EVALUATING SPINACH EXTRACTS FOR ANTIMICROBIAL ACTIVITIES AGAINST BACTERIAL FOODBORNE PATHOGENS

A Thesis

by

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ABSTRACT

The exploration of natural antimicrobials for food preservation has received increased attention due to consumer awareness of natural food products and a growing concern of microbial adaption to conventional preservatives. Leaves of some plants like spinach are identified as important dietary sources of nutrients, antioxidants, antimicrobials, and several bioactive substances. In addition, bioactive compounds of spinach have been reported to inhibit the growth of *Escherichia coli* and *Bacillus* spp. These secondary metabolites like phenolics are altered by nitrogen uptake by plants, owing to importance of nitrogen in plant metabolism, its limited availability or deficiency resulting in reduced growth and a lower yield of plants. At the same time, a shift from N-based to C-based compound content is usually observed. Spinach accessions grown under low and high nitrogen concentrations were received into the Food Microbiology Laboratory (Texas A&M University, College Station, TX) and were stored at -80°C. Twenty grams of each sample were weighed aseptically and 100% methanol was used for extractions, and then these extracts were concentrated through solvent evaporation. Antimicrobial assays were performed to determine the inhibition of spinach extracts against a target pathogen (*E. coli* O157:H7, *Salmonella*, *Listeria monocytogenes*) using the micro-broth method. Micro-plates (96 well) were prepared and incubated for 24 hrs. at 35°C and checked spectrophotometrically to determine the difference in sample-specific light transmission/turbidity as a function of extract and pathogen. The total phenolic content and flavonoid content of spinach extractions were determined by using Folin-Ciocalteu reagent and aluminum chloride colorimetric methods respectively. Results indicate no statistically significant differences in bioactive compounds between low and high nitrogen groups. Though minimum inhibitory concentration (MIC) values were determined to be the same for each isolate, the doubling time at sub-minimum inhibitory concentrations statistically differed between

each isolate. *E. coli* O157:H7 P18, *E. coli* O157:H7 EDL 932*, L. monocytogenes* LIS0072*, and L. monocytogenes* LIS0104 had longer doubling times compared to rest of the isolates when treated with spinach extracts. Results obtained indicated that there were great differences in the doubling time among the different genera/species studied.

DEDICATION

First and foremost, I would like to dedicate this to my parents for their love and support throughout my life. Thank you both for giving me strength to reach for the stars and chase my dreams. My friends deserve my wholehearted thanks as well.

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1. INTRODUCTION: LITERATURE REVIEW

1.1. INTRODUCTION

A pathogen is a disease-causing agent commonly referred to as germ, including biological agents such as viruses, bacteria, or fungi. Human foodborne illnesses are caused by several species of bacteria isolated from a wide variety of foods, including *Salmonella enterica*, *Listeria monocytogenes, Escherichia coli* O157:H7, and other members of the Shiga toxin-producing *E. coli* (STEC). Food products, especially ready to eat (RTE) foods, are easily accessible to pathogen contamination at multiple points in the manufacturing process, resulting in a great deal of concern for the widespread damage due to foodborne diseases (80). Natural antimicrobials from different sources are used to preserve food from spoilage and inhibit the growth of pathogenic microorganisms. Increasing demand for natural antimicrobials has opened new opportunities for the use of natural preservatives derived from plants, animals, or microflora (81). Typical examples of investigated antimicrobial compounds are lactoperoxidase (milk), lysozyme (egg white), saponins and flavonoids (herbs and spices), bacteriocins, and chitosan (shrimp shells derivative) (83). Antimicrobial compounds present in foods can extend the shelf life of unprocessed or processed foods by reducing microbial growth (88). In addition, plants (herbs and spices, fruits and vegetables, seeds and leaves) are the primary sources of essential oils and phenolic compounds that exhibit preservation properties (1). The antimicrobial activity of phenolics and flavonoids is also well documented (5,6). Studies have suggested that the phenolic compounds from fruits and vegetables possess antimicrobial and antioxidative properties (2). Furthermore, some natural elements in seasonings and spices are reported to have effective antimicrobial properties (3). Spices and aromatic vegetable components have long been used in food not only for their flavor and fragrance characteristics, but also for their preservative and medicinal properties (4). Due to

their antibacterial, antifungal, and antiviral activity, phenolic compounds and antioxidant biomolecules have been the subject of anti-infective research for many years (7). The toxic effects of the polyphenols on microorganisms are attributed to their adsorption to and disruption of microbial membranes, inactivation of enzymes, and metal ion deprivation (6). Depending on the constitution and concentration, polyphenolic compounds can affect the metabolism and growth of bacteria (9). It is suggested that phenolic compounds can be used as chemotherapeutic agents, food preserving agents, and disinfectants (8). Flavonoids are the primary class of polyphenols possessing multiple biological properties, such as anti-oxidative, anti-inflammatory, anticarcinogenic, and cardiovascular protection (10). The antibacterial activities of flavonoids results through direct killing of bacteria, synergistic activation of antibiotics, and attenuation of bacterial pathogenicity, which could results by cytoplasmic membrane damage (caused by perforation and/or a reduction in membrane fluidity for which hydroxyl group at C-3 is the primary determinant), inhibition of nucleic acid synthesis (caused by topoisomerase inhibition), and inhibition of energy metabolism (caused by NADH-cytochrome c reductase inhibition) (11). Studies have demonstrated inhibitory activity of flavonoids against the efflux pump of methicillin-resistant *Staphylococcus aureus* (MRSA) (12) and synthesis of peptidoglycan in cells of amoxicillin-resistant *E. coli* (AREC) (13). Flavonoids have inhibitory activity against different kinds of beta-lactamases produced by bacteria, which are the key enzymes that provide resistance against penicillin and other beta-lactam antibiotics (13-15).

1.2. APPLICATION OF NATURAL ANTIMICROBIALS FOR FOOD PRESERVATION

Besides the growing consumer awareness about food safety and quality, there is also a high demand for traditional preservatives-free food and greater use of natural antimicrobials. To address the need for natural and safe alternatives, several natural antimicrobials have been used in the food

industry for years (10). Earlier studies have shown that natural plant extracts can enhance the overall quality and shelf life of the food products (10, 16, 17, 18). Furthermore, they can also be used in various food systems such as meat (turkey, beef, and chicken), seafood (19), fresh and minimally-processed produce, and packaging films (20) in combination with other processing hurdle technologies (bacteriocins, organic acids, low storage temperature, and packaging) to improve the overall microbial quality and safety of the food products (10, 21, 22). It has been reported that adding various natural antimicrobials of animal, plant, and microbial origin to fresh-cut fruits and fruit juices reduces or inhibits pathogenic and spoilage microorganisms (23). Leafy vegetables like spinach are known to synthesize several bioactive compounds like proteins, peptides, phenolics, tannins, saponins, cyanogenic-glycosides, terpenoids, alkaloids, steroids, and defensins (39). Screening of tree spinach (*Cnidoscolus aconitifolius)* leaves revealed the presence of alkaloids, terpenoids, anthraquinones, flavonoids, tannins, saponins, steroids and glycosides that inhibited *E. coli* and *Bacillus subtilis* growth on nutrient agar (39). In addition, spinach leaves contain defensins, which are cysteine-rich peptides reported to exhibit antimicrobial activities (40). Spinach (*Spinacia oleracea* L.) extracts made by ultrasound assisted extraction (UAE) were reported to contain effective antioxidants. It was proposed that these extracts might be active against foodborne pathogens like *S. enterica*, *L. monocytogenes,* and *E. coli* O157:H7 by interfering with metabolism (41). On the other hand, the addition of these antimicrobials to food products without adversely affecting the sensory characteristics is challenging. The amounts that are needed to ensure safety (up to 5.0 $\log_{10}$ CFU/g reduction in the most process-resistant pathogenic microorganism like *Salmonella* spp. in dry foods) are often several times higher than those accepted by consumers from a sensory point of view (24). One possible solution is by combining the use of antimicrobials with other methodologies of food preservation to negate the

effects of these compounds on organoleptic properties to gain consumer acceptance (20). Natural antimicrobials of animal, plant, and microbial origin, when added to fresh-cut fruits and fruit juices, were reported to effectively to reduce or inhibit pathogenic and spoilage microorganisms, thus representing a good alternative to the use of traditional antimicrobials (90). Moreover, studies have demonstrated the effectiveness of essential oils and their active compounds to inhibit the growth of pathogenic and spoilage microorganisms in both fresh-cut fruit and fruit juices. That effectiveness depended on the pH of the fruit product, kind and concentration of used essential oils or active compound, and microorganism type. When active compounds of herbs and spices were incorporated into an alginate-based edible coating and applied on fresh-cut apples, effectiveness for reducing populations of inoculated *E. coli* O157:H7 during storage time was observed. Essential oils such as clove oil had antimicrobial properties and were able to restrict the proliferation of *L. monocytogenes* in food products. For this reason, clove oil has good potential as an antilisterial substance in food preservation as it may be more acceptable to consumers (91). In addition, extracts from cinnamon were used in apple juice to suppress the growth of *E. coli* O157:H7 at 8°C and 25°C. Cinnamon in combination with sodium benzoate or potassium sorbate reduced the number of *E. coli* O157:H7 more than 5.0 log CFU/mL during storage at 8°C for 14 days and 25°C for 3 days. In fact, cinnamon in food systems with other extrinsic and intrinsic factors (for example, acidulation with organic acids) might provide a hurdle effect to control *E. coli* (96).

1.3. FOODBORNE INFECTIONS AND STATISTICS

Foodborne infections sometimes called "foodborne disease", "foodborne illness", or "food poisoning", are not uncommon, costly-yet preventable-public health problems. In the United States 31 of the most important known agents of foodborne disease cause 9.4 million illnesses, 55,961

hospitalizations, and 1,351 deaths each year (93). Although only a small subset of illnesses are associated with recognized outbreaks, data from outbreak investigations provide insight into the foods and pathogens that cause illnesses and the settings and conditions in which they occur. During 2009–2015, Foodborne Disease Outbreak Surveillance System (FDOSS) received reports of 5,760 outbreaks that resulted in 100,939 illnesses, 5,699 hospitalizations, and 145 deaths from all 50 states, the District of Columbia, Puerto Rico, and CDC reported outbreaks. Among 2,953 outbreaks, norovirus was the most common cause of outbreaks (1,130 outbreaks [38%]) and outbreak-associated illnesses (27,623 illnesses [41%]), followed by *Salmonella* with 896 outbreaks (30%) and 23,662 illnesses (35%). On the other hand, outbreaks caused by *Listeria*, *Salmonella*, and Shiga toxin-producing *Escherichia coli* (STEC) were responsible for 82% of all hospitalizations and 82% of deaths reported (93,80).

1.4. NATURALLY OCCURRING PHENOLS

With increasing consumer demand for foods containing lower levels of chemically synthesized food additives in the last two decades, there has been an increase in the incidence and detection of foodborne diseases. Moreover, plant-, animal- and microbially-derived antimicrobial compounds were proven to be a potentially natural substitutes for chemical food preservatives (43). Phytochemical screening of spinach has been reported to identify compounds that inhibit the growth of *E. coli* and *Bacillus* spp. through secondary metabolites (44). Studies previously reported that multidrug resistant *S. aureus* was sensitive to whole extracts from plants (45). Also, common foodborne bacteria are sensitive to plant- derived phenolics (46).

Phenolics are one of the most diverse groups of secondary metabolites that are found in edible plants and a wide variety of fruits, vegetables, nuts, seeds, stems and flowers as well as tea, wine, propolis, and honey, and represent a common constituent of the human diet. Usually they

are involved in plant growth and reproduction and provide resistance from pathogens, predators and protect crops from disease and pre-harvest seed germination (47). There are different classes of polyphenols known as tannins, lignins, and flavonoids. Each class of polyphenols possesses unique chemical characteristics that distinguish them. Studies on extracts derived from plants have shown that orange/yellow pigments like flavonoids, red pigments like anthocyanins, and colorless phenolics are abundant in antioxidant and antimicrobial properties (45). Flavonoids are commonly found polyphenols and are ubiquitous in human consumed vegetation. Dietary flavonoids have grabbed attention because they have a variety of beneficial biological properties, which may play an important role in the maintenance of human health. Flavonoids are potent antioxidants, free radical scavengers, and metal chelators which inhibit lipid peroxidation and exhibit various physiological activities including anti-inflammatory, anti-allergic, anti-carcinogenic, anti-hypertensive, anti-arthritic and antimicrobial activities. Consumption of beverages rich in phenols, fruits, and vegetables has been reported to be associated with a reduction of the risk of cardiovascular diseases in epidemiological studies.

The flavonoid family is the largest group of polyphenolic compounds among 9,000 natural compounds identified in nature (48). Flavonoids are the most abundant polyphenols in our diets. Higher concentrations of flavonoids can typically be found in the outermost layers of fruits and vegetables (e.g., skins, rinds, peels, netting) since the biosynthesis of flavonoids will be stimulated by sunlight (ultraviolet radiation). Flavonoids can be divided into six sub-classes according to the degree of oxidation of the oxygen heterocycle: flavones, flavonols, isoflavones, anthocyanins, flavanones, and catechins and anthocyanidins. Polyphenols extraction can be performed using a solvent like water, hot water, methanol, methanol/formic acid, methanol/water/acetic or formic acid, etc. Therefore, depending on the external conditions applied, total polyphenol amounts

detected from the same plant and their corresponding antioxidant and antimicrobial activities may vary widely. Scanning electron microscopy (SEM) studies on spinach leaf extracts, oregano and thyme essential oils showed these compounds reduced *E. coli* and *S. aureus* to almost non-detectable numbers on treated surfaces (49). In addition, thymol, eugenol, and carvacrol have been found to cause disruption of the cellular membrane, inhibit ATPase activity, and release intracellular ATP and other constituents of several microorganisms such as *E. coli*, *E. coli* O157:H7, *L. monocytogenes*, *Lactobacillus sakei*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, and *S. aureus* (56,57).

1.4.1. Sub-classes of the common flavonoids

Flavones: apigenin, luteloin, diosmetin

Flavonols: quercetin, myricetin, rutin

Flavanones: naringenin, hesperidin

Catechins (flavanols): (-) epicatechin, gallocatechin, (+) catechin

Anthocyanidins: pelargonidin, malvidin, cyanidin

Isoflavones: genistein, daidzein

Phenolic acids: chlorogenic acid, tannic acid, ferulic acid (50)

Although previous studies were focused on the mutagenic and genotoxic activity of phenolics, later epidemiological studies have indicated phenolic compounds may play an important role in the prevention of several common diseases, including foodborne diseases (51).

The exploration of natural antimicrobials for preserving a food receives increased attention due to consumer awareness of natural food products and a growing concern of microbial resistance towards traditional preservatives. The use of antimicrobial agents like phenolic compounds would provide additional benefits, including dual-function effects of both preservation and delivery of

health benefits. Determining the antimicrobial effect of phenolics on food borne pathogens is necessary before concluding them as a potent antimicrobial. Also, results from antimicrobial assays will be helpful to include phenolics as one of the hurdles in hurdle technology to ensure the food product is safe to consume.

1.4.2. Mechanism of action

A previous study stated that antimicrobial action of phenolic compounds was linked to the inactivation of cellular enzymes, which was correlated to the rate of penetration of the substance into the cell or caused by membrane permeability changes (52). Antimicrobial activity of specific phenolic acids on beneficial gut bacteria and pathogens has been observed while evaluating the antimicrobial properties of pure phenolics and plant extracts. Species of *E. coli* include non-pathogenic, pathogenic and commensal bacterial strains that generally inhabit the human gut. *E. coli* O157:H7 is a foodborne bacterium that causes enterohemorrhagic infection and occasionally kidney failure. Thus, *E. coli* strains are often used in antimicrobial screening studies, in conjunction with other beneficial and pathogenic bacteria. In general, variations in antimicrobial activities among bacteria may reflect differences in cell surface structures between Gram-negative and Gram-positive species. *Lactobacillus* spp. and *S. aureus* are reported to be more susceptible to the action of phenolic acids than are Gram-negative bacteria such as *E. coli* and *P. aeruginosa*. *lpxC* and *tolC* genes in that have undergone mutations seem to improve the phenolic acid antimicrobial mechanisms of action against Gram-negative species (86).

In addition, substitutions in the benzene ring of the phenolic acids and the saturated side-chain length influenced the antimicrobial potential of the phenolic acids against the different microorganisms, but in several ways. Phenolic acids seemed to show greater antimicrobial potency than their corresponding precursors such as the monomers (þ)-catechin, (-) epicatechin and dimers

B1 and B2. Hence, microbial metabolism of dietary phenolics (flavonols, flavan-3-ols, flavones and anthocyanins) could lead to more potent microbe-inhibitory compounds (phenolic acids) that selectively influence intestinal bacteria species. These could affect the diversity and metabolic activity of the intestinal microbiota, including the transformation of phenolics in the gut. Studies in future must consider diversity and complexity of the human microbiota are required in order to confirm the potential microbiota modulating effects of phenolic acids (55).

1.5. FACTORS AFFECTING THE CONCENTRATIONS OF BIOACTIVE COMPOUNDS IN PLANTS DURING PRE- AND POST-HARVEST OPERATIONS

1.5.1. Pre-harvest factors

To ensure the high content of bioactive compounds in the plant, sufficient nutrients must be available in the soil along with favorable environmental conditions for the plant needs (24). Several experiments have been undertaken to examine different fertilizer types on the phenolic content of plants such as chamomile and chicory cultivars (25). There are a few studies that reported a strong relationship between the level of soil macronutrients (i.e., nitrogen, phosphorus, and potassium) and the resulting levels of phenolics, flavonoids, and bioactive compounds. Also, studies suggest that production practices, including nutrient and water management, influence the concentration of bioactive compounds (26). Studies have suggested nitrogen applications in the form of fertilizers decrease phenolic levels in *Arabidopsis thaliana*, *Lycopersicon esculentum* and several fruit-bearing species (31, 32). Also, the negative correlation between nitrogen application and total phenolics could be explained by the protein competition model (PCM) or the growth differentiation balance (GDB). According to the PCM the biomass increases in response to elevated nitrogen nutrition whereas the phenolic concentrations will decline because increased protein demand for growth will decrease partitioning to phenolics. Also, increased dry matter

accumulation will dilute phenolic concentrations (33, 34). The GDB theory suggests that biomass accumulation and secondary metabolites are negatively correlated (35).

1.5.2. Post-harvest factors

Postharvest handling conditions, like the length of storage period and the storage environment temperature, have a significant impact on the concentrations of bioactive compounds. Compounds like ascorbic acid quickly decrease during storage in many plant products, whereas carotenoids and flavonoids appear to be more stable (27). The temperature of storage must be considered as the ruling factor to retain the maximum biochemical composition in harvested produce (87). Though a lower temperature decreases metabolic rates and thereby slows plant deterioration, a holding temperature that is too low may cause chilling or freezing injury to the crop (28). The duration of storage is also of importance since the concentrations of bioactive compounds changes over time. Light conditions during storage generally affect concentrations of these compounds by photosynthesis, which may be supported in light-stored leaves. A compound like ascorbic acid increases with light being available at 10,000 lux for 42 hrs (29). Carotenoids, on the other hand, may decrease to a greater extent in vegetables stored in light (30). Blanching, cooking and drying involves heat treatment, therefore expected to destroy ascorbic acid. The extent of damage will, however, depend upon the extent and duration of exposure to these factors (30). Factors like pre- and post-harvest conditions along with favorable environmental conditions affect the availability of bioactive compounds in plant extracts.

1.6. EFFECT OF NITROGEN CONCENTRATION IN SOIL ON AVAILABILITY OF BIOACTIVE COMPOUNDS IN PLANTS

Studies suggest within the carbon allocation hierarchy, as available soil nitrogen increases phenolics represent a lower priority (58). The content of secondary metabolites was significantly

modified by environmental influences (59, 60). Factors like light, nitrogen (61, 62) were key components. Nitrogen is a crucial plant macronutrient which aids the biosynthesis of amino acids and proteins (63). Nitrogen in the form of nitrate gets absorbed into the plant and then is reduced to ammonium followed by rapid incorporation into amino acids through glutamine synthetase/glutamate synthase (GS/GOGAT) enzymes (64). Considering nitrogen's importance in plant metabolism, its limited availability or deficiency results in reduced growth and a lower yield of plants (64). At the same time, a shift from nitrogen-based to carbon-based compound content is usually observed (64). These C-based metabolites include mainly phenolics, such as phenolic acids, flavonoids and anthocyanins (65), as well as coumarins (66). This effect of N deficiency on phenolic content is typical because depletion of other macronutrients, such as potassium, does not elevate the phenol content (67). Phenylpropanoid synthesis is mediated by phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), forming *trans*-cinnamic acid and ammonium ions from phenylalanine. Under conditions of decreased N availability, these ammonium ions can be assimilated via the GS/GOGAT system, thus recycling N. Ammonium may also be formed by photorespiration and protein catabolism and therefore efficient removal of excess NH_4^+ is needed to prevent damage (68). Available evidence has shown that pre- and post-harvest factors influence bioactive compounds availability in leaves, fruits and vegetables. However, this study focused on determining the effect of nitrogen on availability of total phenolic antimicrobial efficacy of whole spinach extract on the human foodborne pathogens, future studies should focus on isolated phytochemicals as it will improve our understanding of mechanism of action responsible for various beneficial effects.

2. MATERIALS AND METHODS

2.1. EXTRACTION PROCEDURE

Samples derived from spinach plant accessions (from Texas A&M AgriLife Research & Extension Center at Uvalde) treated with both high (60 ppm) and low nitrogen (9 ppm) treatments were received into the Food Microbiology Laboratory (Department of Animal Science, Texas A&M University, College Station, TX) and prepared for extraction after preserving at -80°C. To calculate w/v (%) concentration:

$$\text{w/v (\%)} = \frac{\text{mass of solute (g)}}{\text{volume of solution (mL)}} \times 100$$

Following freezing, a sample of 20 g was weighed aseptically and solubilized in 100 mL methanol (100%, reagent grade). The solubilized samples were subjected to blending using an electrical blender. Finely ground samples were centrifuged at 3,220 x g for 10 min. The supernatants were concentrated using a rotary evaporator at 55°C and transferred into a 50 mL centrifuge tube. The supernatants after evaporation were collected and again centrifuged at 3,220 x g for 10 min to remove any undissolved compounds. The supernatants were then stored at -80°C before subjecting to freeze-drying. The samples were then transferred to the freeze-drying apparatus (Labconco 4.5 Plus Freezone Benchtop Freeze Dryer) and lyophilized for two days at -50°C. To the freeze-dried sample 2 mL of water with 0.1% formic acid (v/v) was added to make it a 10X concentrate.

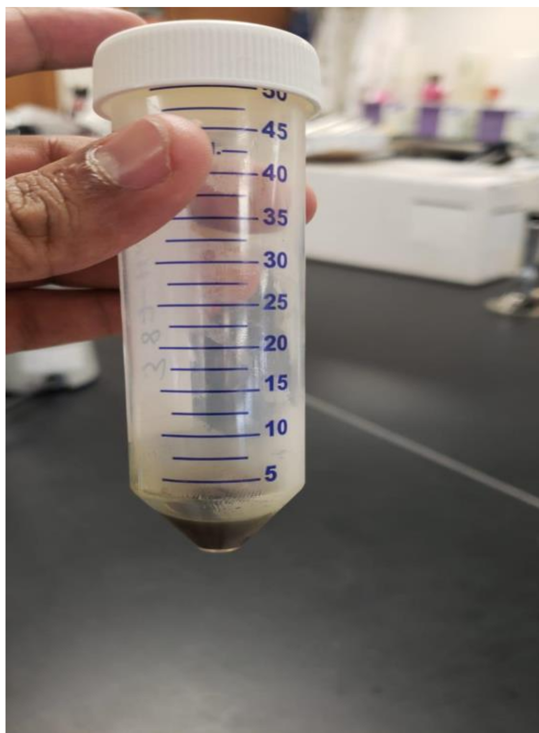

Figure 1**:** Photo of 10X concentrate of spinach extract**.**

2.2. DETERMINATION OF TOTAL PHENOLIC CONTENT

The total phenolic content of spinach extractions was determined by using Folin-Ciocalteu reagent (36). Gallic acid was used as a reference for plotting a calibration curve. A volume of extract (100 μL) was mixed with 0.5 mL of the Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and neutralized with 1.0 mL of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 30 min for color development. The absorbance of the resulting blue color was measured at 765 nm using a double beam UV-VIS spectrophotometer (E-Chrom Tech Co., Ltd., Taipei, Taiwan; UV Analyst-CT 8200). The total phenolic contents were then determined from the linear regression equation of a standard curve prepared with gallic acid; the content of total phenolic compounds expressed as mg/g gallic acid equivalent (GAE) of fresh weight.

2.3. DETERMINATION OF TOTAL FLAVONOID CONTENT

The total flavonoid content was determined according by aluminum chloride colorimetric method (37). Briefly, plant extracts were dissolved in 2 mL deionized water. The resulting solution (0.5 mL) was then mixed with 1.5 mL of 95% ethyl alcohol, 0.1 mL of 10% aluminum chloride hexahydrate ($AlCl_36H_2O$), 0.1 mL of 1 M potassium acetate (CH_3COOK), and 2.8 mL of deionized water. The reaction mixture was incubated at room temperature for 40 min, and absorbance was measured at 415 nm against a deionized water blank on a UV-VIS spectrophotometer. Quercetin was used as a standard. Using a seven-point standard curve, the total flavonoid contents in spinach extract were determined in triplicate. The data were then converted into mg quercetin equivalents (mg QE)/100 g fresh weight.

2.4. PREPARATION OF INOCULUM

The growth characteristics of *E. coli* O157:H7*, Salmonella* and *L. monocytogenes* isolates were evaluated. The isolates were selected from the culture collection of the Food Microbiology Laboratory. The strains then were first passed into tryptic soy broth (TSB) (Becton, Dickinson and Co., Franklin Lakes, NJ) incubated at 37°Cfor about 20-24 hrs. A second pass was done to the fresh TSB and then incubated for 18-24 hrs. at 37°C. Following, a loop from the cultivated TSB tube was transferred to a TSA petri dish for 24 hrs. at 37°C. An isolated colony from the TSA petri dish was transferred to TSB at 37°Cfor 18 to 24 hrs. A loop was used to transfer the cultivated TSB to a tryptic soy agar slant and incubated for 18-24 hrs. at 37°C to preserve strains. The isolated colonies from TSA slants were transferred to TSB to determine the overnight growth (24 hr.) by following the dilution scheme (10^{-0}-10^{-8}).

2.5. MINIMUM INHIBITORY CONCENTRATION (MIC) DETERMINATION

The overnight cultures of *E. coli* O157:H7, as well as *Salmonella* and *Listeria monocytogenes* isolates, were serially diluted in 9.0 mL double-strength TSB (2X TSB) to a final target concentration of 5.0 $\log_{10}$ CFU/mL to be used as an inoculum. Each pathogen in the double-strength TSB was diluted in 0.1% PW for the pathogen inoculation and spread on the surfaces of Petri dishes containing sterilized TSA. These inoculated TSA dishes were then incubated for one to two days at a temperature of 35°C before the enumeration of the colony. The 100 µL of spinach extract was added to each well of sterile 96-well plate (Thermo Fisher Scientific, Waltham, MA. Inoculum (100 µL) were lastly added into each well. Negative controls containing spinach extract but no microorganism were included for optical density at 620 nm (OD620) baseline adjustment (38). Positive controls were prepared to include pathogen isolate and TSB, but no spinach extract. After preparation of the plate, OD620 at 0 hrs. measurements were taken, and then the plate was incubated for one day at 35°C, and then the OD620 was re-recorded. After the adjustment of the baseline, the spinach extract where the corresponding test wells showing <0.05 increase in OD620 from 0 to 24 hrs. were considered a pathogen inhibitory concentration. The lowest concentration of the spinach extractions producing pathogen inhibition was identified across the triplicate replicates as the MIC. OD620 measurement at sub-MICs were recorded to analyze the inhibitory effect of spinach extract on the growth rates of bacterial isolates.

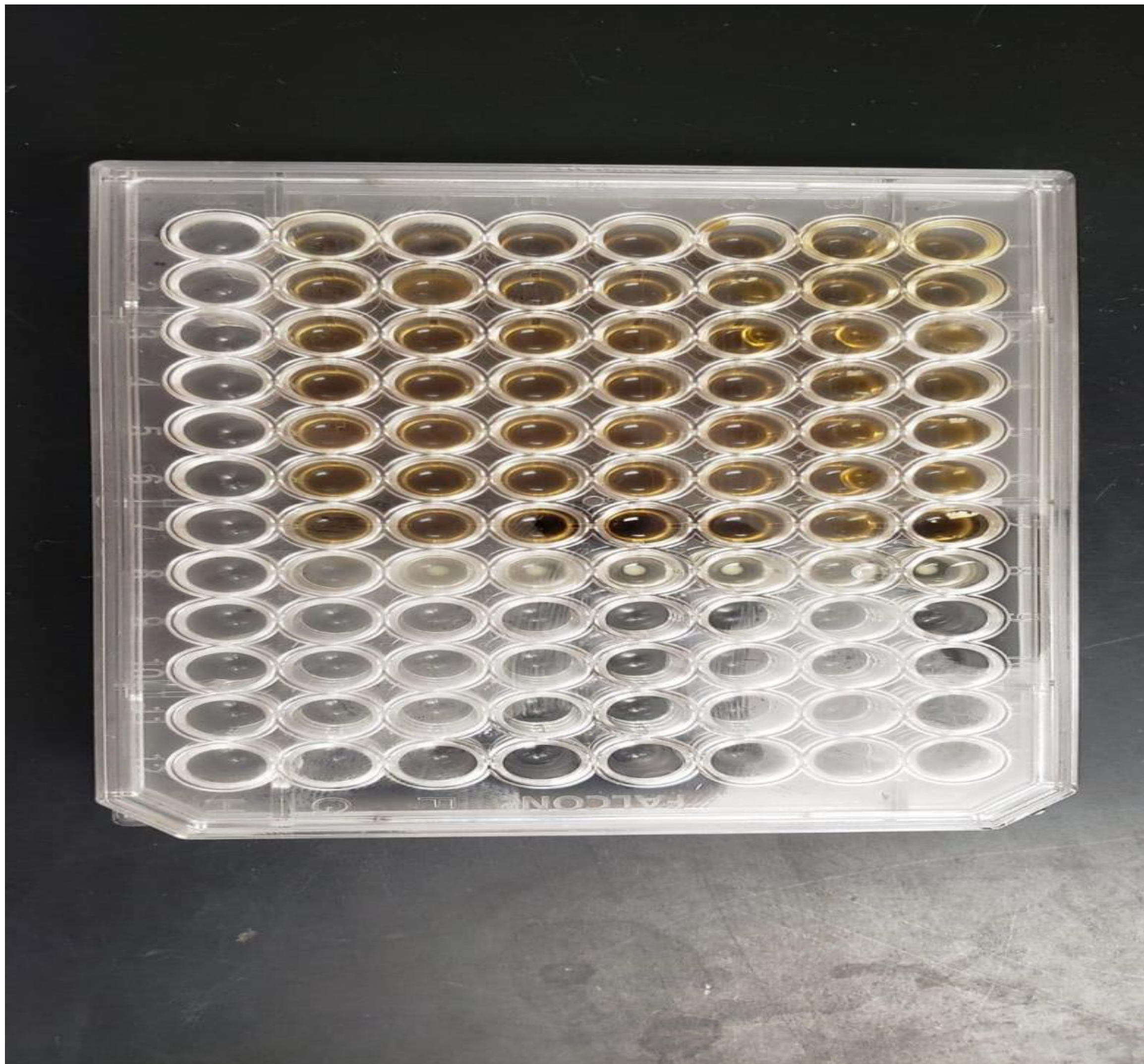

Figure 2**:** Photo of 96 well plate used for MIC determination**.**

2.6. SUB-MINIMUM INHIBITORY CONCENTRATION AND DETERMINATION OF DOUBLING TIME

The growth kinetics of all strains were determined in the presence of phenolic compounds at sub-MIC at 37°C. The overnight cultures of *E. coli* O157:H7, as well as *Salmonella* and *L. monocytogenes* isolates, were serially diluted in 9.0 mL 2X TSB to a final target concentration of 5.0 $\log_{10}$ CFU/mL to be used as an inoculum. 100 µL 0.5 MIC (sub-MIC) of spinach extract were added to each well of a sterile 96-well plate (Thermo Fisher Scientific). Inoculum (100 µL) was

then added into each well. Negative controls containing spinach extract but no microorganism were included for optical density at 620 nm (OD620) baseline adjustment (38). Positive controls were prepared to include pathogen isolate and TSB, but no spinach extract. After preparation of the plate, OD620 at 0 hrs. measurement was recorded and then the plate was incubated for one day at 35℃, and then the OD620 re-recorded. Generation times were estimated graphically from growth curves by plotting the average OD620 for each time point and manually drawing a straight line through the straightest portion of the growth curves and determining the time (hrs.) required for the OD620 to double. A minimum of four time points was used from each growth curve to determine the generation times (69).

2.6.1. Calculation of doubling time

1. Measure growth (cell counts, spec absorbance) in replicate batches.

2. Natural log transform the count data, fit y=mx+b line equations to the linear parts, discard the lag and stationary phases.

3. mu = slope (m) of the ln transformed growth data (mu={lnNt-lnN0}/t, from Euler's equation)

4. 0.693/mu = T_{sub} D (doubling time), or G.

2.7. STATISTICAL ANALYSIS

All the assays were performed in triplicate (*N*=3). All measurements were done separately for ten varieties of spinach samples treated with both high (60 ppm) and low nitrogen (9 ppm) concentrations were collected from the same time points of two different years. Model summary statistics and analysis of variance (ANOVA) via JMP v10.0.0 (SAS Institute, Inc., Cary, NC) were conducted to test the adequacy of models to determine the antimicrobial activities of spinach leaf extracts. One-way ANOVA was performed to determine the effect of nitrogen levels on contents of phenolic compounds and flavonoids. The growth rates of bacterial isolates at sub minimum

inhibitory concentrations of spinach extract were analyzed for significant differences between nitrogen treatments. Analysis of variance was used to determine the significance of main/model effects and the interactions of main/model effects. Statistical significance was determined at $P < 0.05$ and post-hoc test using Tukey's honestly significant difference (HSD) was performed to see if the p-value indicated a significant difference among the means of doubling time of isolates**.**

3. RESULTS AND DISCUSSION

3.1. TOTAL PHENOLICS AND TOTAL FLAVONOIDS IN SPINACH EXTRACTS BY NITROGEN TREATMENT

Summary statistics were collected to describe the basic features of the data in the study and to provide information about sample data and measures (Table 1). Key findings in the summary statistics include the measures of total phenolics (TPC) and flavonoids (FC) contents in the high nitrogen and low nitrogen groups.

Table 1. Total phenolics and total flavonoids in spinach extracts by different nitrogen treatment

Source	N	Mean***	Std Dev	Min	Max
TPC (mg GAE*/g)					
High nitrogen	24	2020.53	297.52	1550.00	2607.14
Low nitrogen	24	1912.5	336.65	1057.14	2464.28
TFC (QE**/g)					
High nitrogen	24	476.69	95.81	327.77	768.05
Low nitrogen	24	507.16	106.50	337.5	736.11

N = Number of spinach samples in both first and second replicates

*GAE (Gallic Acid Equivalents)

**QE (Quercetin Equivalents)

***Values indicate the mean of total phenolics and total flavonoids determined in two replications of ten varieties of spinach.

3.2. TOTAL PHENOLICS AND TOTAL FLAVONOID CONTENT BY NITROGEN TREATMENT

There was no statistically significant effect (*P*=0.2449) of nitrogen treatment on the total phenolic compounds in spinach extracts (Table 2). This outcome is in contrast with the prior literature which demonstrated the effect of fertilizers, mainly nitrogen-containing fertilizers, on increases in the content of the phenolic compounds. Soil nitrogen affects both anthocyanins and flavonoids content, and generally a higher polyphenolic content is observed when less nitrogen fertilizer is added to the soil (58). In addition, the concentrations of total phenolics and also those of the compound groups of phenolics in the first true leaves were generally higher in seedlings grown at very low and low nitrogen than seedlings grown at moderate nitrogen, indicating higher nitrogen supply inversely affected the content of phenolic compounds (64).

Table 2. Analysis of variance of total phenolics in spinach extracts by nitrogen treatment

Source	DF	Sums of Squares	Mean Squares	F Ratio	*P*>*F*
Model	1	140060.6	140061	1.3877	0.2449
Error	46	4642812.5	100931		
C. Total	47	4782873.1			

As was the case for phenolic compounds, there was no statistically significant effect (*P*=0.3029) of nitrogen treatment levels on the total flavonoid content in spinach extracts (Table 3). This is in contrast with the previous studies that demonstrated the increase in the total flavonoids in plants with a reduction in nitrogen availability. The study reported that total flavonol content of tomato

leaves was increased in response to reduced nitrogen availability. Total flavonol content varied from 50.5 μg/g in the high-nitrogen regime to 117.0 μg/g in the low-nitrogen regime (71). On the other hand, the effects of nitrogen supply on nitrogen metabolism in leaves of *Chrysanthemum morifolium* Ramat. were examined in five different stages throughout the growing season. Results suggested flavonoids content was observed to have a negative correlation for nitrogen supply (89).

Table 3**.** Analysis of variance of total flavonoids in spinach extracts by nitrogen treatment

Source	DF	Sums of Squares	Mean Squares	F Ratio	*P>F*
Model	1	11138.74	11138.7	1.0854	0.3029
Error	46	472066.18	10262.3		
C. Total	47	483204.93			

3.3. MINIMUM INHIBITORY CONCENTRATION AND SUB-MINIMUM INHIBITORY CONCENTRATION

MIC and sub-MIC experiments were conducted using the whole extract of spinach. MIC was obtained at 5X concentration of spinach extract in which concentration of phenolics were determined to be 9832.58±1595.01 ppm. These results are comparable to an alcoholic extract of spinach where the MIC was determined to be 10,393 ppm (79). At MIC the OD620 absorbance values were determined to be unchanged after 24 hrs. of incubation at 37°C. However, at sub-MIC levels the effect on each bacterium's growth was determined to be different. The sub-MIC (0.5 MIC) of spinach extracts has shown some ability to extend the lag phase and increase the doubling time of the bacterial growth curve. From figures 2.1-2.9 it is evident that the bacterial growth

curves differed between treatment and control groups. Knowing how bacteria behave when they are not dividing is of great importance in food preservation, for the maximum extension of shelf-life and relationship to lag phase extension (88). For the determination of shelf life, the duration of the lag phase is important. The length of this phase depends on a variety of factors, such as the nutritional and physical composition of the food, environmental factors, initial viable count, physical state of the cell related to the history, and time necessary for the synthesis of the essential coenzymes. The prolonging of the length of the lag phase is an important aspect in the development of the methods to increase shelf life (70, 89).

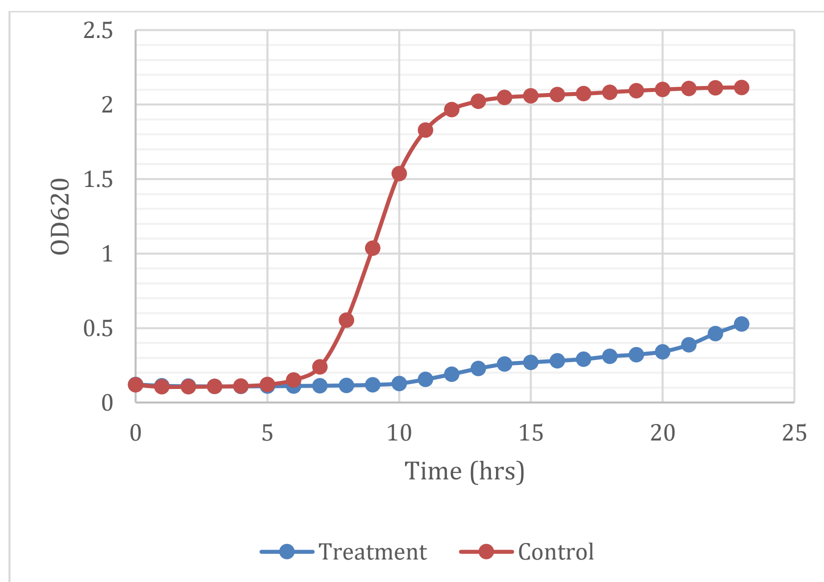

Figure 3**.** The antimicrobial activity of spinach extract at 0.5 MIC against *Listeria monocytogenes* LIS0104 in 2XTSB at 37°C**.** OD620 values for treatment group indicate the mean of two replicates of ten samples.

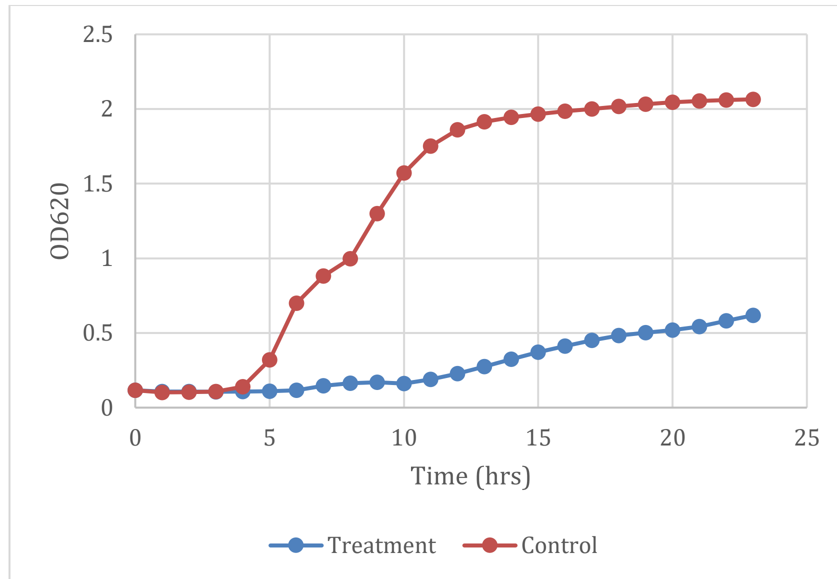

Figure 4. The antimicrobial activity of spinach extract at 0.5 MIC against *Listeria monocytogenes* LIS0072 in 2XTSB at 37℃. OD620 values for treatment group indicate the mean of two replicates of ten samples

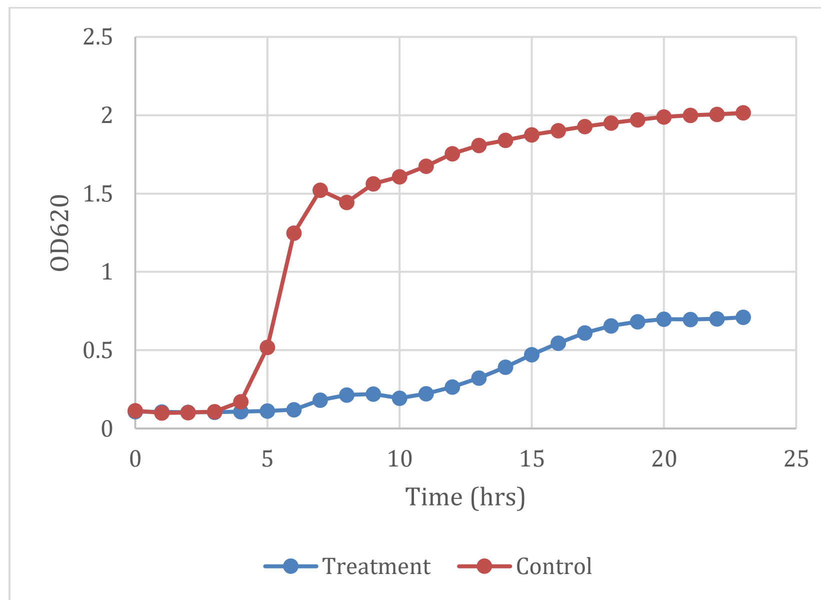

Figure 5**.** The antimicrobial activity of spinach extract at 0.5 MIC against *Listeria monocytogenes* LIS0104 in 2XTSB at 37℃. OD620 values for treatment group indicate the mean of two replicates of ten samples

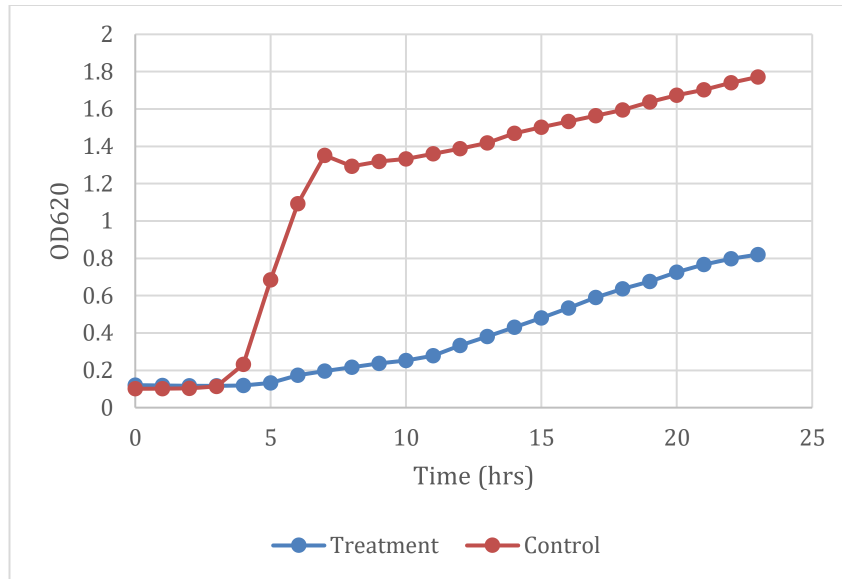

Figure 6**.** The antimicrobial activity of spinach extract at 0.5 MIC against *Escherichia coli* O157:H7 P18 in 2XTSB at 37°C. OD620 values for treatment indicate the mean of two replicates of ten samples

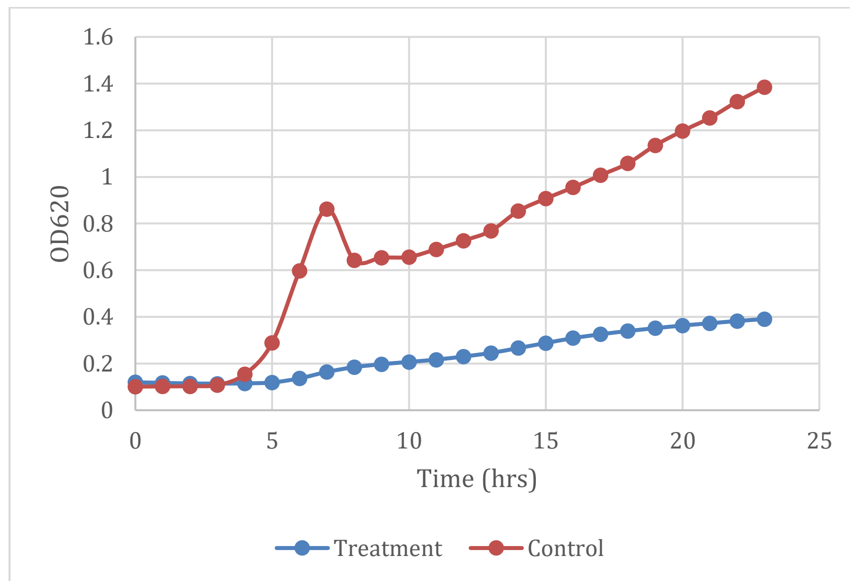

Figure 7. The antimicrobial activity of spinach extract at 0.5 MIC against *Escherichia coli* O157:H7 EDL 932 in 2XTSB at 37°C. OD620 values for treatment group indicate the mean of two replicates of ten samples

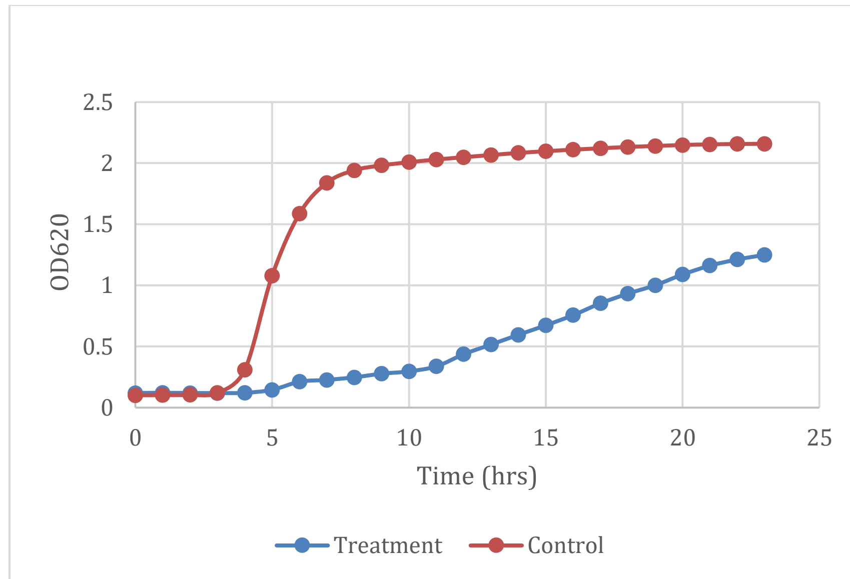

Figure 8. The antimicrobial activity of spinach extract at 0.5 MIC against *Escherichia coli* O157:H7 K3999 in 2XTSB at 37°C. OD620 values for treatment group indicate the mean of two replicates of ten samples

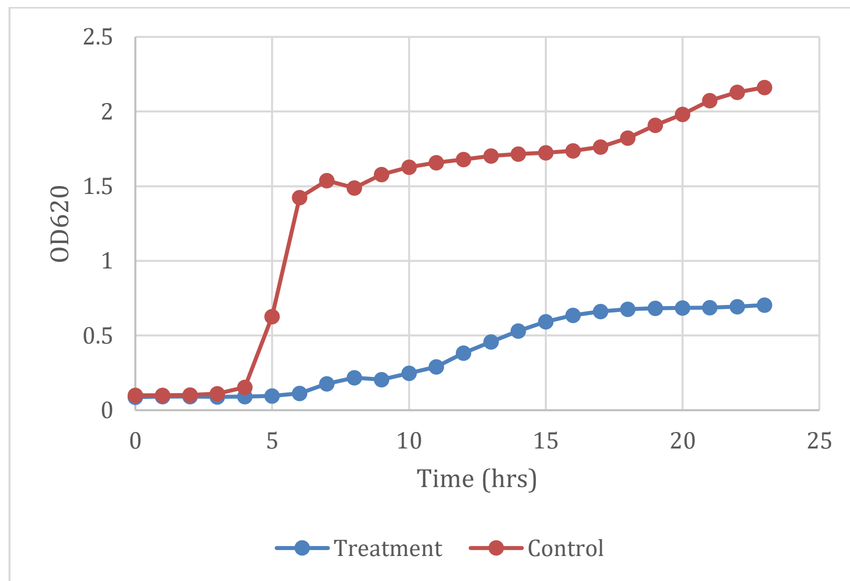

Figure 9. The antimicrobial activity of spinach extract at 0.5 MIC against *Salmonella enterica* Agona ATCC 701 in 2XTSB at 37°C. OD620 values for treatment group indicate the mean of two replicates of ten samples

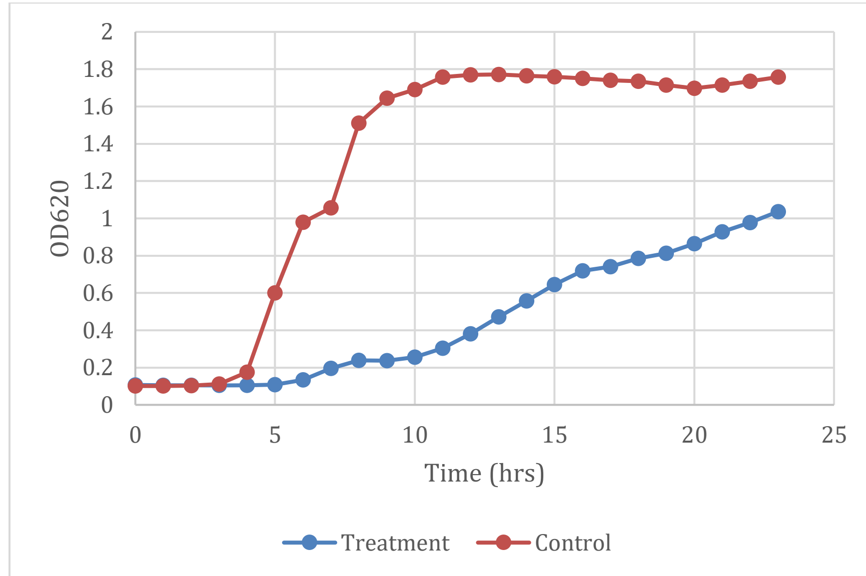

Figure 10. The antimicrobial activity of spinach extract at 0.5 MIC against *Salmonella enterica* Montevideo BAA-710 in 2XTSB at 37°C. OD620 values for treatment group indicate the mean of two replicates of ten samples.

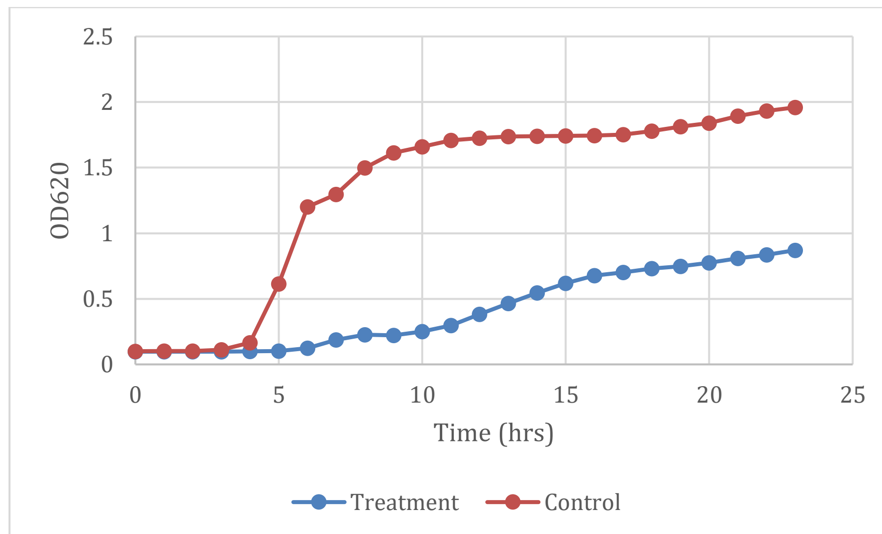

Figure 11. The antimicrobial activity of spinach extract at 0.5 MIC against *Salmonella enterica* Cerro H2007-3 in 2X TSB at 37°C. OD620 values for treatment group indicate the mean of two replicates of ten samples.

3.4. DOUBLING TIME OF THE BACTERIA BY ISOLATE, NITROGEN LEVEL, AND TREATMENT GROUPS AT SUB-MIC LEVEL OF PHENOLICS

To determine the antimicrobial efficacy, the effect of spinach extract on doubling times of isolates were observed. There was a statistically significant effect ($P<0.001$) of the main effects of bacterial isolate, nitrogen input, and treatment group at sub-MIC level of phenolics on the doubling time of isolates (Table 4).

Table 4**.** Analysis of variance of isolate, nitrogen input during spinach production, and treatment group at sub-MIC level of phenolics on bacterial doubling time

Source	DF	Sums of Squares	Mean Squares	F Ratio	$P>F$
Model	26	2093.55	80.52	20.4702	<0.001*
Error	693	2725.98	3.93		
C. Total	719	4819.54			

Statistical significance was determined in two of the main effects, isolate and treatment, indicating that the doubling time of bacteria differed as a function of isolate and treatment group. No statistical significance was determined for the interaction terms isolate x nitrogen level ($p=0.0882$) and isolate x treatment ($p=0.0714$).

Post-hoc testing by Tukey's HSD was performed to determine differences between isolate means as analysis of variance (ANOVA) F test is significant. *Salmonella* Agona ATCC 701 had a statistically lower mean doubling time as a function of treatment ($P=0.001$) when compared to *E. coli* O157:H7 K3999*, L. monocytogenes* LIS0072 and LIS0104. *L. monocytogenes* LIS0104 had a

statistically significant higher mean score (*P*=0.001) when compared with *Salmonella* Agona ATCC 701 and *Salmonella* Cerro H2007-3. Results obtained indicated that there were great differences in the doubling time among the different genera/species studied. The differences were probably because of variation in the genetics and metabolism of these bacteria, which influences their response to antimicrobial compounds (73).

Table 5**:** Least squares means of doubling time for isolates demonstrating significant differences (*P*=<0.001).

Isolate	*Doubling Time (hrs.)*
Escherichia coli O157:H7 P18	2.68ABC
Escherichia coli O157:H7 EDL 932	3.01AB
Escherichia coli O157:H7 K3999	2.31ABC
Listeria monocytogenes LIS0072	3.04AB
Listeria monocytogenes LIS0089	2.22ABC
Listeria monocytogenes LIS0104	3.15A
Salmonella enterica Agona ATCC 701	1.90C
Salmonella enterica Cerro H2007-3	2.13BC
Salmonella enterica Montevideo BAA-710	2.40ABC

Means not sharing a capitalized letter (A, B, C) differ at *p*=0.05.

Even though the effect of nitrogen level on doubling time of the bacteria was not statistically significant in the model, the nitrogen level showed an inverse relationship with the doubling time of the bacteria, indicating that low nitrogen level decreased the bacterial growth rates (Table 6.)

This antibacterial activity can be attributable to the higher total phenolic and total flavonoids contents (74, 75) in the plants grown in low nitrogen levels (71, 76, 77).

Table 6. Least squares means for doubling time (hrs.) by nitrogen level (*P*=0.179).

Level	*Doubling Time (hrs.)*	*95% CI Lower Limit*	*95% CI Upper Limit*
High Nitrogen	2.44	2.23	2.64
Low Nitrogen	2.63	2.43	2.84

There was a statistically significant effect of treatment group on the doubling time of bacteria in the overall model. The treatment group had a significantly longer doubling time compared to control group. This is consistent with prior research that demonstrated that the alcoholic extract of spinach (variety Mashhad) showed more potent antibacterial activity on *E. coli* O157:H7. Also, the effect of its alcoholic extract was comparable with erythromycin on *L. innocua* at disc diffusion test. The high amounts of flavonoids and terpenes, unsaturated fatty acids and inorganic materials are probably the most important inhibitory agents of spinach on gram-negative bacteria such as *E. coli* O157:H7 (79).

Table 7**:** Least squares means for doubling time (hrs.) by treatment level (*P*=<0.001).

Level	*Doubling Time (hrs.)*	*95% CI Lower Limit*	*95% CI Upper Limit*
Treatment	4.14	3.93	4.34
Control	0.93	0.73	1.14

Even though statistically not significant, least-square means for the interaction of the effects of isolate x nitrogen level demonstrated the effect of nitrogen level on the doubling time of various isolates. The doubling time of some isolates increased to a greater extent in low nitrogen levels, while under high nitrogen conditions, the doubling times of most of the isolates were shorter (Figure 12).

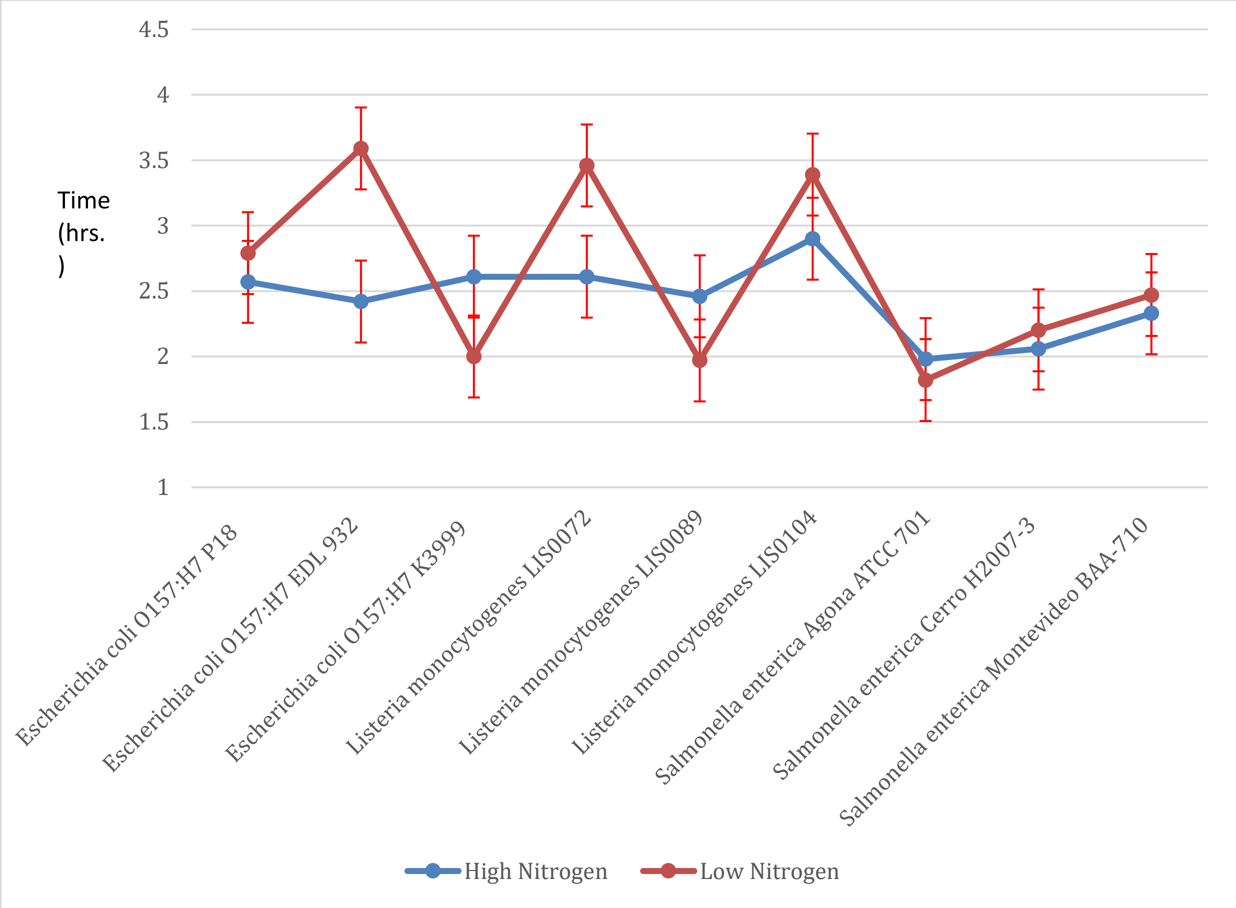

Figure 12**.** Least squares means for doubling time of bacterial isolates by nitrogen level**.** Values indicate means of doubling time among isolates by the function of nitrogen in two replicates of ten samples; error bars depict model standard error.

4. CONCLUSION

Overall, the findings from the research indicate that nitrogen supplemented to spinach growing fields during production had no impact on total phenolic and flavonoid content in experimental spinach plants and resulting extracts. However, future studies must consider the quantity of nitrogen applied as treatment which in our case was 20 lbs. per acre, Calcium nitrate. This study indicates that there was antimicrobial property for spinach extracts, namely spinach-derived phenolic compounds. However, the concentrations where the antimicrobial property was demonstrated were high. Also, there was a statistically significant effect of isolates on the doubling time of bacteria in the overall model. Isolates *E. coli* O157:H7 P18 and EDL 932, as well as *L. monocytogenes* LIS0072 and LIS0104, had longer doubling times compared to rest of the isolates. These results are consistent with the prior literature that demonstrated heterogenous effects of key antimicrobial components like phenolic compounds, ranging from bacterial growth stimulation to antibacterial activity and dependent on bacterial strains. Since this study lacks purification of compounds from spinach extract, there is need for future studies focusing on purification of compounds to determine the antimicrobial property of each purified compounds separately.

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