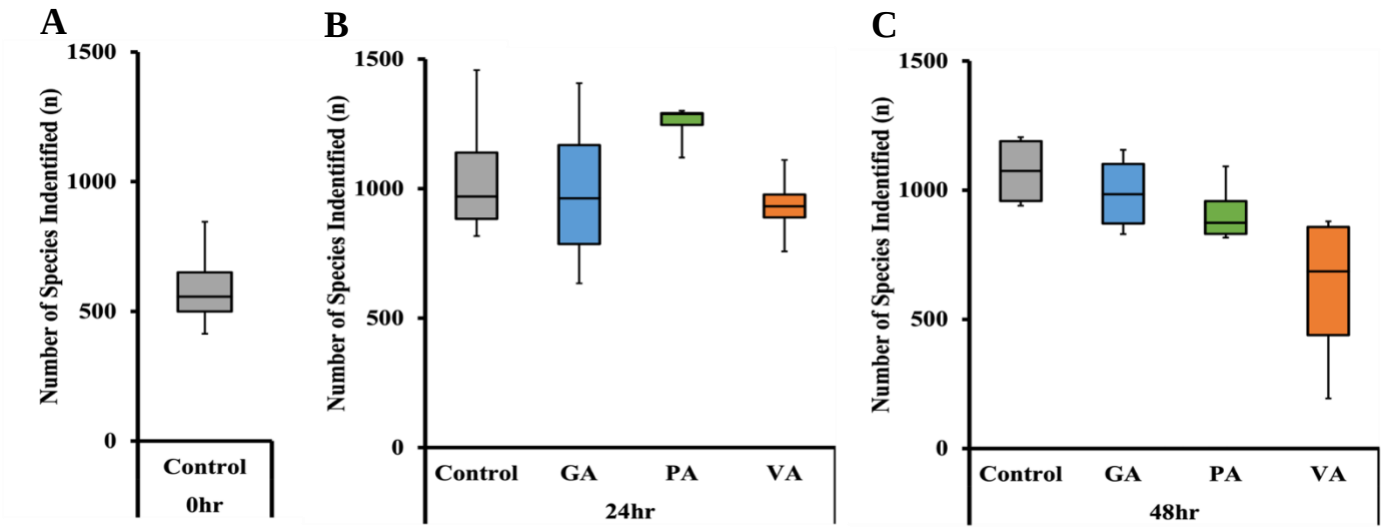


Figure 4-5. Alpha diversity analysis at the species level using Shannon Index from 16S-rRNA gene sequencing data at 0hr (**A**), 24hr (**B**) and 48hr (**C**) for determining diversity.

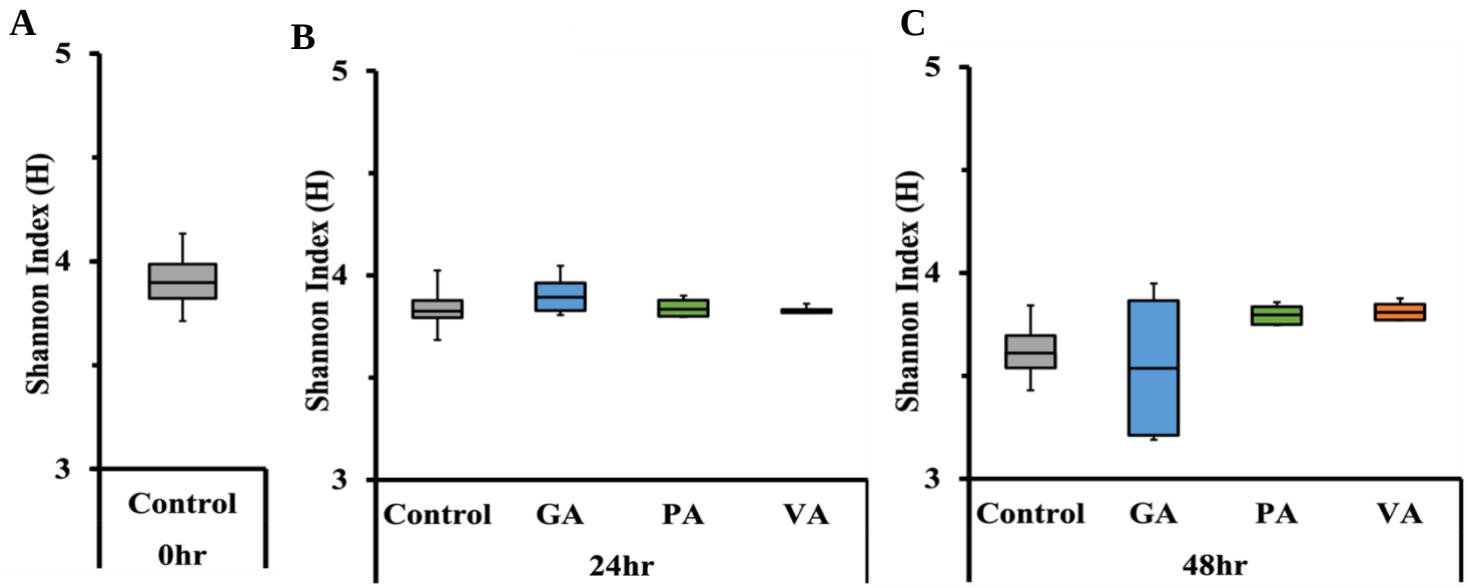


Figure 4-6. Alpha diversity analysis at the species level using Inverse Simpson Index from 16S-rRNA gene sequencing data at 0hr (**A**), 24hr (**B**) and 48hr (**C**) for determining evenness.

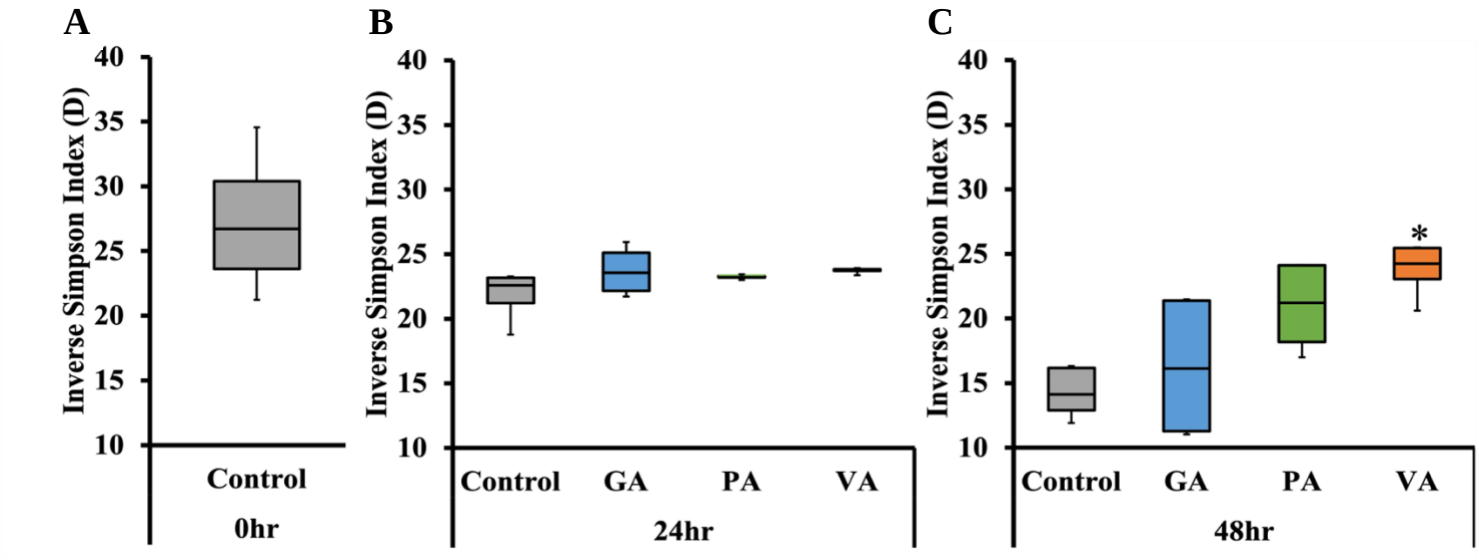


Figure 4-7. Multivariate Venn diagram demonstrating species commonness between sample OTUs by comparing controls at different timepoints (**A**), as well as the control, GA, PA and VA treatment groups at 24hr timepoint (**B**) and control, GA, PA and VA treatment groups at 48hr timepoint (**C**).

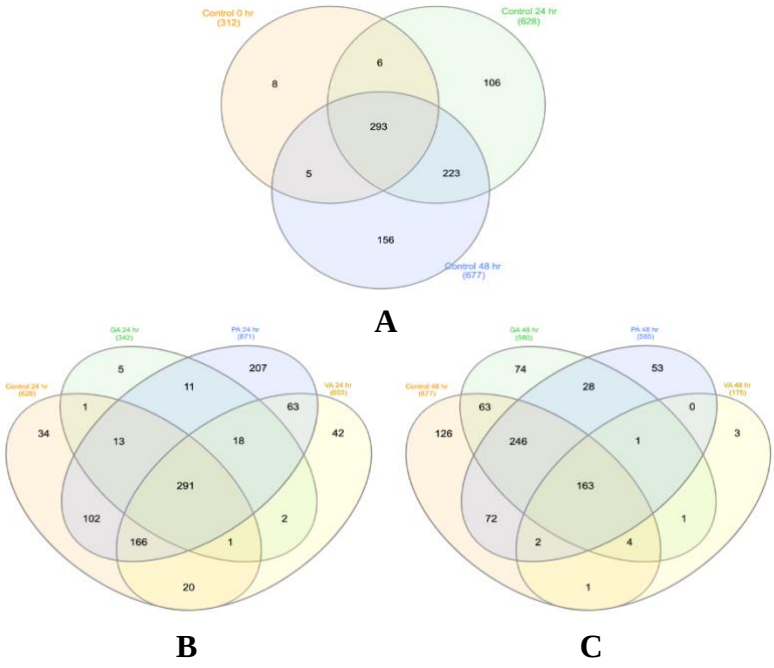
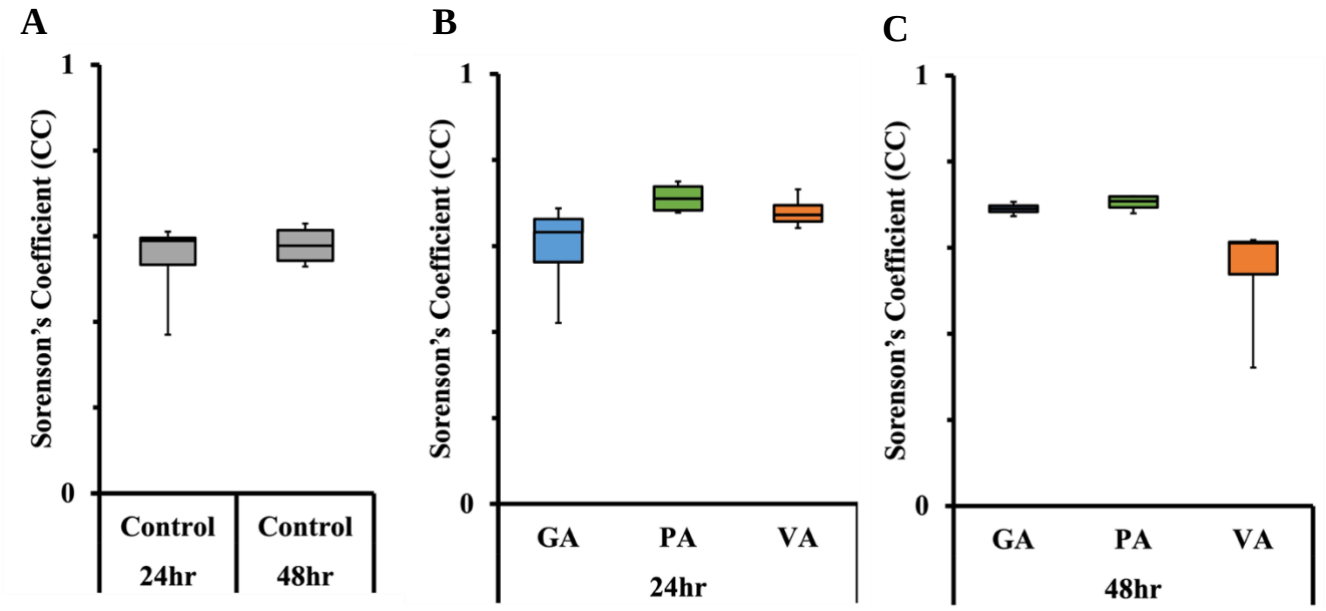


Figure 4-8. Beta diversity analysis at the species level using Sorenson's Coefficient from 16S-rRNA gene sequencing data calculated by assessing overlap between population of species found in untreated controls at 0hr with those at 24hr and 48hr (**A**), as well as assessing overlap between each treatment category and their respective untreated control within the same timepoints, namely 24hr (**B**) and 48hr (**C**)



Chapter 5: Phenotypic and morphological alterations of *S.* Typhimurium after treatment with individual phenolics to predict mechanism of action

Introduction

Salmonella enterica serovar Typhimurium (ST) remains one of the most relevant bacterial pathogens in the USA given their prevalence in multiple contamination routes, making it a major causative agent of disease, hospitalization, and death, in addition to their clinical relevance as an increasingly antibiotic resistant pathogen for which treatment options are becoming increasingly limited (Angelo et al., 2016; Gut et al., 2018; Scallan et al., 2011). Though some cases are self-limited, the invasive capability of the pathogen allows it to survive intracellularly, leading to prolonged infections that can progress into a systemic infection, requiring more specialized medical intervention (Mastroeni & Grant, 2011). Discovery of novel antimicrobial compounds with the purpose of controlling pathogenic bacteria has also been faced with several challenges exacerbated by the increasing trends in antibiotic resistance, which places an additional pressure on the discovery, testing, releasing and implementation of antimicrobials that have long-term use (Silver, 2011). In addition to this, current knowledge surrounding the importance of preserving the integrity of the local probiotic and commensal communities of bacteria that make up the microbiome of an animal host (Konstantinidis

et al., 2020). Current pipelines for discovery of novel antimicrobials often focuses on antibiotics derived from fungi or other bacteria belonging to the Actinomycetes, which are often further modified chemically in order to improve their spectrum of efficacy, but antibiotic resistance has led to these modifications being required for the antibiotics to remain effective (Hutchings et al., 2019). An alternative source of bioactive molecules with antimicrobial potential has been found in plants, particularly in the polyphenolic compounds that make up a major portion of plat extracts (Savoia, 2012).

Plant-derived compounds like polyphenols are comprised by a diverse array of molecules with numerous functional groups that modify a single or multiple benzene rings (H. Al Mamari, 2022). The modification of benzene rings with hydroxyl, methoxy and carboxyl functional groups allow polyphenolic compounds to interact with bacteria through various pathways but have also been found to both directly and indirectly interact with proteins and toxins, in addition to other cell structures and macromolecules of bacteria (Makarewicz et al., 2021). The use of plant-derived polyphenolic compounds for the inhibition of both Gram-positive and Gram-negative pathogenic bacteria has gained notice and has been well reported in the past, including for pathogens like ST. Though polyphenolic compounds belonging to groups such as flavonoids, tannins and catechins have received much attention for their antimicrobial potential, many researchers have focused on compounds belonging to phenolic acids. These comprise at least one-third of the polyphenolic compounds found in plants, are readily solubilized without need for toxic solvents, and have been proven to have antibacterial potential (A. Borges et al., 2013; Kaurinovic & Vastag, 2019). The low

molecular weight of phenolic acids compared to other polyphenols has been cited as a factor that potentially allows for more interactions with bacterial structures, resulting in greater antimicrobial effects (Ravichandran et al., 2011). Gallic acid (GA), protocatechuic acid (PA) and vanillic acid (VA) have shown antimicrobial potential against ST in previous research, in addition to the ability to reduce the expression of specific virulence genes *in vitro*, however, the mechanisms of action that confers the observed antimicrobial and antivirulence activity of these compound remains to be fully elucidated (Alvarado-Martinez et al., 2020).

Considering the mechanisms of action that have been proposed for phenolic acids in the past, the current study focuses on assessing the effects of GA, PA and VA on various parameters of bacterial physiology in ST. This research evaluated changes in bacterial function associated to acid resistance and cell division, in addition to evaluating changes in morphological and phenotypic characteristics of ST related to cell structure and integrity of the cellular envelope.

Materials and Methods

Bacterial strain and their growth conditions

Salmonella enterica serovar Typhimurium (ATCC 14028) (ST) was used for all experiments performed in the current study. ST was grown in Luria-Bertani (LB) (Becton, Dickinson and Co., NJ, USA) agar at 37 °C under aerobic conditions (Thermo Fisher Scientific Inc., Marietta, OH, USA). All experiments described were performed in LB broth (Becton, Dickinson and Co., NJ, USA) and were incubated under aerobic

conditions for 24 h in 37 °C with continuous aeration at 150 rpm through the use of a shaking incubator**.**

Compounds and stock solution preparation

GA (Acros Organics, Geel, Belgium), PA (Sigma-Aldrich, MO, USA) and VA (Alfa Aesar, MA, USA) were purchased in solid powder form. Stock solutions of gallic acid and protocatechuic acid were prepared by dissolving in sterile deionized water, while vanillic acid was prepared by dissolving in 30% ethanol (Pharmco-Aaper, CT, USA). Stocks for all compounds were prepared to a concentration of 10 mg/mL. Phosphate buffer saline (PBS) was prepared to a pH of 7.2.

Working solutions of the fluorescent D-amino acid (FDAA) 7-hydroxycoumarincarbonylamino-D-alanine (HADA) (Tocris Bioscience, MN, USA) was prepared following the manufacturer recommendations (Peters et al., 2019). Briefly, A 50mM stock solution of HADA was prepared by dissolving 2.1mg of HADA powder in 127.77μL of DMSO.

RNA extraction and cDNA synthesis

Samples were prepared for total RNA extraction by inoculating a bacterial suspension of ST to a final concentration of 10^4 colony forming units (CFU)/mL and incubated overnight with sublethal concentrations of each compound. They were individually treated with the sublethal concentrations (below calculated MIC, namely 3.0, 1.5 and 1.0 mg/mL for GA, PA and VA, respectively) of each compound and incubated. RNA was extracted following the previously described methods with certain

modifications (Peng et al., 2015). Briefly, samples were collected through centrifugation at 14,000 × *g* for 15 min to collect bacterial pellet containing total RNA, with TRI Reagent® (Molecular Research Center Inc., OH, USA) being used as the extraction agent for homogenizing bacterial pellet through cell lysis, allowing for a chloroform extraction to separate the RNA. RNA was suspended in molecular-grade RNase-free water and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., OH, USA) for standardization. This RNA was used as the template for cDNA synthesis with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA), in accordance with the manufacturer indications. The cDNA synthesis protocol was set to incubate for 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min.

Quantitative RT-PCR assay

Changes in the relative expression of genes associated with various cellular functions of ST were measured using qRT-PCR. The qRT-PCR reaction was prepared in accordance with the manufacturer indications for the PerfeCTa SYBR Green Fast Mix protocol (Quanta Bio, MA, USA), and performed using the Eco Real-Time PCR system (Illumina, CA, USA). Cq values were recorded and used to calculate the RQ, but was represented as the Log-fold change. Cycle protocol was set to 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, 15 s at 55 °C and 10 s at 72 °C. Custom primers were designed to target conserved regions of genes associated with regulation and activation of various proteins and enzymes responsible for enacting cellular functions

in ST. Relative expression of these genes was determined by normalizing and comparing to an untreated control, in addition to having using a *16S-rRNA* housekeeping gene (Table 5-1).

Measuring membrane damage and permeability using fluorescent dyes

Damage to the cell membrane of ST that might have led to increased permeability of the membrane during treatment with phenolic acids was evaluated through the use of a Cytation 3 fluorescence microplate reader (BioTek, VT, USA) to measure RFI and later fluorescent microscopy was performed using the Zeiss AxioObserver Florescence microscope (Carl Zeiss AG, Jena, Germany). Nucleic acid dyes SYTO9 and PI from a BacLight^{TM} Bacterial Viability Kit (Invitrogen Molecular Probes, CA, USA) were used as indicated by the manufacturer, with some modifications. Briefly, ST cultures set to a final OD_{600} of 0.1 (10^8 CFU/mL bacteria) in LB broth were individually treated with previously determined lethal doses from each phenolic acid, which were calculated based on the MBC of each compound, while an untreated negative control was also prepared. These bacterial solutions were individually treated for 0, 4, 8 and 24 h, at which times 1 mL aliquots were taken from the total suspension. At the given timepoints, aliquots were prepared as per the manufacturer recommendations by spinning down the samples, washing with and resuspending in a 0.85% NaCl solution. SYTO9 and PI were both added to the samples and incubated for 15 min, after which the fluorescence of both dyes was measured in a black 96-well plate with a fluorescent plate reader set to an excitation of 485 nm with

emissions of 530 mm and 630 nm for SYTO9 and PI, respectively. In addition to the plate reading, 5 μL aliquots from each sample at 4, 8 and 24 h timepoints were taken and fixed using 10% formaldehyde for preparing slides that were later observed using fluorescent microscopy. Pictures taken with the Zeiss AxioObserver Florescence microscope were minimally processed using the default Zeiss ZEN software from the same company.

Scanning electron microscopy

Preparation of samples for observing changes in the cell shape of ST through SEM was performed as described previously (Farkas et al., 2017; Peng, Tabashsum, et al., 2018), with some modifications. Briefly, ST cultures in LB broth were individually treated for 24 h with sublethal concentrations (below calculated MIC, namely 3.0, 1.5 and 1.0 mg/mL for GA, PA and VA, respectively) of phenolic compounds, in addition to an untreated control. Bacteria were pelleted, washed and resuspended in PBS, after which samples were placed in a polycarbonate membrane filter (GTTP 0.2 μm) (MilliporeSigma, MD, USA) and fixed with 2.5% (*v/v*) glutaraldehyde (Electron Microscopy Sciences, PA, USA) for 1 h. Filters were washed with deionized (DI) water and later dehydrated by sequentially soaking with increasing concentrations of absolute ethanol (10%, 20%, 50%, 80% and 100%) for 5 min each. Filters were kept overnight over anhydrous magnesium sulfate to eliminate residue moisture. Filters were prepared for SEM by sputter-coating with gold and observed using the Hitachi SU-70 FEG Scanning Electron Microscope (Hitachi High-Tech Corporation., Tokyo, Japan).

Fluorescent HADA probe labeling

Changes in synthesis of peptidoglycan (PG) cell wall was evaluated in ST individually treated with either GA, PA or VA. Integration of old and novel PG was evaluated through measuring accumulation of FDAA HADA probe, which is a fluorescent PG analog. A bacterial suspension was prepared in LB broth to contain a final bacterial load of 10^8 CFU/mL (OD_{600} 0.1) and a prepared to contain sublethal concentrations of either GA, PA or VA. HADA labeling was performed as has been previously described, with some modification (Peters et al., 2019). Briefly, two sets of samples were prepared, one containing an initial dose of HADA that was added to each sample, achieving a final concentration of 250μM, while the other had a second dose of HADA added after the 24h incubation with each phenolic acid dose and allowed to integrate for 30min. Preparation of samples for measuring fluorescence was done by centrifuging samples at 14,000 × g for 15 min to collect bacterial pellet, which is washed with PBS X 1 twice to eliminate residues of media and unbound HADA. Bacterial pellets were resuspended and OD_{600} was measured to adjusted to 0.1 in order to account for signal differences caused by differences in bacterial densities. Relative fluorescence intensity (RFI) was measured using a fluorescent microplate reader at an excitation of 350nm and emission of 460nm.

Statistical Analysis

Data were analyzed using a Student's t-test to determine the significant difference ($p < 0.05$) between the untreated control and the separate treatment with the individual compound.

Results

Changes in relative gene expression of ST genes

Treatment with GA, PA and VA lead to notable changes in the expression of specific genes and was evaluated using qRT-PCR (Figure 5-1). The genes tested could be classified as being associated with either acid resistance, cell division or cell structure, though in many cases the functions of these genes overlap between categories. Associated to acid resistance, *cfa*, *lpxO* and *rpoS*, were evaluated, with *cfa* showing a statistically significant ($p < 0.05$) reduction for samples treated with PA (1.27 Log). Treatment with VA showed an significant ($p < 0.05$) upregulation for *cfa* (2.15 Log), *lpxO* (2.52 Log) and *rpoS* (2.04 Log). GA showed a numerical downregulation of *lpxO* (0.57 Log) and *rpoS* (0.67 Log), but were not statistically significant. Further analysis of genes associated with cell division, GA and PA significantly ($p < 0.05$) downregulated the expression of *minD* by 1.09 and 2.07 Log, respectively, while VA had an upregulation effect by 1.70 Log. PA was also abe to downregulate *ftsZ* by 1.01 Log. In samples treated with VA there was a significant upregulation of other division genes like *ftsZ* (2.66 Log), *mraZ* (2.91 Log) and *murD* (2.87 Log). GA, PA and VA exhibited a numerical increase in *envC* of 4.08, 1.49 and

3.79 Log. Genes associated with maintaining cell structure showed to be significantly upregulated by VA, as seen for the cases of *lapB* (2.98 Log) and *mreB* (3.73 Log). GA showed a numerical upregulation in *mreB* of 1.35 Log.

Plasma membrane permeability and damage

Changes in outer membrane permeability were measured using BacLight™ Bacterial Viability Assay, which uses SYTO9 and propidium iodide (PI) as indicators of membrane integrity. SYTO9 freely crosses the cell membrane and intercalates with DNA, emitting a green fluorescence, while PI is used as the main indicator for membrane damage, since it mainly crosses the bacterial membrane when there is increased permeabilization, allowing it to compete with SYTO9 for DNA intercalation within the bacterial cytoplasm, leading to the emission of red fluorescence that can be measured spectrophotometrically in units of relative fluorescent intensity (RFI) and microscopically (Figures 5-2 and 5-3). There was no significant difference between control and treatments at 0 h. Significant ($p < 0.05$) differences between the RFI levels of the control and treatment were seen in GA at subsequent timepoints, with an increase of 2584, 2123 and 2345 RFI at 4, 8 and 24 h. Overall RFI for GA decreased at 8 and 24 h, but remained significantly higher as compared to the control. The RFI for PA and VA experienced an increased to 1851 and 4062 RFI, respectively, at 4 h, which was significant ($p < 0.05$) as compared to the control. At 8 h, the RFI for both PA and VA decreased, with only the latter remaining slightly numerically higher than the control. At 24 h, both RFI values increased to 2441 and 3212 for PA and VA, respectively, as

well as remaining significantly higher than the control. The results from fluorescent microscopy showed an untreated bacterium to mostly take up SYTO9, as evidenced by the prevalent green coloration of the bacteria, though the presence of slightly more yellow bacteria at increasing timepoints signals a low take-up of PI. In treated bacteria, an increasing PI buildup could be seen at 4 h particularly for GA and VA, while at 8 h all bacteria exhibited a PI buildup and higher counts of red bacteria. At 24 h, all treated bacteria had comparatively more presence of red bacteria than green.

Reduction in cell wall synthesis

A fluorescent plate reader was used for measuring the RFI of samples labeled with HADA, either at the beginning or towards the end of the treatment bout with either GA, PA or VA (Figure 5-4). When given only an initial dose of HADA, after 24 h (Figure 5-4A), there was an uptake of HADA by the untreated control that resulted in an RFI of 3.79×10^6, which in the case of the sample where HADA was added later there was an increase to 6.81×10^6 RFI (Figure 5-4B). The corresponding bacterial load at that time was also calculated in Log CFU/mL using microdilution assay and plating in fresh LB agar (Figure 5-4C). The corresponding bacterial load for the untreated control was 8.70 Log CFU/mL. In the case of samples treated with GA, there was a lower RFI values for the samples where new HADA was added, as compared to the control, with 3.5mg/mL being the lowest and yielding a value of 2.19×10^6, while the corresponding bacterial load was also lower with a value of 6 Log CFU/mL. In the case of PA new HADA uptake was lower than the control at 2mg/mL, with an RFI of 4.98

x 10^6, however, these samples had a slightly higher bacterial load, as shown by a 9.93 Log CFU/mL concentration. VA treatment also showed HADA uptake to be lower at 1.5mg/mL, with an RFI of 4.86 x 10^6, along with its bacterial load, which was 3.55 Log CFU/mL. At 0.5mg/mL VA also displayed the lowest RFI recorded or 2.08 x 10^6, despite having a bacterial load close to that of the control, that being 8.21 Log CFU/mL.

Morphological alterations of ST

Physical changes in ST were examined using scanning electron microscopy (SEM), which allows for a detailed visualization of the bacterial outer membrane after treatment and a later comparison to the untreated control (Figure 5-5). The untreated control exhibited the usual rod shape associated with ST. However, a distinct inward collapse of the cell membrane, that could be visualized as a hole, was observed at the polar ends of rods treated with GA and PA. On the other hand, VA did not exhibit the same morphological defect as its other two phenolic counterparts; however, cells from this sample featured a vast number of rods in the middle of binary fission, with a clearly formed division ring.

Discussion

Plant extracts have been long used and tested for their antimicrobial potential against common pathogenic bacteria, while also being proposed as both alternatives to conventional antibiotics and/or as adjuvants that can restore antibiotic function (Ali et al., 2022; Salaheen, Peng, et al., 2017b). The efficacy of raw plant extracts has been partly attributed to the wide variety of potentially bioactive compounds found in

them, which are believed to function synergistically and exert multiple pressures against the bacteria (Gonelimali et al., 2018). Though full plant extracts might be more potent and efficacious (Rasoanaivo et al., 2011), plant extract fractions have been used as methods for further studying the mechanisms of action behind specific bioactive molecules in the raw extracts (Mogana et al., 2020). Further, identifying specific compounds that drive the activity of plant extracts is a crucial step for identifying the mechanisms of action behind the antimicrobial activity of these compound, which provides valuable information that informs the proper future use of these compounds, as well as helps in identifying more potential use cases for these compounds (Saritha et al., 2015). The current study focused on assessing the effects of GA, PA and VA on specific functions associated to bacterial physiology of ST, namely, structure, cell division and environmental acidity response, and use these parameters to elucidate more information regarding the mechanism of action behind these compounds.

Given the interest in understanding more about how these compounds affect important cellular functions, there was an initial qRT-PCR screening of genes identified in the literature to be important mediators, regulators or encoders of important proteins and enzymes responsible for multiple facets of cell survival. Though previously there was mention that the genes being tested for fell within the categories of mediators of acid resistance, cell division or cell structure, it is important to note that these functions often overlap in specific genes (Guan & Liu, 2020b). Many mechanisms involved in acid resistance, also involve the regulation of

metabolic activity, which affects bacterial growth, but also involves modifications of the cell envelope to increase rigidity. On the other hand, many genes associated with maintaining cell structure are also crucial for the process of cell division (Mahone & Goley, 2020). Previous research that has delved more profoundly into studying the effects that phenolic acids have on Gram-negative bacteria have cited a decreased metabolic activity, inhibition of enzyme function and cell membrane damage as common outcomes of exposure to these compounds (Liu et al., 2020). Though these could all contribute to cell death, outer membrane damage has been consistently documented, which warrants further study, since the outer membrane of Gram-negative bacteria is of extreme importance for survival in adverse environments and resistance to antibiotics (Gootz, 2006).

Relative expression of genes associated to acid resistance was measured for *cfa, lpxO* and *rpoS,* though *cfa* could serve dually as a structure gene since it is responsible for the formation of cyclopropane fatty acids (CFAs), which are formed in response to acid stress to fortify the outer membrane (Karlinsey et al., 2022). Out of all the treatments, PA had a reduction, while VA saw an uptick in expression for *cfa,* which begins to suggest different modes of action between these compounds. When looking specifically at genes related to cell division, VA showed upregulation in multiple genes, namely, *ftsZ, minD, mraZ* and *murD*. Genes for *ftsZ, minD* and *mraZ* are associated with the regulation, formation and colocalization of the bacterial divisome (Eraso et al., 2014; Park et al., 2018). Overexpression in some of these genes have been associated with abnormal cell arrangement, such as that of

filamentous cells and mini-cells. In the case of GA and PA, they both showed activity in downregulating *minD*, which studies have shown that when knocked out in a bacteria could also lead to the formation of minicells that do not contain chromosomal material and present defects on the main mother cell (Rampley et al., 2017). On the side of structure genes, *mreB* serves as a pseudo-cytoskeleton, though it is also involved on the synthesis of new cell walls during cell division (Varma & Young, 2009), while *lapB* functions to assemble LPS (Cian et al., 2020). Both of these were again upregulated during the treatment with VA, suggesting that though VA has been considered to be the most lethal of the three phenolic acids tested, at sub-lethal conditions it could be inducing or activating these bacterial survival mechanisms.

Further assays were performed on the cell membrane, given its importance as the first defensive barrier against antimicrobials and is responsible for conferring resistance to a variety of antibiotics, as well as to multiple environmental stressors (Page, 2012). Research on food contaminated with *E. coli* has discovered that the communities found in these environments resemble stationary phase bacteria that are well adapted to a lower pH, higher temperatures and osmotic pressure (Berry, 2000). However, one of the characteristics of these bacteria is an increased re-enforcement of the cell envelope (Jaishankar & Srivastava, 2017). On the other hand, resistance to cationic antibiotics that target outer membranes has been reported to be usually mediated through the modification of lipopolysaccharides (LPS), leading to an alteration of the net charge of the outer membrane (Poirel et al., 2017). Phenolic acids

have long been hypothesized to damage bacterial outer membranes through mechanisms mediated by electrostatic and ionic interactions, or by passive diffusion to the cell interior, but the exact interaction that leads to this outcome has remained undefined (Gutiérrez-Larraínzar et al., 2012). Previous studies have reported this effect to be dependent on the relative acidity and oxidation of the compound in the solution at the time of contact with the bacteria, which will affect whether it will be able to penetrate the outer membrane and the extent to which it will interact with bacterial lipids and proteins located in the cytoplasm and periplasmic space (Gutiérrez-Larraínzar et al., 2012; Slonczewski et al., 2009b). Measuring PI RFI served as an indicator to measure changes in membrane permeability as a result of treatment and the time of exposure. This experiment demonstrated a significant increase for all treatments at the 4 and 24 h timepoints when compared to the untreated control at that same time, with GA and VA accounting for the highest reported RFI reads, though at 24 h PA had similar value as GA. The reasons behind the decrease in RFI at 8 h is unclear, but could be related to a delay in growth and cell death because of the treatments, yielding a lower cell count that resulted in a lower RFI signal for PI, but leaves open the possibility that this occurred as a result of some acid tolerance response mechanism. Previous research on membrane-permeable organic acids suggests that small molecules such as phenolic acids readily cross the outer membrane and damage the cell from the inside through cytoplasm acidification, which is known to be a factor in increasing membrane permeability (Krulwich et al., 2011). Another review on weak acid interactions with bacteria suggested that when

weak acids disassociate, there is an accumulation of anions in the periplasmic space that can lead to a disruption of membrane function and metabolic processes, despite the various resistance mechanisms that ST has developed to counteract weak acids (Hirshfield et al., 2003). The time that this mechanism takes to activate and take effect is unclear, but a decrease in PI uptake at 8 h could be a result of the activation of these tolerance mechanisms, but an increased exposure time overwhelms the bacteria leading to a resurgence of PI uptake at later timepoints such as the one seen at 24 h.

Further investigation on alterations to membrane integrity were examined through microscopy. Through fluorescent microscopy, there was a visible representation of the increasing membrane permeabilization as the exposure time to the treatments increased, evidenced by the increasingly red coloration of treated cells as time passed. Additional tests in the cell envelope involved using an FDAA called HADA which could label the peptidoglycan layer of ST as it formed and intercalated new subunits. When comparing the integration of HADA that was added at the beginning of the treatment there was a slight difference between the treated and the untreated control, with the untreated control having a higher intercalation rate based in the higher RFI value. However, when new HADA was added to the solution, the untreated control was able to integrate more HADA than the one present initially, suggesting a continued activity in cell wall synthesis. In the case of GA treated samples, even after the addition of the new HADA, RFI remained lower than the control suggesting a lower activity in new cell wall synthesis. The effects of phenolic

acids on cell wall synthesis have been reported before, but mainly in tandem with discussions also including the cell membrane and envelope as a whole (Aldulaimi, 2017; Pancu et al., 2021). However, it is important to also note that in the case of PA, there was a recorder higher RFI for original and new HADA, but this could have been influenced by the higher bacterial load present at the end of this experiment. Conversely an opposite effect was seen in the lower concentration of VA, where there was a bacterial load similar to that of the control, but low integration of new HADA at these sublethal concentrations.

Further investigation on this effect was conducted through SEM, which allows for a detailed viewing of cell morphology. Images taken of GA and PA treatment showed bacteria that shared a similar morphology, suggesting a similar mode of action of the compounds and a similar cellular response, even though the lethal dose required for achieving cell death was significantly higher for GA, as explained previously. The most notable cellular defects that were observed in bacteria treated with GA and PA were inward dents located at the polar ends of the rod-shaped cell, while the central body of the cell remained relatively unchanged as compared to an untreated control. The reason for this change is yet to be determined, as this kind of phenotype has not been reported previously for bacteria treated with phenolic acids. Possible explanations for this particular morphology could be related to damage or alterations to proteins more commonly collocated at cellular poles, such as osmotic pressure regulators and division complexes (Varma & Young, 2009), or could be related to changes in the integrity of bacterial cytoskeletal proteins (Bulmer et al.,

2012; Doble et al., 2012). The morphotype observed for VA was different than the other phenolic acids, as these did not show clear evidence of membrane damage, or cell structure change; however, the overwhelming majority of bacteria observed were found to be mid-division. These contained a clearly formed division septum, but separation of the cell membrane was arrested as the daughter cell remained attached. These findings suggest a perturbation of the cell division process that allows for septum formation but prevents finalization.

Conclusions

Given the results of the current study, researchers can begin to gain a better understanding regarding the mechanisms of action behind certain plant-extracts and how they might be being driven by specific compounds. The safe application of novel antimicrobial compounds requires a better understanding of the ways that bacteria interact with them. This also opens the opportunity for targeted application of these compounds in specific scenarios. The results discussed here corroborate previous theories surrounding the mechanism behind GA, PA and VA, but expand on other areas in which these may be active, particularly when considering the unique phenotypes observed after treatment with these compounds.

Table 5-1. Primers used in determining gene expression of ST strains.

Gene	Protein	Primer Sequence (5'-3')
16S-rRNA	16S-ribosomal RNA protein	F: GGTCTTGACATCCACAGAAGAA
		R: TCACTGGCAGTCTCCTTTGAG
cfa	formation of cyclopropane fatty acids	F: GGGATCGAGCATACGGGAAA
	(CFA)	R: GGAATGCGAGCGTCTGGATA
lpxO	putative dioxygenase for synthesis of lipid	F: CGACGCCGGATTCAATACCT
		R: TAGGGGTCGCGATGTTTTCC
lapB	LPS synthesis	F: CGATCGCGCCATTCGTATTC
		R: CATATAGTCGCGCCCCAGTT
mraZ	negatively regulates proximal part of the	F: TCGTTTCATCCCACAGCTCA
	division	R: ATGAACCCGGTAGAACGTCG
ftsZ	contractile Z ring structure and	F: CTTCCAGCAGCGGAGAAGAA
	colocalization	R: CTAACGACGTGCTGAAAGGC
zipA	cytoplasmic membrane anchor for the Z	F: GAACGTTCCCGGATTGACCA
	ring	R: GCGTAAAGAGGCGGTCATCA
envC	activates amidase for peptidoglycan	F: AGGCTTCCTGCTGCTTATCG
	cleavage	R: ACAGGCGCGTAATGAACGTA

minD	regulates Z ring formation and location	F: GGCGCTCTATTTTGCCGATG
		R: TGCTGAGCATGTCGCCTTTA
murD	cell wall synthesis	F: GTCTGGGCTTAACCGGACTC
		R: CGTCACGCGAGTATCCATCA
mreB	membrane-associated dynamic filaments	F: AAGTTGCCGTGATCTCCCTG
		R: ACTTCGATCTCGCGGACTTC

Figure 5-1. Evaluating the relative expression of genes associated with acid resistance, cell division and cell structure, in samples treated with either GA, PA or VA and normalized to an untreated control using a 16-rRNA gene as the housekeeping gene. Significance is denoted as a star (*) ($p < 0.05$).

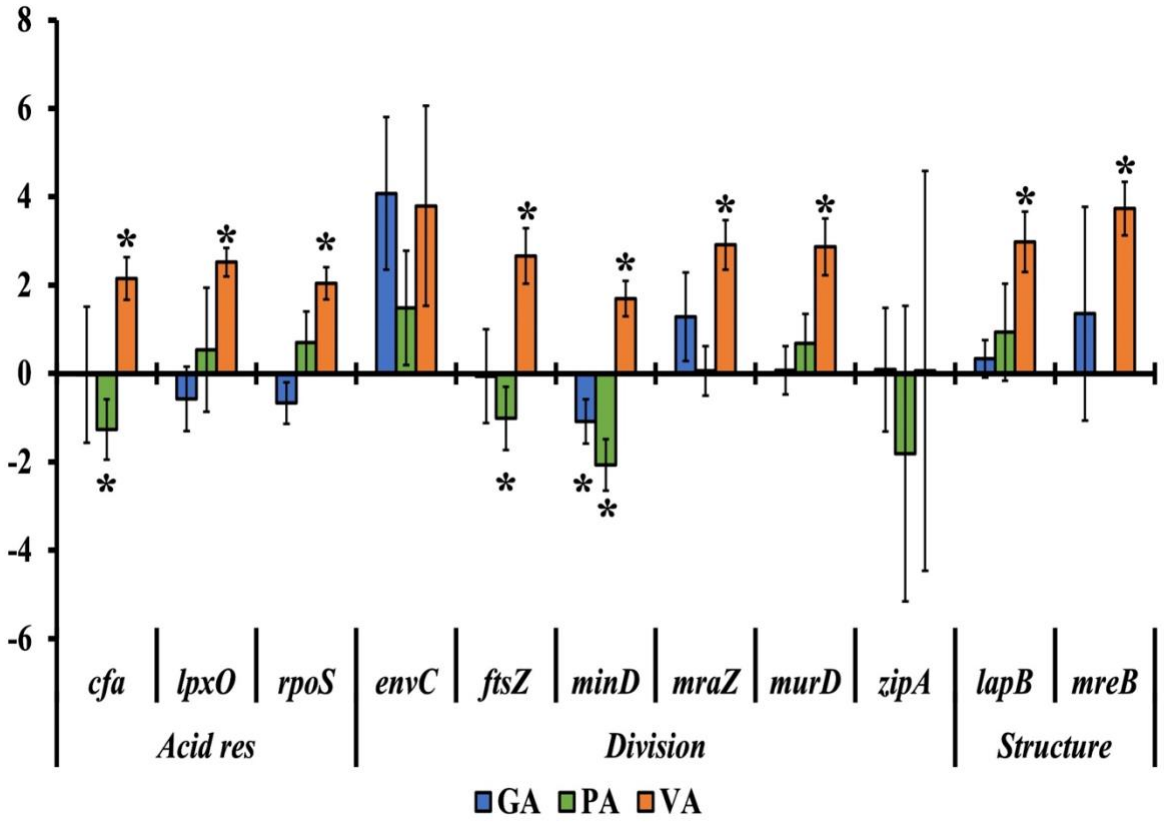


Figure 5-2. Measuring permeability of cells using fluorescence microplate reader measurements of relative fluorescent intensity (RFI) for untreated and treated ST with GA, PA and VA. Letters (a-d) denote significant difference between treatment groups ($p < 0.05$).

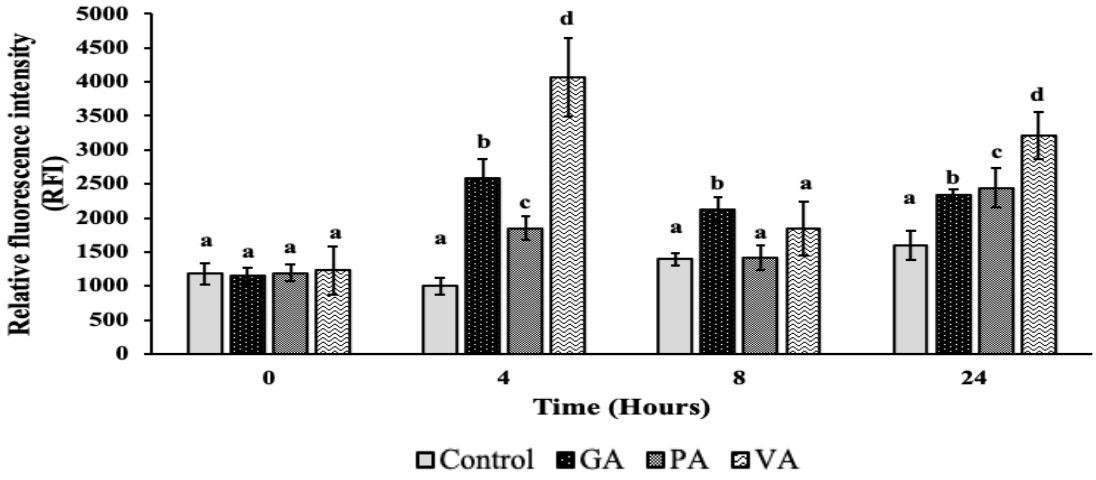


Figure 5-3. Observing changes in membrane permeabilization through fluorescent microscopy with dyes SYTO9 and PI for negative control (**A1–A3**), GA (**B1–B3**), PA (**C1–C3**), VA (**D1–D3**) over 4 (1), 8 (2) and 24 (3) h of treatment periods.

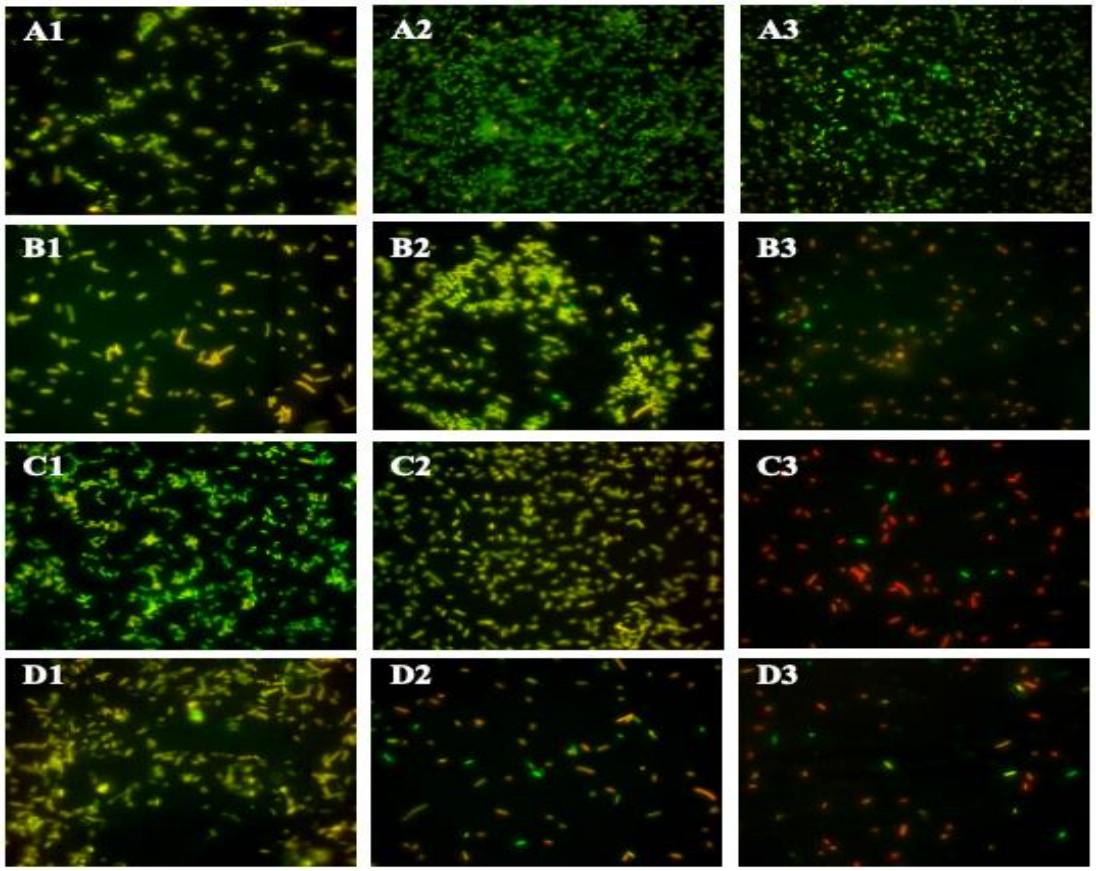


Figure 5-4. Fluorescent microplate reads for quantifying HADA integration during peptidoglycan wall synthesis. Measurements were taken for samples that were allowed to integrate HADA since the beginning of the trial (A), following a 24h incubation period, at which final HADA dose before the end of the trial was added (B). The corresponding bacterial load for these samples was calculated from aliquots of these samples (C).

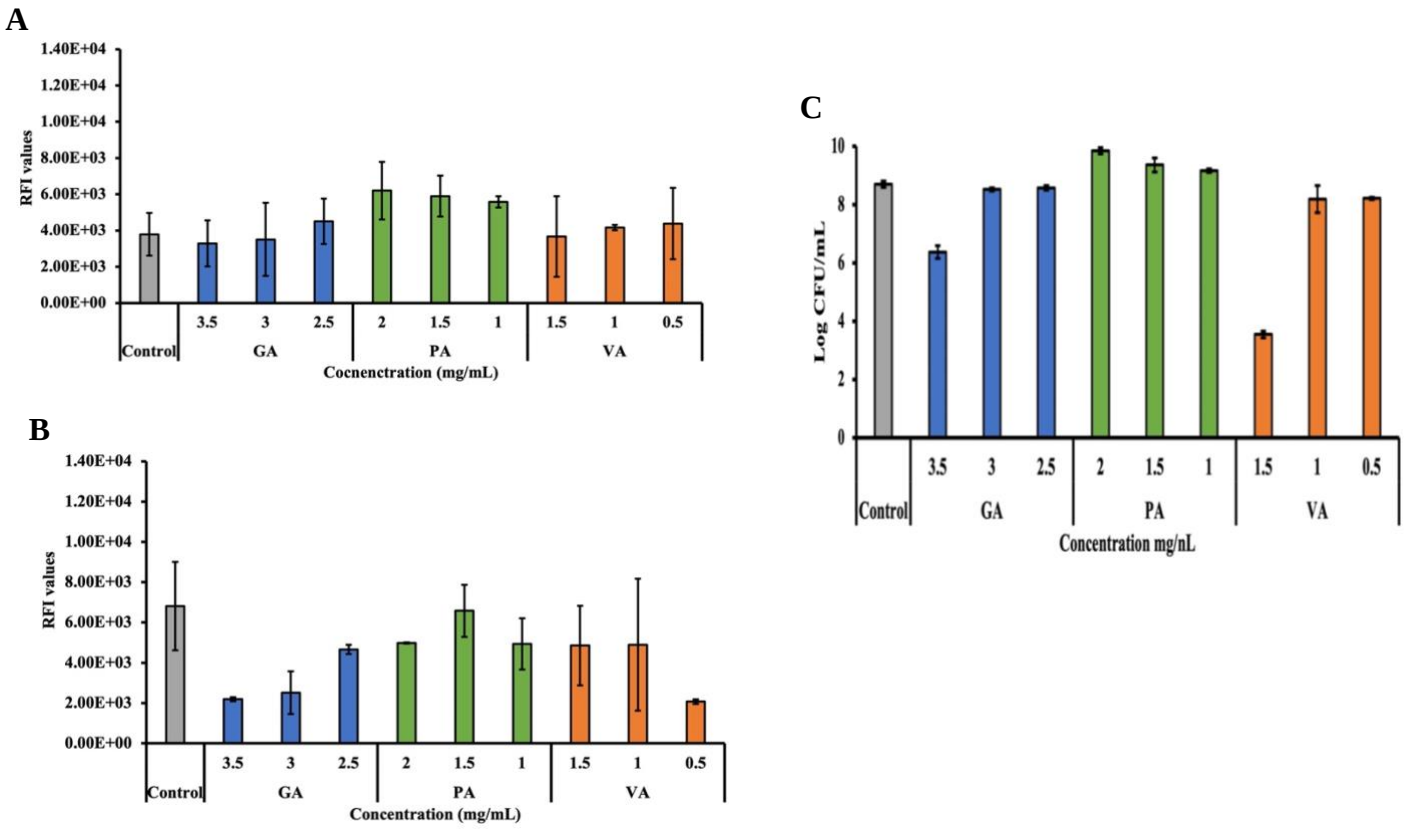
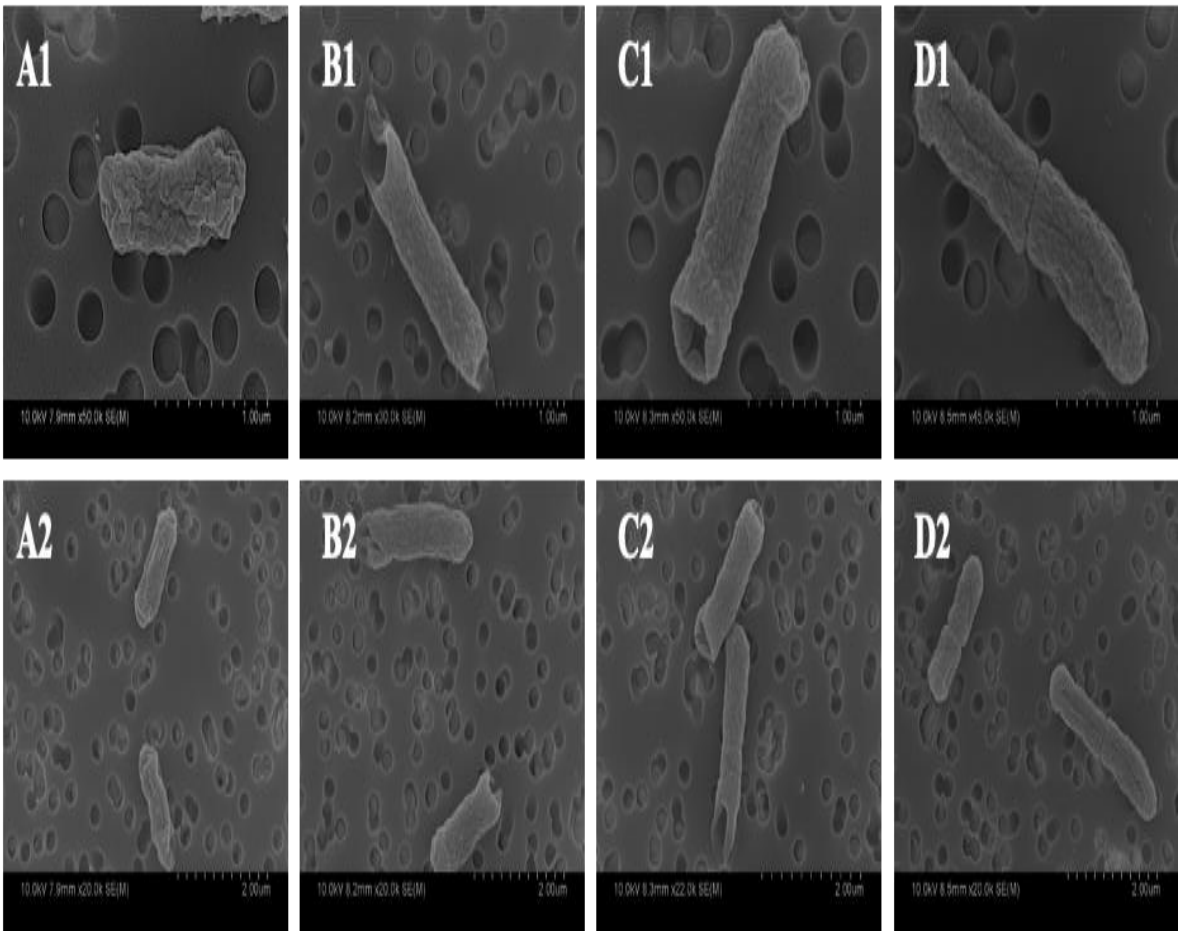


Figure 5-5. Scanning electron microscopy (SEM) images of untreated ST as negative control (**A1,A2**), as well as treated ST with GA (**B1,B2**), PA (**C1,C2**), VA (**D1,D2**), taken at 1μm (**A1–D1**) and 2μm (**A2–D2**) magnification.



Overall Conclusions

1. Phenolic acids, which are commonly found in plant-extracts and make up a significant portion of them have antimicrobial activity against *Salmonella enterica* serovar Typhimurium (ST).

2. Gallic acid (GA), protocatechuic acid (PA) and vanillic acid (VA) have the capability of inhibiting the growth of ST *in vitro* to undetectable levels

3. In addition to antimicrobial potential, genes associated with inducing virulence were found to be reduced after treatment with these compounds, especially in the case of the master regulator *hilA*.

4. Consecutive exposure to these phenolic acids and continuous passages did not show an increase in resistance to these compounds

5. When tested in an infection model containing an epithelial cell line infected with ST, GA, PA, and VA showed the capability to reduce invasion of ST

6. Optimization of phenolic acids to enhance antagonistic effects against ST without affecting host cells requires the adjustment of pH and concentration of the compounds.

7. When further tested for activity in more complex environments, GA showed the capacity to remain more bioactive at a wider range of conditions as it managed to reduce the growth of ST in a chicken simulated gut model, while VA managed to reduce ST to numbers below the initial inocula.

8. 16S-rRNA gene sequencing analysis showed phenolic acids to be capable of protecting commensal and probiotic bacteria found in the cecum fluid, while showing inhibitory potential against common groups of pathogenic bacteria other than ST.

9. Purposed mechanisms of action for GA, PA, and VA suggest impairment and permeabilization of the bacterial cell membrane

10. A downregulation in genes such as *mind* suggests that treatment could be affecting cell division, which in turn reflects in structurally compromised bacteria, as well as bacteria that lack proper cell wall and cell membrane synthesis.

11. Additional morphological and phenotypic changes that were seen involved the formation of dents at the polar ends of bacteria treated with GA and PA, while the ones treated with VA had an arrested divisome, finding themselves in duplet, mid division.

Future directions

1. Expand research to other phenolic acids within plant extracts to increase prospects.

2. Test for synergy between plant compound and traditional antibiotics.

3. Expand the scope of transcriptomic changes observed in bacteria treated with phenolic acids to find more pathways of interest.

4. Continue to test these compounds within various application contexts, from food preservation, to treatment, to subtherapeutic use.

5. Explore delivery methods.

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