ABSTRACT

Title of Document: THE ROLE OF ORGANIC MATRICES (DRIED TURKEY MANURE) IN THE CONTAMINATION AND SURVIVAL OF *SALMONELLA* SPP. ON BABY SPINACH LEAVES

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Microbiological safety of fresh produce has attracted attention due to constant produce outbreaks. Manure contaminated with enteric pathogens has been identified as a major pre-harvest contamination source. This study investigated the survival of *Salmonella* in dust particles generated from dehydrated turkey manure, and how this could serve as an airborne contamination of leafy greens. Laboratory trials of the UV resistance of *Salmonella* in manure dust were also conducted to assess its protective effect; along with greenhouse studies to determine the survival of *Salmonella* in manure dust on spinach leaves. Results indicate survival times were inversely related to moisture content and particle size of manure dust. The presence of manure particles substantially enhanced the

pathogen's survival under UV and reduced its inactivation on spinach leaves. This study presents data that could be used to assess the potential role of aerosolized manure as a pre-harvest risk factor for contamination of leafy greens.

THE ROLE OF ORGANIC MATRICES (DRIED TURKEY MANURE) IN THE CONTAMINATION AND SURVIVAL OF *SALMONELLA* SPP. ON BABY SPINACH LEAVES

By

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Dedication

I dedicate my graduate experience and this piece of work to the wonderful family God has blessed me with to support and encourage me in my academic pursuits. To my loving parents, Titus & Victoria Oni, and my wonderful siblings – Esther, Debbie, Nike and Junior; this is for you all.

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My heartfelt appreciation goes to my family and friends who were a source of encouragement throughout my program – the food you brought when I spent nights in the lab, the messages you sent that tickled and made me laugh – all went a long way in helping me put one step ahead of the other. Mrs. Funke Ajayi; thanks for being such a great blessing! Very special thanks go to my fiancé, Dayo, who constantly believed in me, giving me wings to fly when I didn't think I could manage a jump. You are truly the man of my dreams.

Finally and most importantly, I give glory to the Almighty God whose profound mercies, grace and love gave me all that I needed to complete this study.

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Chapter 1: Introduction

1.1 Overview

In the last three decades, there has been an increasing association between fresh produce and foodborne disease outbreaks, and this has led to various research investigations about potential pre- and post-harvest sources of contamination. *Escherichia coli* O157:H7 and a number of non-typhoidal *Salmonella* strains are among the most dominant bacterial agents associated with contamination of both fresh and minimally-processed vegetables *(16, 28, 62, 104)*. The Centers for Disease Control and Prevention (CDC) used the Foodborne Outbreak Surveillance System to compile and analyze data from a twenty-four year period (1973 to 1997), and reported that the proportion of foodborne outbreak–associated illness due to the consumption of fresh produce jumped from 1% in the 1970s to 12% in the 1990s (Fig I – 1) *(87)*.

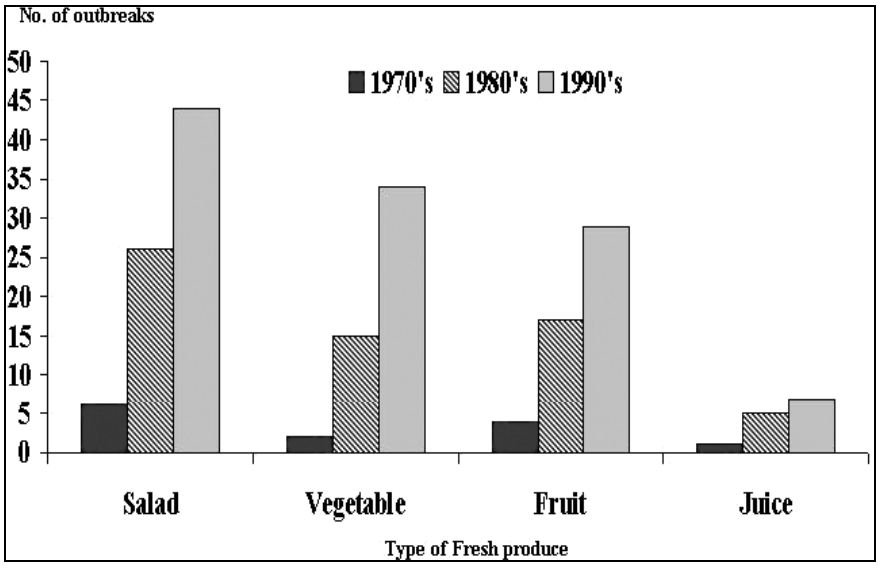

Figure I -1: Number of produce-associated outbreaks by specific vehicle (n=190), 1973 through 1997 *(87)*

Within a ten-year span – 1996 to 2006, 72 produce-related outbreaks were documented; resulting in more than 8,500 reported illnesses and several deaths *(37)*. Between 1998 and 2006, leafy greens were among the top three produce items that caused outbreaks, accounting for 30% of all produce–related outbreaks *(16)*. So far in 2012, there has been a product recall linked to contaminated or suspected contaminated leafy greens about every one to two months (Warriner, K., electronic mail from info@foodseminarsinternational.com). These records are in spite of the limitations of the passive nature of surveillance and the knowledge that not all food-related illnesses are reported. As expected, substantial research has gone into investigating outbreak patterns and seeking potential sources of pathogen contamination, transmission and survival in both pre-harvest and postharvest settings. While significant attention has been given to sanitation practices and technology for post-harvest processing at the industry and consumer levels, there is still room for improvement in advancing on-farm food safety through pre-harvest practices, as research addressing pre-harvest produce safety is still in its early stages. Currently, there is relatively limited information on the potential for in-field pathogen contamination of produce items from sources such as water, air, manure, vectors or a combination of one or more of these sources. It is therefore no surprise that the emerging field of pre-harvest produce food safety has continued to garner a lot of attention. This research study seeks to augment the knowledge base about pre-harvest contamination of produce via aerosolization of manure.

Animal manure contaminated with the enteric pathogen *Salmonella* has been identified as one of the major sources of contamination before plant harvest *(28)*. However, specific

contamination modes are yet to be identified, and this research aims to investigate the propensity of dried manure particles, of a size capable of being airborne, as a potential transmission vehicle on produce farms. Aerosol dispersion of manure as a pre-harvest contamination source had previously been identified, along with other on-farm contamination routes *(28)*. However, the potential impact of airborne *Salmonella* blown onto vegetable leaves from adjacent fields has not been investigated. On the Eastern shore of Maryland, for example, poultry facilities are interspersed with fresh produce farms, and although there is no direct contact between the poultry birds and the produce field, films of wind-driven 'manure dust' can be seen on the vegetables leaves and are possible sources of contamination. This occurrence is also particularly evident after the application of dried poultry compost as a soil amendment to other crops such as grains and soybeans on nearby fields as illustrated in (Fig. I -2) below.

Figure I - 2: An example of how manure-spreading operations on farms could generate dust causing unintended aerosolization to near-by farms especially during high wind events. [Image sourced from http://informedfarmers.com/fertilizing-pest-control/]

1.2 Hypothesis of Research

The hypothesis for this research is that the association of *Salmonella* cells with dry manure can protect them from lethal UV solar radiation and enhance their survival. Subsequently, the manure particles could become aerosolized and serve as a vehicle for pathogens thereby contaminating agricultural environments. This ultimately increases the chances of phyllosphere contamination and presents a public health risk.

Although the USDA requires that at least 120 days elapse between the application of non-composted manure and the harvest of organic crops with edible portions which have been exposed to soil particles *(97)*, it is possible that this regulation is being breached in instances where unintended aerosolized transmission to geographically distant fields occur.

Harsh physicochemical conditions, such as dehydration and UV radiation, have been known to prevent the survival and growth of pathogens present on the aerial tissue surfaces of plants *(23)*; however, the role of dried manure particles in aerosol dispersion and how this influences pathogen survival remains unknown.

1.3 Study Approach

This study was conducted in three phases: the first phase sought to evaluate the survival capabilities of two serotypes of *Salmonella enterica* – Typhimurium and Enteritidis - in dust generated from dried turkey manure. The second phase looked at the potential protective effect which manure dust has on *Salmonella* cells when exposed to UV

radiation. And lastly, the survival of *Salmonella* present in manure dust particles on growing spinach leaf surfaces was investigated. Each phase was set up to simulate field conditions as much as was practicable.

1.4 Potential Impact of Study

This study seeks to create awareness about the potential risks associated with the airborne contamination (poultry manure particles) of spinach in pre-harvest settings. It also seeks to assess the potential hazards of interspersing poultry facilities with leafy green fields, as well as the possible risk of using poultry manure as a soil amendment in fields upwind from leafy green cultivation sites. Since current production and processing facilities cannot be relied upon to ensure pathogen-free fresh produce, more measures are needed to prevent pre-harvest contamination. Possible contamination of vegetables will largely depend on the survival capabilities of the pathogens in animal manure, soil or the phyllosphere of crops; therefore assessment of these capabilities is important if effective produce safety guidelines are to be put in place.

Chapter 2: Literature Review

2.1 The Problem

The agro-food chain industry has encountered major food safety issues in recent times due to lapses and inadequate control measures along the farm to fork continuum, or knowledge gaps as to the specific origin and dissemination of contaminants. Due in part to better outbreak surveillance systems, an increasing number of outbreaks caused by foodborne pathogens have been linked to fresh produce, many of which have resulted in illnesses, fatalities and numerous hospitalizations *(13, 22, 28, 90)*. Even though produce safety issues have been recognized and documented for several decades, outbreaks have continued to occur, indicating that there are still knowledge gaps as to how contamination routes can be sufficiently controlled *(93)*. For example, the 2006 outbreak of *E. coli* O157:H7 linked to contaminated salad spinach resulted in at least 3 deaths and 205 confirmed illnesses, with 31 victims developing hemolytic uremic syndrome (HUS) *(20)*. Although, trace-back records and epidemiological investigations helped identify the specific growing region where the contamination occurred, the US Food and Drug Administration (FDA) was unable to identify the precise means by which the bacteria were disseminated to the spinach *(36)*. In response to these outbreaks, many microbiological research efforts are being made to investigate effective ways to detect and mitigate contamination of fresh produce, both before and after harvest. Prevention of microbial contamination of fresh produce is however favored over dependence on post-contamination remedial actions.

Fresh Produce Consumption and Global Food Safety

The necessity of investigating the produce safety problem remains critical because the challenges of supplying safe and nutritious food to an ever-expanding world population cannot be adequately met if food safety issues are not proactively addressed. Current projections have estimated that the global population will reach eight billion by 2025-2030, and will possibly reach nine billion by 2045-2050 *(4)*. Produce safety is of primary concern in the global sphere of food safety due to increased consumption arising from growing awareness that fresh fruit and vegetables are essential components of a healthy and balanced diet. This resultant increase in consumption has been encouraged in many countries by health organizations in order to protect against illnesses that range from gum disease to more serious ones like cardiovascular diseases. Consumption of fresh fruits and vegetables in the US has been and is currently still on the increase. According to the Economic Research Service of the USDA, vegetable production is expected to rise more rapidly than the population growth over the next decade, due mainly to persistent emphasis on produce's role in health and nutrition, and the subsequent increased consumer demand *(95)*. Unfortunately, fruits and vegetables consumed raw are increasingly being recognized as vehicles for transmission of human pathogens *(12)*. The Food Safety Modernization Act (FSMA) which was passed into law early 2011 is recognized as being the most sweeping reform of U.S. food safety regulations in more than 70 years. The reform is designed to shift safety of the food supply from responding to contamination to preventing it. Incorporated into FSMA are Prevention Standard Provisions which include Produce Safety Regulations and Guidance. Produce Safety

Standards, which would greatly help farmers comply with safe practices, would be issued by the FDA in the near future *(82)*.

2.2 How Produce Contamination Occurs

2.2.1 Focus on Pre-harvest Contamination

Fresh produce can be contaminated at any point during its production cycle: growth on the farm, harvesting, processing, marketing, distribution or final preparation by the end user *(87)*. Traditionally, the contamination of produce is divided into two sectors: pre-harvest and post-harvest. Pre-harvest contamination occurs primarily at the site of production on the farm up to the point of harvest, while post-harvest contamination occurs during the chain of operations that take place once the field crops have been harvested. The difference between both sectors can be blurred somewhat with the increased conduct of the initial post-harvest steps taking place during harvesting (e.g., field packaging operations or initial trimming and coring operations during harvest). This study focuses on pre-harvest contamination modes.

Sources of Pre-harvest Contamination

There is growing amount of information on the potential for in-field contamination of produce from direct sources such as water *(81)* and manure *(54)*, or other less-direct sources such as wild-birds and rodents *(72)* or vector-borne pathogens (e.g. transmission via insects, flies and nematodes) *(57, 83)*.

Internalization of pathogens into plant tissue is another relevant issue that is being examined *(28, 33, 49, 86, 104)*; whether and how this occurs through the roots and plant vascular tissues or through plant surfaces, cracks and crevices is an area of ongoing research.

Although several contamination modes exist in the leafy green production chain, animal manure and contaminated irrigation water have been highlighted as the two most important contamination sources *(39)*.

2.2.2 Use of Manure Associated With Contamination

The presence of animals or their fecal matter on or around produce fields can spread fecal contamination via multiple routes such as contaminated irrigation water, aerosolization, or even direct contact of the animals with the growing crops. This is particularly important when specific animals act as reservoirs for the pathogens. Likewise, produce harvested from the soil or produce that come in close contact with improperly composted manure can become contaminated with fecal pathogens which can remain viable in the feces for extended periods *(9, 51)*. Post-contamination survival of pathogens on plants has been shown to be greatly enhanced by factors such as proximity of edible portion of plant to the soil, concentration of pathogen in contamination vehicle (soil, manure or water), and time lapse between manure application and harvest *(28, 39, 50, 51)*. As such, pre-harvest contamination of fresh produce will largely depend on the survival capabilities of pathogens harbored in soil, manure and on plants *(84)*. Since it is almost impossible to eliminate enteric pathogens from the environment, many mitigation

strategies have focused on preventing contamination in the field instead of trying to eliminate it from already-contaminated products. Occasionally, the sources of enteric pathogens associated with outbreaks are directly linked to fecal contamination of the agricultural environment *(28, 51)*. A substantial body of research focuses on the persistence, spread and survival of *Salmonella* and other pathogenic bacteria in manure and its subsequent transmission onto fresh produce, especially leafy vegetables, as these tend to have elevated levels of bacteria due to their large surface area *(16)*. Animal manure, when dehydrated, generates dust-like particles which could become airborne, thereby facilitating unintentional transfer from the source of the manure to leafy green farms in proximal locations *(16)*. This particular mode of transmission and the survival abilities of the pathogens in the transmission vehicle have not been studied to any great degree.

2.3 Post-harvest Contamination of Fresh Produce

2.3.1 Factors Affecting Contamination after Harvest

A series of physiological changes occur in produce once they are harvested from the farm, especially when processed as "fresh-cut" *(28)*. Fresh-cut vegetables differ in surface morphology, tissue composition and metabolic activities, thereby producing diverse ecological niches *(14)*. These changes are largely brought about by a chain of operations carried out on the plants, some of which include mechanical disruptions such as cutting, shredding, dicing, and peeling. When these disruptions occur during production of minimally-processed produce, cross-contamination via wash (processing) water could occur *(61)*. Also, pathogens could be spread through use of knives used

during cutting or slicing procedures or via direct contact of contaminated peel with edible peeled parts. Aside from the possibility that the cut-surfaces make the produce more susceptible to attachment and entry of pathogens, these surfaces ooze significant amounts of nutrients which are easily utilized by microorganisms *(15, 28)* thus encouraging attachment and survival even after these processing operations are completed. Accordingly, there are three major factors that affect produce post-harvest contamination: attachment of pathogens to produce via various mechanisms, cross-contamination during processing of fresh-cut produce, and survival and growth of pathogens on produce during storage *(28)*.

2.3.2 Limitations of Post-Harvest Control Measures

The mere fact that outbreaks continue to be associated with the consumption of fresh and fresh-cut produce is an indication that mitigation strategies addressing post-harvest contamination are not enough to curb the contamination problem. The efficacy of both physical and chemical interventions to reduce or eliminate pathogen load on produce after harvest has been explored in many studies. Chlorine (sodium hypochlorite) is one of the most commonly used chemical sanitizing agents in the fresh produce industry *(28, 98)*. When used in recommended concentrations (50 to 200 ppm) *(79)*, it helps reduce the microorganism load on the surfaces of produce and minimize cross-contamination during wash operations by curtailing the redistribution of pathogens *(28, 79)*. However, numerous studies have shown that chemical sanitizers like chlorine have limited effectiveness in eliminating pathogens from the surface of produce, mainly because its primary purpose is to prevent cross-contamination during post-harvest processing. The effectiveness of chlorine as a sanitizer depends on the freely available chlorine in the

wash water, the pH of the wash water, the amount of organic matter present and the storage temperature after washing *(28, 87)*. This is illustrated in the investigation of a 1999 outbreak of salmonellosis linked to cilantro consumption *(19)*. Further research after the outbreak showed an increased growth of *Salmonella* Thompson on chopped cilantro leaves stored at room temperature as compared with the un-cut cilantro leaves. In general, the use of chlorine in wash water can be expected to only yield a 1-2 log reduction in bacterial counts *(91)*, a level of control that is insufficient for effective pathogen elimination. Formation of microbial biofilms on plant tissue surfaces has also been said to play a role in the increased resistance of bacteria to aqueous sanitizers *(24)*. Once these type of barriers are in place, contact between the chemical sanitizer and the pathogens is considerably reduced, because the sites on the plant where microorganisms are harbored – cut surfaces, pores, nutrient-dense indentations such as leaf veins and other grandular trichomes – become inaccessible *(14, 28, 45, 61, 79, 91)*. The strong attachment of the pathogen to the plant tissue also limits the effectiveness of wash procedures *(24)*.

2.3.3 The Internalization Dilemma

The ability for washes to eliminate pathogens from fresh and fresh-cut produce is further hampered by the potential for pathogen internalization into plant tissue. Internalization may perhaps offer a partial explanation for the inability of sanitizers to efficiently eliminate pathogens in leafy greens. Many studies have demonstrated the internalization of *E. coli* O157:H7 in leafy vegetables and seedlings *(49, 79, 88, 98)*, while a few others indicated no internalization occurred at all *(54)*. In a number of studies, internalization of *E. coli* O157:H7 and *Salmonella* was restricted to seedling or root tissues and did not

occur in mature leaves *(44, 49, 98)*. A feasible explanation for this is that most mature plants have been able to evolve both their physical and chemical defense mechanisms thereby inhibiting internalization of transient saprophytes *(94)*. However, the integrity of produce tissues cannot be guaranteed as mechanical and biological disruption of various parts of the plants – roots, leaves - during or after harvesting is a common phenomenon, and sometimes interferes with the plant's natural protective defenses. Direct entry of pathogens into plant tissues through stomata openings is another area that has received attention. Figure (II-1) shows the stomata openings on the abaxial (underside) of a spinach leaf. These openings are potential entry points for pathogens.

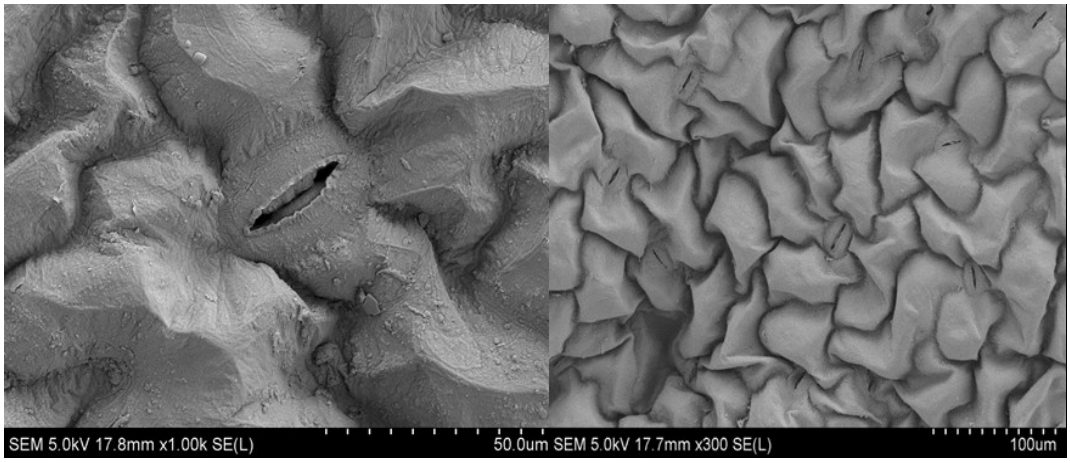

Figure II -1: SEM images of the abaxial side of a spinach leaf showing the stomata openings

Salmonella enterica has been shown to penetrate leaf samples from crops grown in soil contaminated with *Salmonella (40).* In several pre-harvest studies, inspection of inoculated spinach leaves, some researchers using high resolution electron microscopy, suggests internalization of *E. coli* O157:H7 and *Salmonella* within plant stomata *(60, 68, 85, 102).* One of the studies investigated how *Salmonella* gets internalized into lettuce

leaves via light and chemotaxis induction *(60)*. *Salmonella* cells were seen to crowd around stomatal openings, (Fig. II - 2) suggesting that *Salmonella* penetration requires open stomata. There is greater potential for internalization on the abaxial surface of leaves than the adaxial surface due to the greater number of stoma present on the underside of leaves.

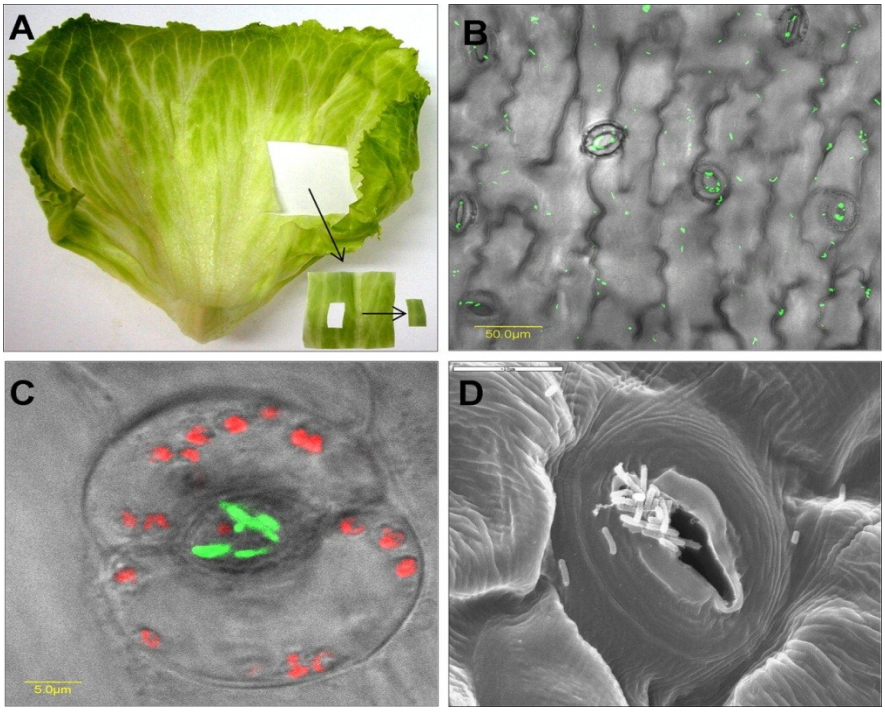

Figure II - 2: Interactions of *S. enterica* serovar Typhimurium with lettuce leaves. (A) *S. enterica* serovar Typhimurium incubated with lettuce leaf for 2h and examined by confocal microscopy. (B and C) Microscopic images of GFP-tagged *Salmonella* (green) showing both diffuse and stomatal-associated attachment (B), and a higher magnification of a single stoma harboring *Salmonella* cells are shown (C). Red fluorescence indicates autofluorescence of the chlorophyll of guard cells. The fluorescent images were overlaid with the transmitted light image obtained using Nomarski differential interference contrast. (D) SEM image showing the complex topography of a single stomatal region and multiple bacteria (potentially *Salmonella*) residing within the stomatal space. Bars, 50 µm (B), 5 µm (C), and 10 µm (D) (Reproduced from Kroupitski *et al*, 2009) *(60)*

These observations encourage further studies into the risks that could be associated with pathogens being deposited on leaf surfaces via airborne transmission. If dust particles generated from manure could provide protection to *Salmonella* cells, then the potential for subsequent internalization may possibly be enhanced. And even when the manure dust particles are not able to enter the stomata themselves, they could act as a shield to *Salmonella* cells possibly located underneath them, giving protection against adverse environmental conditions and aiding longer survival on leaf phyllosphere.

Limitations of other Interventions

In terms of physical interventions employed for pathogen inactivation on fresh produce, processes such as the use of UV light, irradiation, thermal treatments, e.g. water-assisted microwave heating, and high pressure processing have been used *(26, 28)*. These alternative measures have drawbacks ranging from limited consumer acceptance to undesirable alterations in organoleptic properties, particularly in the use of high pressure treatments *(8)* and irradiation *(25)*. Dose-dependent UV treatments, while generally effective, depend on product-type. More pathogen reduction is observed in produce with smoother surfaces such as apples, while produce with rougher surfaces tend to block light paths and prevent accessibility *(11)*. Also, some of these alternative methods are expensive, thereby limiting their usage within the produce industry. Prevention strategies available to the final consumer which could help combat any lingering bacterial contaminants are limited for produce eaten without any form of cooking (kill-step for bacteria) *(87)*. The indicated limitations of processes currently used to combat

contamination of fresh produce are part of the reason why it is necessary to seek a deeper understanding of what occurs on the farm. The knowledge gained could help find new ways to prevent contamination. Limiting the initial burden of the pathogens before harvest is a prerequisite to successful post-harvest control measures, i.e. post-harvest technologies are insufficient in the absence of effective pre-harvest controls.

2.4 The Pathogen – Salmonella

Salmonella are a gram-negative, facultatively anaerobic, rod-shaped bacilli commonly found in the gastrointestinal tract of humans and animals. They are non-spore-forming, motile bacteria belonging to the *Enterobacteriaceae* family. The *Salmonella* genus consists of two species, *Salmonella bongori* and *Salmonella enterica*, and includes over 2,500 known serotypes, all of which are considered potential human pathogens.

The ability of *Salmonella* to persist outside its host is perhaps the most critical trait that enables it to sometimes contaminate fresh produce *(61)*. This resilient pathogen is one of the most commonly identified etiological agents associated with fresh produce outbreaks *(45, 62)*. Invasive infections caused by *Salmonella* can be severe and potentially life threatening, especially in the very young, elderly and immuno-compromised patients *(87, 92)*. Over the last decade, there have been significant reductions in the incidence of many foodborne illnesses caused by pathogens such as *E. coli* O157:H7 and *Campylobacter* spp.; however, *Salmonella* infections have not experienced similar declines despite significant public health efforts (Figure II - 3).

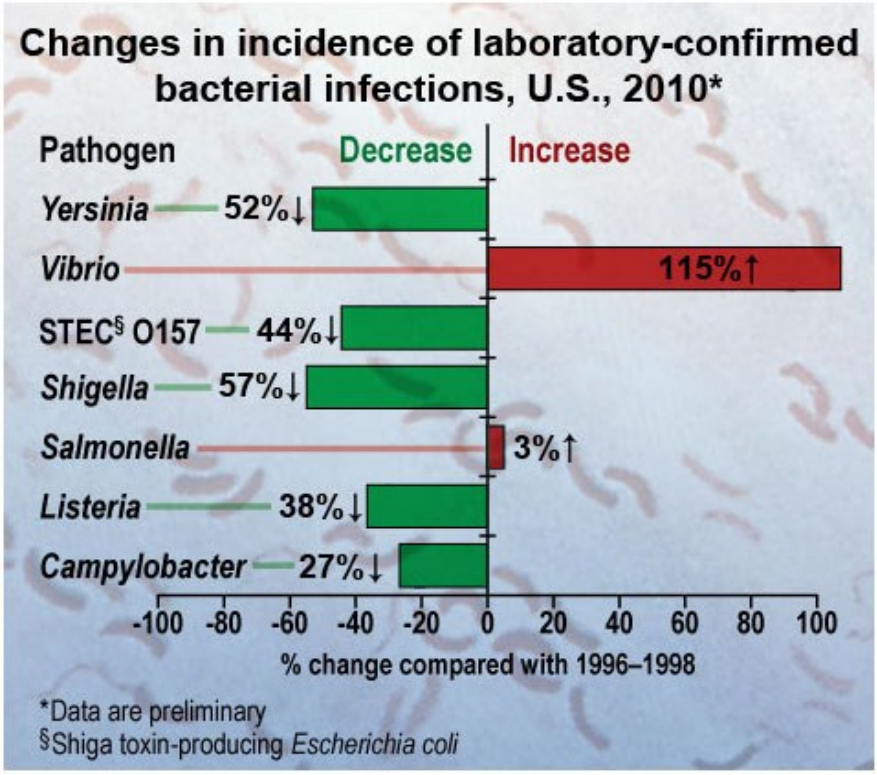

Figure II-3: *Salmonella*, the most commonly diagnosed and reported bacteria associated with foodborne illness, continues to be a challenge. Causing about 1.2 million U.S. illnesses annually, it is the most common cause of hospitalization and death tracked by FoodNet since surveillance began in *1996 (21)*

Salmonella is quite difficult to control due to its high tolerance for environmental stresses such as UV radiation, its ability to form biofilms, its widespread distribution, multiple drug resistance and its remarkable adaptability *(45)*. The infectious dose has been found to be as low as <10 – 100 cells, depending on the strain type, the bacteria's physiological state and the characteristics of the host *(39)*.

During the period from 1973 to 1997, the CDC reported that among the 103 produce-associated outbreaks where a known pathogen was identified, 48% were caused by *Salmonella (87)*. In 2008, the CDC reported that, of the total number of outbreak-related foodborne illnesses documented for that year, 1,276 resulted in hospitalizations, and

Salmonella spp. was the most common cause (62%) of these hospitalizations *(22)*. It was also the second most common etiologic agent, causing 23% of 479 outbreaks with a single confirmed etiologic agent. In this particular report, Enteritidis was the most common serotype among the 108 *Salmonella* outbreaks where a serotype was reported *(22)*. The CDC report also highlighted the observation that *Salmonella* in vine-stalk vegetables were the pathogen-commodity pair responsible for the most outbreak-related illnesses for the study year (2008) *(22)*. Recent advances in diagnostics and control technologies as well as regulatory changes are currently being implemented in an attempt to reduce the incidence of salmonellosis in the food chain. One of the ways this is being done is a critical examination of the routes of introducing and disseminating *Salmonella* in the primary production chain.

2.5 The Vehicle of Contamination – Manure

2.5.1 Sources

Manure is believed to be the major vector of transmission of pathogens to produce growing on fields *(50, 103)*. The major producers of manure are poultry (chicken and turkey), cattle and swine operations *(101)*. Feces from these ruminant and poultry animals have been identified as principal reservoirs for *Salmonella enterica* and Shiga-toxin-producing *E. coli* (cow manure) *(41, 51, 59)*. These and other enteric pathogens such as *Listeria monocytogenes*, *Yersinia* spp., *Campylobacter* spp., which are shed in the feces of healthy farm animals as well as some wild animals can persist in the environment for extended periods *(38, 79)*. The use of poultry and cattle manure in fresh produce farming practices has received attention and research has been done on the persistence, spread and survival of some of these pathogens in manure and their subsequent transmission onto

fresh produce, especially leafy greens *(24, 39)*. The application of untreated (non-composted) or improperly composted manure to agricultural fields poses a microbiological risk to edible produce *(9, 51)*. This risk increases in organic farming settings as animal manure or compost is largely used as fertilizer *(51)*. In a recent review by Critzer and Doyle, it was pointed out that a lot of the interactions that occur between foodborne pathogens and the fruits and vegetables they contaminate are just beginning to be elucidated *(24)*. *Salmonella* Typhimurium has been found to be more persistent in soil than other bacterial pathogens *(50, 51)*, and could be ideal for studying soil-manure-pathogen interactions.

The diagram below (Fig II - 4) shows a schematic representation of the complex nature of the transmission of pathogens through the food chain using vehicles such as manure, insects (vectors), soil, food and water, as well as the potential role humans play in the contamination conundrum.

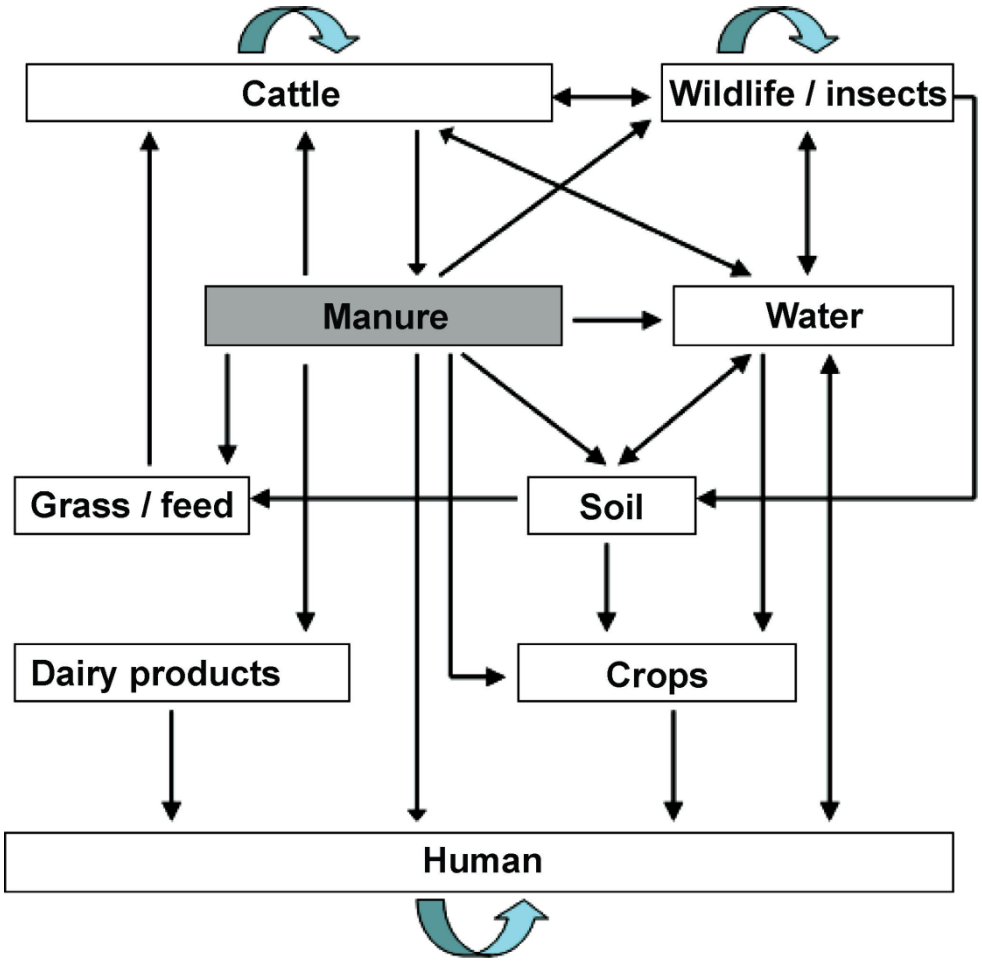

Figure II-4: Schematic representation of pathogen cycling through agricultural and environmental niches *(39)*

2.5.2 Use of Manure in Farming Practices

Animal manure is used around the world as an organic fertilizer, most especially in areas where arable farming co-occurs with livestock farming *(39)*. The appropriate use of manure as an organic fertilizer is encouraged as a means of promoting sustainable recycling of essential nutrients required to maintain good soil quality *(9)*. The role of composting in manure usage on farms is an important one. Although both conventional

and organic farmers frequently use animal manure for fertilization, manure use in organic food production is more prevalent since organic food producers are not allowed to use any type of synthetic fertilizer *(100)*. There have been concerns over the possible increased risk of organic crops becoming more contaminated as a result of greater manure usage. However, several investigations have not demonstrated that organic produce is significantly more contaminated than conventional produce *(2, 55, 71)*. One limited Norwegian study found a slightly higher occurrence of fecal indicators in organic lettuce *(64)*. Conventional food production does not have the requirements which organic standards have: that animal manure be composted according to specific procedures *(100)*. The United States Department of Agriculture (USDA) requires that at least 120 days elapse between the application of non-composted manure and the harvest of organic crops with edible portions which have been exposed to soil particles; or 90 days if composted *(97)*. Many studies have supported this requirement and reported significant inactivation of pathogens when raw manure is stored for a sufficiently long period of time. One study however suggested that the 120-day rule might be inadequate because of extended survival of *Salmonella* in soil beyond this period *(51)*. While both organic and conventional systems are compelled to use manure which has been composted or previously stored, there is no guarantee that the composting process completely eliminates indigenous pathogens in the manure. Results from preliminary studies conducted by the Center for Veterinary Medicine (CVM) of the Food and Drug Administration (FDA) show that *Salmonella* and *E. coli* can survive in litter or manure-treated soils for up to three months - a time frame that would easily span the life of many plants *(32)* . In the United States, several million tons of poultry litter or manure are

generated annually by poultry farms, and a good portion of this is estimated to be spread on croplands as a way of disposal *(32)*.

2.6 Knowledge Gap

2.6.1 Known versus Unknown

As the knowledge base about produce safety widens, it has come to light that an important component to comprehensive farm-to-fork strategies to reduce foodborne illness incidences is the reduction of human pathogen contamination on the farm *(29)*. More comprehensive data on the fate and transport of pathogens in production areas is critical for devising effective strategies aimed at minimizing contamination of produce at the pre-harvest stage.

Extensive research is being conducted by government, industry and academia to investigate sources of produce contamination in both pre- and post-harvest settings. These include detection of pathogens on produce while still on the farm, understanding pathogen transmission, dissemination and survival in different transmission vehicles, assessment of potential intervention technologies and microbial ecology of produce. These measures are increasingly allowing the conduct of formal risk assessments. In spite of this, how and where in the farm-to-fork chain produce becomes contaminated is often unknown. This emphasizes the need to better understand the factors that influence dissemination and survival of foodborne pathogens on the field.

From reviewing available literature, it is apparent that the risks associated with airborne pathogens in produce cultivation settings is a research area of growing interest and

several research efforts have addressed the role of airborne contamination of produce on the field. However, not much research efforts have gone into exploiting avenues for airborne contamination of produce on the field with dried fecal material as a vehicle.

2.6.2 Aerosolization as a Mode of Contamination

The environment in which fresh produce is grown could have a major influence on the microbiological quality of the product. Previous research has been done on dust generated during livestock operations and manure management systems and attempts have been made to assess the hazard this dust presents to workers and individuals in nearby communities, animals (cattle hides) as well as croplands in the vicinity *(30, 67, 99, 103)*. Aside from livestock operations, land application of manure is another event which could potentially spread contamination. Manures with a low moisture content, such as chicken litter or dewatered feces, are normally applied to fields using a manure slinger or spreader *(30)* (see illustration in Fig 1 – 1 in chapter 1). Expectedly, application methods that launch solid manures into the air could establish a potentially risky situation since pathogens could become aerosolized and get transported to downwind receptors *(30)* (e.g. agricultural fields).

A recent study attempted to determine if spinach grown in close proximity to cattle feedyards can become contaminated with microorganisms from the feedyard and, if so, how far the contamination can travel *(103)*. Soil, dust samples obtained using air samplers and spinach bundles placed at strategic locations, were analyzed for the presence of *E. coli O157* and *Salmonella*. Data from this study indicated that feedyard environment could significantly contaminate exposed spinach leaves fifty yards away

within a 24-hour period. *Salmonella* and *E. coli* O157 were recovered from the spinach samples but not from the dust or soil samples, leaving open the question of how exactly the pathogens got on the leaves. The researchers stated that further research is necessary to confirm pathogen carriage and contamination via dust. This particular study used already-harvested spinach and restricted the duration of contamination investigation to a maximum of 24 h environmental exposure.

Another research group evaluated dust and soil samples from a cattle feedyard and observed that 6.7 and 11.1% were positive for *E. coli* O157 and *Salmonella*, respectively *(67)*. Observations from these studies support the concern that produce grown in the vicinity of cattle feedyards can become contaminated with pathogens by environmental sources, and there is a good chance that aerosolized dust is a transmission vehicle.

In some animal production facilities, solid and liquid manures are commonly stored in piles or holding ponds *(30)*. Further procedures are then carried out such as composting, mechanical dewatering, anaerobic biogas production, or a combination of any of the aforementioned procedures. Aerosols can be generated during any of these operations, and produce fields within the vicinity are at risk of contamination. More research is needed to determine the extent to which dust generated by livestock operations can contaminate fresh produce.

2.6.3 Survival of *Salmonella* in Manure and on Produce

The survival capability of enteric pathogens in manure and manure-amended soils largely dictates whether or not vegetables grown using such manure will become and remain contaminated. Survival of *Salmonella* has been shown to be greatly enhanced by factors such as proximity of edible portion of the plant to the soil, concentration of pathogen in contamination vehicle (soil, manure or water), and elapsed time between manure application and harvest *(28)*. *Salmonella* inoculated into animal manure was shown in several laboratory and field studies to survive for up to 300 days, although higher initial bacterial inoculation doses correlated with longer survival periods *(10, 42, 45, 51)*. In a 1999 study, *Salmonella* present in cattle manure was able to survive for at least 60 days at 4°C and 20°C, but was undetectable after 19 days at 37°C *(46)*. This observation would indicate that incubation temperature plays a significant role in the duration of survival. Microbial competition is another factor that could influence the survival of enteropathogens in manure or slurry (liquid manure). *E coli* O157:H7 was found to have survived longer in autoclaved soil amended with manure than in non-autoclaved soil, suggesting antagonistic interactions with indigenous soil microorganisms *(53)* . Quite recently, the influence of aerobic vs. anaerobic conditions on survival of *Salmonella* was studied by Semenov *et al (84)*, and it was observed that there was no significant difference between the survival of *Salmonella* serovar Typhimurium under aerobic vs. anaerobic incubation and storage conditions. This observation was unlike the estimated survival times of *E. coli* O157:H7 in the same study, which showed that *E. coli* survived longer under anaerobic than under aerobic conditions *(84)*.

In a 2004 study, *Salmonella* was detected on leafy green vegetables (lettuce and parsley) for up to 63 and 231 days respectively, after coming into contact with contaminated soil *(51)*. Apparently, pathogen transfer in this particular scenario had occurred through direct contact of aerial tissues with the soil or via splashes of rain or irrigation water. Pathogens on aerial tissue surfaces such as leaves have been known to encounter harsh physicochemical conditions such as UV sun rays and dehydration which can impede their growth and survival. Whether or not these conditions can cause sustained damage of the pathogen cells is an area for active research, as is the role of dried manure in aiding the survival of these produce-associated pathogens.

2.6.4 *Salmonella* Survival in Dry Matrices

This research study proposed using moisture content of dried manure as a variable in investigating the survival of *Salmonella* in manure dust particles. As such, background knowledge about survival mechanisms of *Salmonella* in dry matrices is helpful. Characteristically low-water-activity foods would not ordinarily support the growth of vegetative pathogens like *Salmonella (77)*. However, it is recognized that *Salmonella* can survive for long periods in low-moisture food products or other matrices given certain conditions. Survival could depend on factors such as storage temperature, physical and chemical composition of the host matrix, the test media and the media used to recover damaged cells *(77)*. The type of strain and serotypes tested could also play a role in survival capabilities - certain serotypes of *Salmonella*, such as serovar Senftenberg 775W, have been reported to have significant desiccation tolerance *(80)*.

After a 1999 nationwide outbreak of gastroenteritis due to consumption of dried squid chips (contaminated with *Salmonella enterica* serovars Oranienburg and Chester) in Japan, a study was conducted to closely investigate the survival capabilities of *Salmonella* and shiga toxin-producing *E. coli (*STEC*).* The study was done using a desiccation model system of selected dry foods to monitor survival of the pathogens after artificial inoculation. Desiccated *Salmonella* cells (24 h drying at 35°C on paper disks) were found to acquire high tolerance to stresses such as storage temperature, heat and ethanol *(47)*. Storage temperature was also found to have a huge impact on *Salmonella* survival. With a water activity range of 0.5 to 0.6, it was reported that, although 100% of selected salmonellae (4/4) and 80% of selected STEC strains (12/15) showed strong resistance to dryness in refrigerated storage conditions (4°C) after 24 h of initial drying, all the desiccated *Salmonella* strains in the dried paper disks were destroyed after 35 to 70 days of storage at 25°C and 35°C. This was in spite of their ability to survive 22 to 24 months of storage at 4°C *(47)*. This means that, in dry conditions, salmonellae may die in 1 to 2 months when stored at room temperature or higher but may survive for up to 2 years when stored at cold temperature. Similarly, in a recent study which examined whether dehydration induces tolerance to other cell stressors, dried *Salmonella enterica* serotype Typhimurium cells were exposed to multiple stresses and their viability was evaluated *(43)*. It was discovered that dehydration does induce cross-tolerance to multiple stresses in *Salmonella.*

2.6.5 Possible Mechanisms of Survival Employed by *Salmonella*

The mechanism by which bacterial cells are able to exhibit desiccation tolerance has been said to reflect a complex array of interactions at the structural, physiological, and molecular levels *(78)*. Although the mechanism(s) of the resistance of *Salmonella* (and some other gram-negative bacteria) to dry conditions remains to be fully expounded, the "water replacement hypothesis" could provide a possible rationalization for an underlying mechanism *(78)*. The underlying principle accounts for how the non-reducing disaccharides, sucrose and trehalose preserve the structural integrity of proteins and hence the function of membranes. This is achieved by the ability of the cell to inhibit structural damage and replace bacterial membranous water in desiccated conditions *(63)*. In view of this, the increased survival after drying in the presence of sucrose might be related to abilities of bacteria to accumulate intracellular sucrose and/or trehalose by *de novo* synthesis, a desiccation resistance strategy commonly employed by some soil bacteria *(63, 106)*. During desiccation, intracellular trehalose is said to help keep the phospholipid bilayer of cell membranes in the liquid crystalline phase. It can also maintain protein in a hydrated form by hydrogen bonding and water replacement *(63)*. Therefore, trehalose is believed to be a very good osmoprotectant under severe water activity reduction *(78)*.

The ability of *Salmonella* to adapt to dry conditions has also been explained in a study done by Mattick *et al (65)*, a research group that used the 'filament formation' theory to interpret their observation. In their study, *Salmonella* strains - *Salmonella* Enteritidis PT4 and *Salmonella* Typhimurium DT104 - were found to form filaments (elongated cells) in matrices that had low a_w values (0.92 to 0.95). The cell elongation is thought to occur via

inactivation or inhibition of cell division proteins and subsequent blockage of septation during cell division. Simply put, filament formation occurs when cells grow without dividing at a_w levels that are suboptimal for growth but not bactericidal (approximately 0.93 to 0.98).

2.6.6 Effect of Water Activity (a_w) and pH on Survival

It has been shown that the heat resistance of completely dried organisms is several times higher than that of the same organisms in a dilute suspension which has an a_w of about 1.0 *(80)*. The heat resistance of *Salmonella* has also been reported to increase with decreasing a_w levels *(7)*. Very low water content in bacterial cells can play an important role in the mechanism of heat resistance of bacteria. This is because low water contents in the cell can inhibit the protein denaturation induced by high-temperature heating through vibration of water molecules to break disulfide and hydrogen bonds of intracellular proteins *(31)*. This phenomenon could partially explain the observed resistance of desiccated *Salmonella* cells in dry food matrices. The degree and strength of the vibration of water molecules in these bacteria are considered to be restricted to a certain extent; therefore, the very low water contents in the desiccated *Salmonella* cells prevent the membrane proteins of the bacteria from denaturation and preserve their integrity even during exposure to extreme stress conditions *(47)*.

Chapter 3: Project Objectives

The ultimate goal of this study was to investigate how unintended aerosolization of dust particles generated from manure could transmit fecal contamination onto leafy greens on the field, and how physicochemical factors like moisture content and UV light affects the pathogens' survival on the manure particles and on the leaf tissue.

In a 2011 comprehensive review by Doyle and Erickson, it was suggested that mitigation strategies can be divided into those aimed at pathogen reduction in the environment and those that focus on reduction or elimination of pathogen contamination in animals or plants *(29)*. Strategies targeted at reducing environmental pathogens could be further subdivided into colonization site on the farm, time of sampling or harvesting and mode of pathogen dissemination and contamination; this research project was designed to investigate the latter part.

Three objectives were pursued in order to investigate the role airborne manure could play in the contamination of leafy greens:

- The first objective involved a study of the survival capabilities of two serotypes of *Salmonella* in an organic matrix (dried turkey manure).
- Secondly, the effect of UV radiation on the survival of *Salmonella* in dried turkey manure was evaluated.
- The third objective investigated the survival of *Salmonella* present in dried particles of manure on spinach leaf surface.

This thesis research reports the results of studies designed to assess the role which dried manure particles of a size capable of being airborne, could play in the pre-harvest contamination of spinach leaves.

Chapter 4: Survival of *Salmonella enterica* in Turkey Manure Dust at Different Moisture Contents

4.1 Background

Contaminated poultry litter has been linked to food safety concerns due to its ability to serve as a reservoir for *Salmonella enterica*. Although, studies have been done to investigate the influence of both internal and environmental growth factors on the survival of *Salmonella* in poultry litter and chicken manure *(46, 56, 76, 89)*, there is still a need to examine the role and extent to which airborne transmission of these matrices in dry, particulate form could play in the pre-harvest contamination of leafy greens. As a first step to assessing this potential source of contamination, it is necessary to appraise the likely survival of *Salmonella* in poultry manure particles of a size capable of being airborne.

In this phase of the research, parameters affecting the risk of windblown manure serving as a vehicle for the transmission of foodborne pathogens was studied using turkey waste (fecal material + bedding) finely ground to particle sizes that would allow aerial dispersal. Moisture content was used as the major variable in studying the survival of *Salmonella* in dried manure. It is well known that appropriate moisture conditions are essential for continued growth of microorganisms in various matrices. The influence of water activity on the survival of *Salmonella* in organic matrices such as cow or poultry manure has also been studied and found to be significant, though mostly in conjunction with other factors such as pH and temperature *(38, 42, 46, 75)*. A few studies which have also attempted to relate total moisture content to water activity in poultry litter have observed an unsurprising linear relationship between log total moisture and water activity

(46). These studies however, were investigating relatively high water activity values ranging from 0.75 to 0.99. There seems to be little known about the survival response of *Salmonella* to extremely dry conditions (a_w as low as 0.40) in particulate poultry manure.

4.2 Study Objective

The major aim of the present study was to investigate how much influence very low moisture content, and accordingly, low water activity, has on the survival of *Salmonella* in dried manure particles capable of being aerosolized. The survival of a mixture of *Salmonella* serotypes in the dried manure matrix was examined using moisture levels of 5, 10, and 15%. Knowledge from this study could help further assess the extent of risk which aerosol dispersion of manure presents to produce fields.

4.3 Materials & Methods

In this experiment, known levels of *Salmonella* were incorporated into dehydrated turkey manure (litter) at different moisture levels (5, 10 and 15%). The inoculated samples were kept in sterile containers and pathogen survival was monitored for several months.
At specified intervals, the turkey manure samples were characterized in terms of pH, water activity and background microflora.

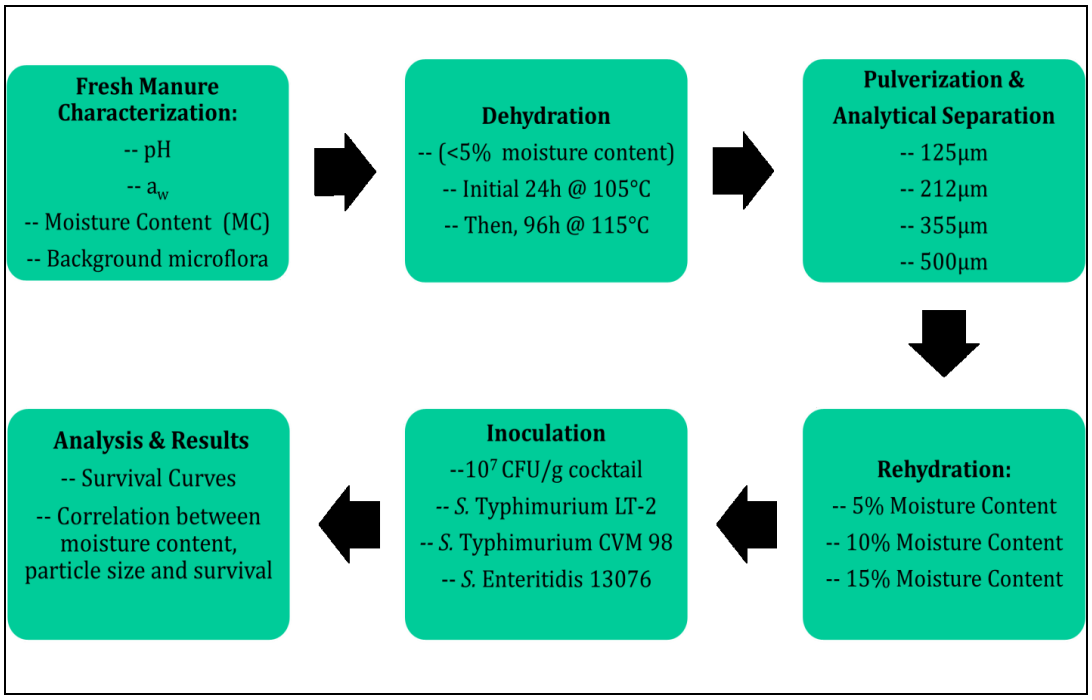

Figure IV-1: Diagrammatic summary of major steps taken in phase 1 experiments

Turkey Manure (litter) Characterization, Dehydration and Processing

Approximately 3.0 kg (wet weight) of fresh turkey litter (fecal material plus bedding in the form of pinewood sawdust) was obtained from a United States Department of Agriculture, Agricultural Research Service (USDA- ARS, Beltsville, MD) poultry facility. The bedding material had a 1-month rotation period. The manure was stored in closed plastic bags at < 4°C prior to a subsequent dehydration process. Fresh samples of manure ("as received") were analyzed for pH, water activity, moisture content (MC) and background microflora - at the start and at the end of each experiment.

pH Measurement

The pH of the freshly collected manure was measured in a water suspension (1:2.5 g/v) at 25°C using a pH meter (Orion pH electrode 9165 BN, Orion Research, Boston, MA, USA). To do this, the suspension was prepared by weighing 10 g (wet weight) of manure into a clean bottle and adding 25 mL deionized water. A stirring rod was used to homogenize the moisture before the pH reading of the supernatant was taken (average of three separate measurements). pH measurements of dehydrated manure were also taken periodically throughout the duration of the entire study.

Water Activity Measurement

Water activity was measured using a water activity meter (Novasina IC-500, AW-LAB, Switzerland), and manufacturer directions concerning measurements were adhered to. See Table (IV - 2) for results.

Moisture Content Determination – Analytical Drying Method

The percent moisture content was gravimetrically determined (i.e., weighed before and after drying) and calculated using the following method: 20 g of fresh manure in a small foil container was dried in a convection oven (Fisher Scientific Oven, Isotemp, Gravity Convection, Model 255G, Pittsburg, PA) at 105°C oven for 24 h. The sample was thereafter re-weighed and the moisture content determined using the following equation:

$$M_n = ((W_w - W_d)/W_w) \times 100$$

where;

M_n = moisture content (%) of manure material

W_W = wet weight of the sample, and

W_d = weight of the sample after drying *(96)*

The aim here was to obtain a dehydrated product of less than 5% moisture content; therefore, the fresh manure was dehydrated in batches in the convection oven. A batch of ca. 150 g was put in an aluminum foil tray and dried in a 105°C oven for 24 h, turned over, then further dried at 115°C for another 96 h. This staggered drying process was necessary to achieve a <5% moisture content without over-heating the manure material. Continuous moisture content analysis (using formula above) helped detect when the manure was down to a final 1.9% MC (see sample calculations in Appendix II & III).

Microbiological Profile

Microbiological analysis of the freshly collected manure was done by presumptive testing for bacteria commonly found in poultry litter (See Appendix I). Duplicate plates of 10^{-1}, 10^{-3} & 10^{-5} dilutions of manure (1.0 g of fresh manure to 9.0 ml of sterile 0.1% peptone water) were spiral plated (Neutec Group Inc., Farmingdale, NY) onto Brain Heart Infusion (BHI) agar plates (used to enumerate total aerobic plate count) and Violet Red Bile Glucose (VRBG) agar (used to enumerate *Enterobacteriaceae*). Coliforms such as *E. coli* (fecal indicator organism), *Enterobacter*, *Klebsiella, Citrobacter* and *Proteus,* as well as other organisms like *Bacillus, Clostridium, Staphylococcus*, *Salmonella*, *Pseudomonas aeruginosa, Yersinia enterocolitica, Listeria, Campylobacter jejuni* and *Aeromonas hydrophila* are generally known to be associated with poultry manure *(27, 56, 70, 89)*. A few of these organisms were presumptively identified by plating appropriate dilutions of manure on suitable selective and differential media (Difco Laboratories,

Detroit, MI), as well as through the use of simple biochemical tests and microscopy. They include *E. coli, Bacillus, Enterobacter, Proteus, Staphylococcus, Klebsiella*, *Shigella* and *Listeria*. Some fungi and yeasts were also presumptively identified. The manure, both before and after dehydration, was tested to ensure that only *Salmonella*-free manure was used for this study.

Processing Of Dried Manure – Pulverization and Analytical Separation

After drying, the manure was once again analyzed for pH level, moisture content, water activity and microbiological profile. The extensive drying process ultimately reduced microflora to mainly *Bacillus* spp., a soil saprophyte and spore-former (see Results section and Appendix I for details).

The dried manure was ground in small batches using a 1000W Ninja® Professional Blender (EURO-PRO Operating LLC, MA). The resulting mixture was passed through a coarse #8 mesh screen with wire diameter 28 inches and opening 0.097 inches (The United Company, Westminster, MD), The less-coarse particles which passed through the screen was set aside, while the material retained in the screen sieve (larger particles) was returned to the blender for further grinding. This process was continued until approximately 1.0 kg (dry weight) of coarsely ground dried manure material was obtained. Large, coarse particles - small wood chips and some unidentified fibrous materials were discarded while the less coarse material was stored in air-tight containers until further use.

The dried manure-mix was once again profiled for background microflora load. Cultivation on Brain Heart Infusion Agar plates revealed that there was still a heavy

presence of microorganisms which might interfere with the *Salmonella* survival studies; therefore the dried manure-mix was further dehydrated in the convection oven for another 48 h at 105°C. This eventually brought the background microflora to a reasonable level – ca. 10^4 CFU/g.

Analytical Separation of Manure into Various Particle Sizes

A standard test sieve ASTM E-11 (Fisher Scientific, Pittsburg, PA) was used to separate the manure mix into different particle sizes of 125 µm (sieve No. 35), 212 µm (No. 45), 355 µm (No. 70) and 500 µm (No. 120).

Figure IV-2: Analytical sieve used to separate dried manure particles

The resultant particles of different sizes (Fig. IV – 3) were stored in separate containers for further analysis. For ease of reference, these manure particles were mostly referred to as 'manure dust' for the remainder of this thesis.

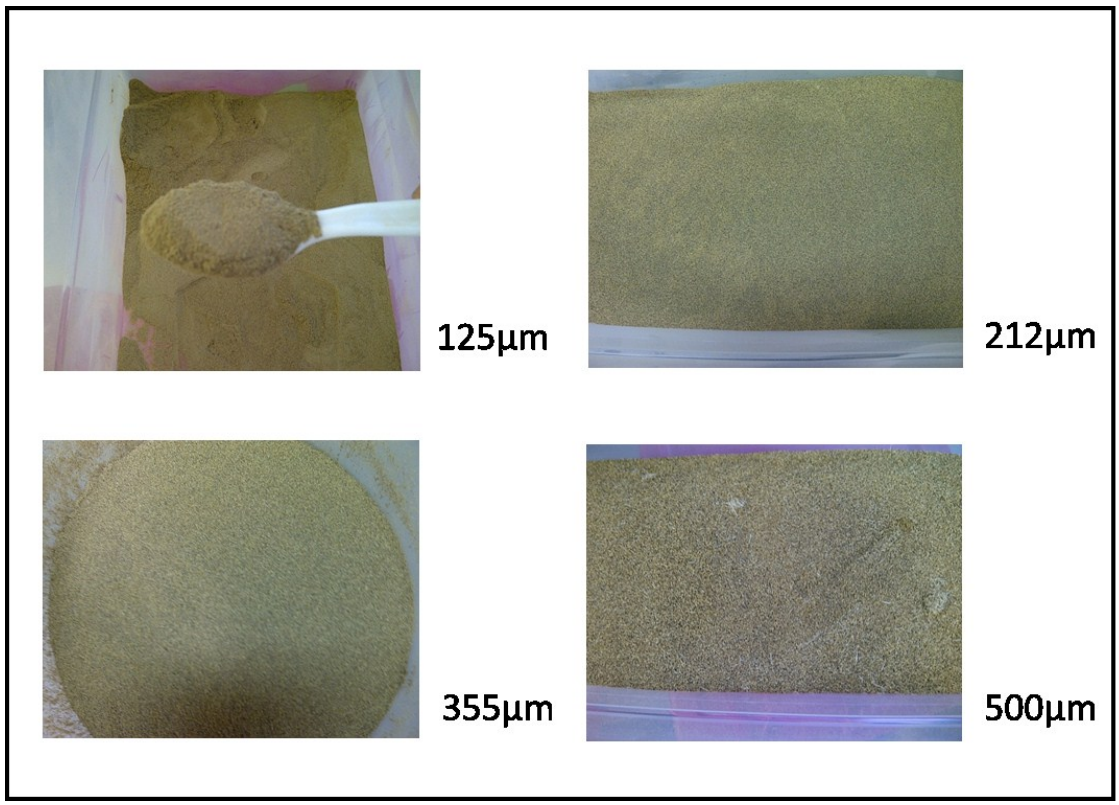

Figure IV-3: Manure Dust of Varying Particle Sizes

Re-hydration of Manure into Various Moisture Contents

Each batch of manure dust of a particular particle size was split into three portions which were later re-hydrated when appropriate. Three days before any experiment with the manure dust would begin; sterile water was used to rehydrate the three portions to 5%, 10% and 15% moisture contents. For example, to rehydrate 140 g (dry weight) of the dust to 5% moisture content, the following calculation was done:

$X\ \% = (W_R - W_D)/W_D)$

where;

X % = % Moisture content

W_R = Rehydrated weight of the sample, and

W_D = Dry weight of the sample

Thus, 5% = W_R – 140 g/140 g

W_R = 147 g

And, amount of water needed to rehydrate dust = 147 – 140 = 7.0 ml.

Using the same method, one would add 14ml and 21ml to obtain 10% and 15% moisture content dust respectively. The rehydrated dust was allowed to equilibrate at room temperature for at least 3 days, after which it was ready for experimental use. Due to uncontrollable factors such as amount of pelleted *Salmonella* inoculum added at the beginning of each experiment, the moisture content of the rehydrated dust had a variability of ± 1%.

Bacterial Strains Used

A cocktail of three *Salmonella enterica* strains were used in this study: *Salmonella* serovar Typhimurium CVM 98, *Salmonella* serovar Typhimurium LT-2 and *Salmonella* serovar Enteritidis KPL 13076. All strains were acquired from the culture collection at a University of Maryland, College Park Microbiology Lab.

Preparation of Inocula

Individual frozen stock cultures of the three *Salmonella* strains were activated by thawing and streaking onto Brain Heart Infusion (BHI) agar (Becton Dickinson, Sparks, MD) and incubating at 37°C for 18 to24 h. A single colony was selected from each plate and streaked onto separate plates of Xylose Lysine Desoxycholate (XLD) agar (Becton

Dickinson) which is selective for *Salmonella*, and incubated at 37°C for 24 h. One pure black colony was then harvested from each XLD plate and grown in five 10ml tubes of brain heart infusion (BHI) broth (Becton Dickinson) and incubated at 37°C for 24 h. The five tubes of each strain were combined into a sterile 50-ml centrifuge tube (BD Falcon, Franklin Lake, NJ), and the cells were pelleted and harvested by centrifugation (3, 000 × *g* for 10 min at 7°C). The cells were washed three times and re-suspended in 3ml of sterile 0.1% peptone water. Finally, equal volumes of each strain were combined, re-centrifuged, and re-suspended in 1ml of sterile 0.1% peptone water to produce the three-strain cocktail with a final concentration of ca. 10^9 CFU/ml. The inoculum was prepared such that it was ready to use on the day that each experiment was scheduled to begin. The inoculum was maintained on ice and applied to the manure dust within 1 h of preparation. After the first experiment, it was decided that a more effective inoculum preparation method could be used, and some modifications were made as follows: after the cocktail of strains were pelleted by centrifugation, a sterile spatula was used to scoop out the bacteria pellet at the bottom of the centrifuge tube, and mixed into 1g of manure dust which had been previously measured out. A few drops of food coloring were added, and the combination was mixed carefully using the back of a sterile plastic spoon. The *Salmonella*-dust inoculum was then placed under the fume hood for an hour in order to eliminate much of the moisture obtained from the liquid *Salmonell*a inoculum. This method ensured there was negligible amount of moisture, if any, added to the 5, 10 and 15% moisture level samples (possible variability of ±1%).

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Procedure for Inoculating Manure Dust

Experiment I -- 125µm Manure Dust

As previously explained, sterile water was used to rehydrate three portions of manure dust to 5%, 10% and 15% moisture contents three days before each experiment began (to allow for equilibration of water and manure dust). To do this for the first experiment:

- 75 ml-capacity plastic sample bottles with screw cap lids (Fig. IV - 4) were labeled so that there were 4 bottles per % moisture level. Three of the bottles were used for the inoculated manure dust and the remaining bottle was used for the un-inoculated control (Total of 4 bottles per moisture level).
- Three portions of ca. 140 g of ground turkey manure dust was transferred to three wide-mouth 2-L bottles with screw cap lids. The moisture contents of the three portions were adjusted by adding the appropriate amount of distilled water to obtain moisture contents (MC) of 5%, 10% and 15%. The water was carefully added in small quantities using a pipette, in order to get it evenly distributed throughout the manure dust. The back of a clean plastic spoon was used to break and distribute globules of water and manure dust which clumped together. To further ensure even distribution, the mixture was run through a blender for 30 s. Determination of moisture content and a_w was carried out at the end of the 3-day equilibration.
- On the day of the experiment, a 35 g portion of 5% MC manure was transferred to a previously-labeled sampling bottle. The same was done for the 10% and 15% MC manures. These three bottles served as un-inoculated controls.

- Working under the fume hood in order to contain the easily-aerosolized dust, 0.3 ml of the concentrated cocktail of *Salmonella* strains was added drop-wise to the remaining 5% MC manure dust (approximately 105 g) with thorough mixing between additions. In order to ensure uniform contamination of the matrix of interest, a few drops of food coloring were added to the culture; this helped monitor the distribution pattern of the culture in the manure dust, and, with the aid of a plastic spoon, even distribution was achieved [a preliminary experiment was conducted to confirm that the food coloring has no unintended effects on the *Salmonella* cells]. The inoculated 5% MC manure was then transferred in 35 g portions to each of the appropriate pre-labeled plastic sampling bottles. The process was repeated with the 10% and 15% MC manures. This inoculation method yielded an initial level of between 10^5 and 10^6 CFU of *Salmonella* per gram of manure dust.
- All the sample bottles were stored at room temperature away from direct exposure to light.

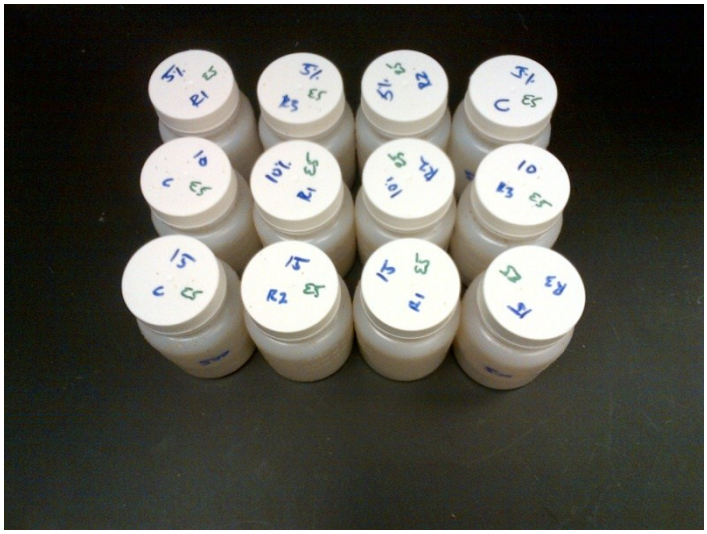


Figure IV-4: Screw-cap plastic bottles used for airtight storage of dried manure particles at 5, 10 and 15% moisture levels

Duration of Experiment

This experiment was carried out four times staggered over 10+ months. In the first experiment, dust particle sizes of 125 µm were inoculated at the three moisture levels mentioned and observed for pathogen survival over 10 months.

Experiment II – IV

Subsequent experiments observed survival of *Salmonella* in manure dust particle sizes of 212 µm, 355 µm and 500 µm, using exactly the same process as the first experiment, but observed over less time due to faster die-off of *Salmonella* in those larger particle sizes. The level of *Salmonella* in the cocktail used to inoculate the manure dust averaged out at 5.4 x 10^9 CFU/g for the four experimental trials.

Sampling

- Pathogen survival was originally to be monitored over 6 months using viable plate count and enrichment methods. However, sampling duration was extended, as *Salmonella* in the manure dust was found to have survived well beyond the originally anticipated sampling period.
- Therefore, samples were removed periodically and assayed for total aerobic plate count (BHI agar), *Enterobacteriaceae* (VRBG agar) and *Salmonella* cells (XLD agar). Sampling times were designated for 1, 2, 3, 5, 7, 14, 28, 56, 84 days post inoculation. On each sampling day, the following procedure was followed:

    - Four bottles representing each moisture content (three inoculated and one un-inoculated), were removed from storage. Working under the fume hood, each bottle was given a vigorous shake for 20 seconds, and 1 g of sample from each bottle was weighed out into a sterile 9.0 ml dilution blank of 0.1% peptone water blank to generate a 10^{-1} dilution. The bottles were returned to storage after the 1-g samples had been removed. 10^{-3} and 10^{-5} dilutions were further made by transferring 0.1 ml of the preceding dilution to sterile 9.9 ml 0.1% peptone water dilution blanks. The number of dilutions plated was adjusted as needed on each sampling day.
    - From each of three sample dilutions (10^{-1}, 10^{-3}, 10^{-5}), predetermined volumes (50µl), including the control sample, were spiral plated on duplicates on XLD, VRBG and BHI Agar plates.

- The plates were incubated at 35°C for 48 h, with enumeration being performed at 24 and 48 h using an automated colony counter (Neutec Group Inc., Farmingdale, NY).

Enrichment Procedures

The lower limit of detection (LOD) has been described as the lowest number of microorganisms that can be detected but in numbers that cannot be precisely estimated *(3)*. In this experiment, when counts fell below the detection level, a two-step enrichment involving an initial non-selective enrichment followed by a selective enrichment was done to help recover and isolate injured cells.

For each sample to be enriched, a 1:10 dilution of 0.1% peptone water homogenate vs. buffered peptone water was made, and incubated for 18-24 h at 37°C. On day 2, this mixture was sub-cultured into RVB (1ml: 9 ml), a selective enrichment media, and incubated for 24 h at 42°C. On day 3, the selective enrichment culture was streaked onto XLD plates after vortexing, and incubated at 37°C. Observations were recorded as positive or negative.

Statistical Analysis

All plates were incubated at 35°C and plate counts were done at 24 h and 48 h using an automated colony counter. The whole experiment was run three times, population density averages in Log CFU/g were taken and survival curves generated. Microbial counts were log transformed prior to analysis. Data were subjected to the Statistical Analysis System (SAS Institute, Cary, N.C.) and analyzed as a two-factor (treatment and time) linear model using the PROC MIXED procedure. Each point value presented in the results

section represents the mean of three values (experiment done three times). Assumptions of normality and variance homogeneity of the linear model were checked, and variance heterogeneity was corrected using the variance grouping technique *(74)*. Whenever effects were statistically significant, mean values were compared using Sidak adjusted p-values *(48)* to maintain experiment-wise error ≤ 0.05. A p-value of <0.05 was considered to be statistically significant.

4.4 Results

A. Characterization of Manure – Fresh and Dehydrated

Freshly collected manure had an initial total aerobic plate count (BHIA) of 10^{10} CFU/g. After 24 h drying, population count was reduced to ~10^{6} CFU/g. However, this level was still too high for the purpose of the experiment and further drying was conducted as previously explained. The table below (Table IV-1) presents a summary of measured profiles.

Table IV - 1: Measurement of various manure parameters

Characterization of Manure					
	Total Aerobic Plate Count (TAPC)	***Enterobacteriaceae* level**	**pH**	**Moisture Content**	**a_w**
Fresh Manure	10^{10} CFU/g	10^{8} CFU/g	6.46	49%	0.97
Dehydrated Manure	10^{4} CFU/g	$< 10^{0}$ CFU/g	6.97	~ 1.9%	0.38

B. Survival of *Salmonella* based on Water Activity (a_w) of Manure Particles

Across all particle sizes, dust with 5% moisture content had the lowest a_w in their respective size categories. Water activity values of all dust particles in all moisture content categories ranged from 0.402 to 0.665.

Table IV - 2: Water activity measurements read at 22°C. Each value is an average of three separate readings.

Water Activity Measurements*			
Dust Particle Size	**Moisture Content**		
	5%	10%	15%
125 μm	0.402 ± 0.001	0.421 ± 0.002	0.430 ± 0.003
212 μm	0.414 ± 0.002	0.431 ± 0.001	0.461 ± 0.002
355 μm	0.513 ± 0.003	0.537 ± 0.001	0.575 ± 0.001
500 μm	0.611 ± 0.008	0.642 ± 0.005	0.665 ± 0.010

*mean and standard deviation values are shown

C. Survival of *Salmonella* based on Moisture Content of Manure Particles

Moisture content effects on survival were analyzed separately in each particle size category. Survival times were inversely related to percent moisture content: the lowest moisture level of 5% corresponded to the slowest inactivation rate. Significant ($P < 0.05$)

differences were observed in the inactivation rates of *Salmonella* based on moisture levels (Fig IV - 5). Manure dust (125 μm) with moisture levels of 5%, 10%, and 15% had respectively achieved log reductions of 2.07, 2.69 and 2.80 on XLD agar by day 14, although enrichments indicated survival beyond 60 days for all moisture levels. Specifically, viable *Salmonella* was still detectable after approximately 291 days in the 5% moisture samples, but not in 10% or 15% moisture samples (Fig IV - 8).

The survival rate in the 10% and 15% moisture levels were mostly not significantly different from each other: out of 28 sampling days, the survival rates were statistically significantly different only 3 times (data not shown).

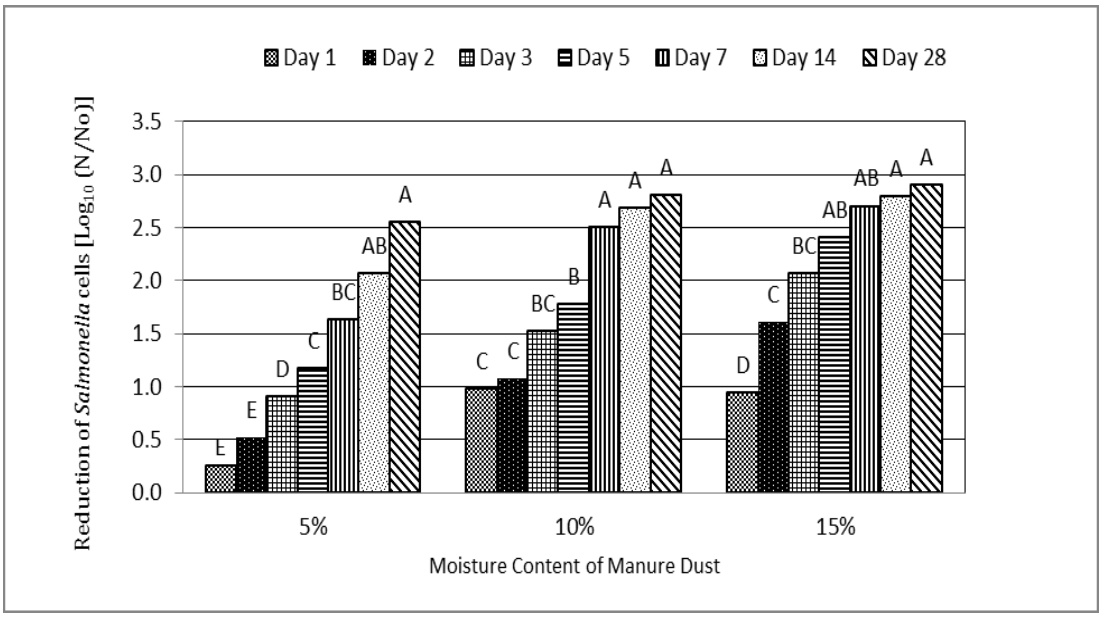
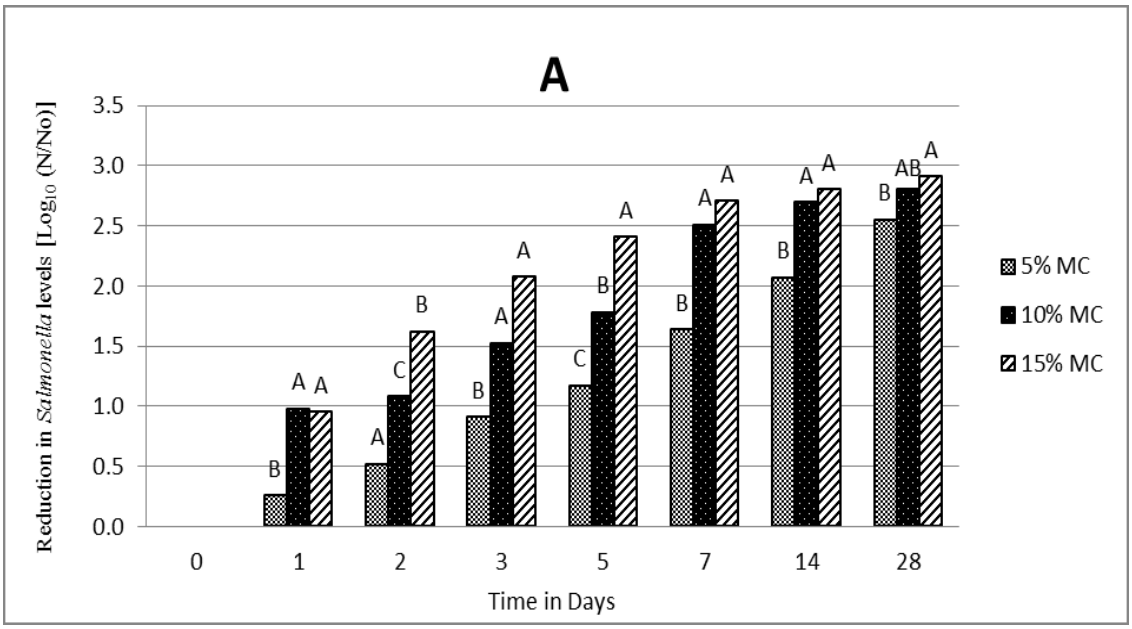
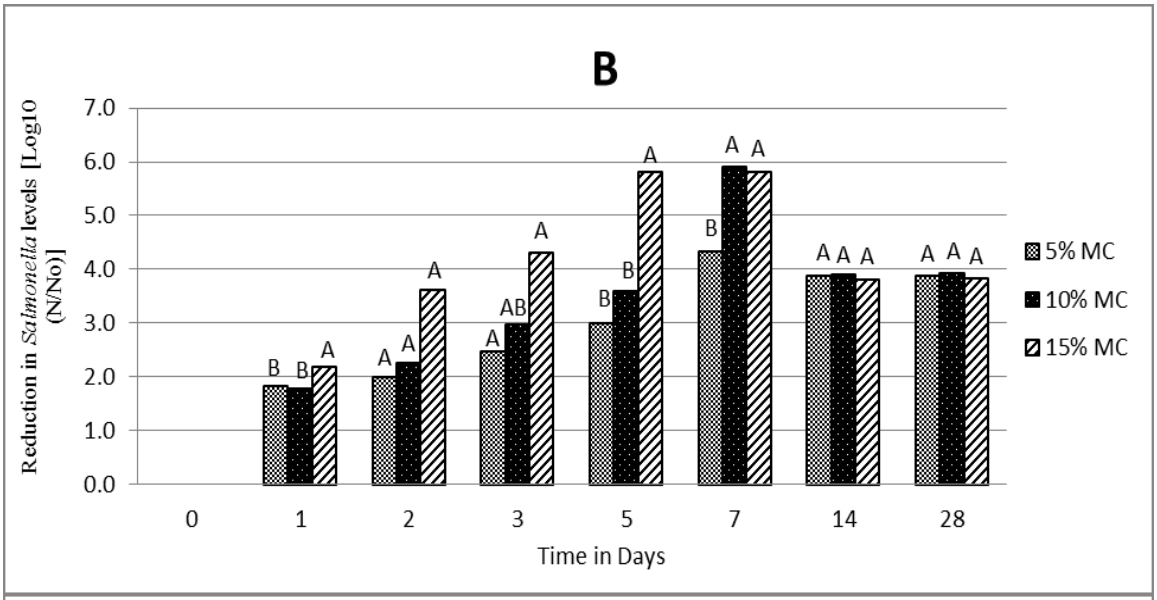


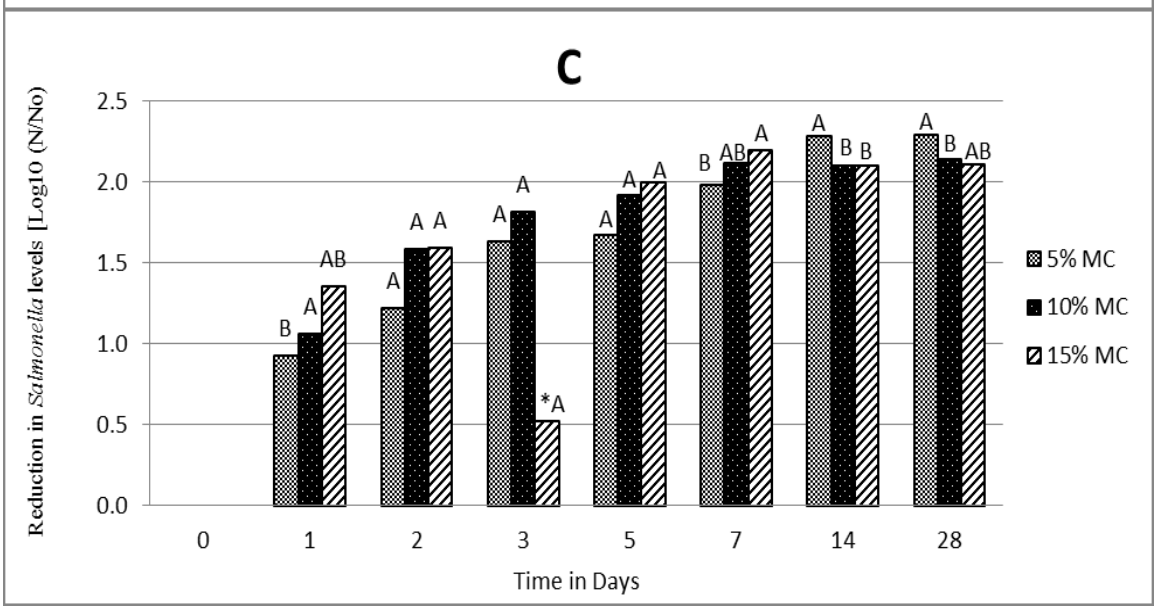
Figure IV - 5: Inactivation of *Salmonella* in125μm manure dust samples according to moisture content. Within each day, different letters above bars indicate significant differences (P < 0.05) in mean populations. Data shown has been normalized.

D. Effect of Manure Particle Size

Notable differences in survival patterns were observed across the four particle sizes (Fig IV - 6). For all four independent trials, the 5% moisture content dust had significantly ($P < 0.05$) higher survival rates than the other moisture levels. *Salmonella* survival was less likely at any given moisture content with increasing particle size. Differences in inactivation rates between moisture levels were more evident in the smallest particle size, 125 μm, compared to the larger particles, especially between Day 2 and 7. Up to Day 7, in all particle-size categories, 5% moisture manure dust had a statistically significantly ($P < 0.05$) lower die-off rate than the other two moisture levels; although, as counts became undetectable via direct plating, the differences between the moisture levels waned, and only enrichment procedures helped determine the persistence of *Salmonella* in the 5% moisture samples beyond Day 28 (Fig IV - 8).







*Not significantly different (P>0.05)

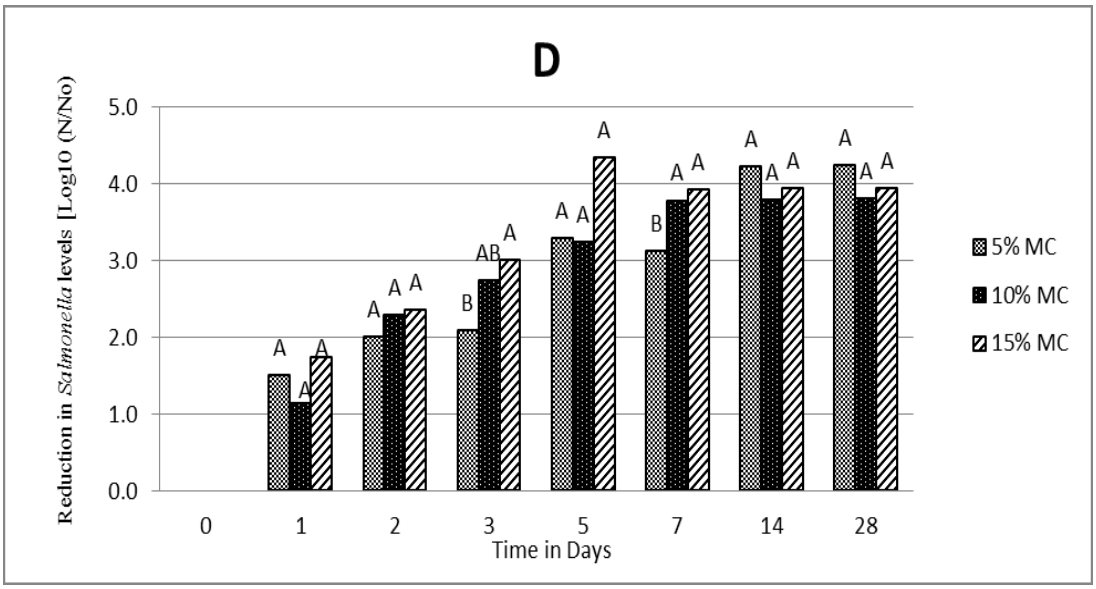


Figure IV - 6: **Mean population log reductions (Log (N/N_o)) of *Salmonella* in varied particles sizes of manure (A -125 µm, B – 212 µm, C – 355 µm, D – 500 µm), showing differences in survival rates at three moisture levels during a 28-day period. Within each day, different letters above bars indicate significant differences ($P < 0.05$) in mean populations.**

Focus on *Salmonella* Survival in Smallest Dust Particle Size – 125 µm

For this particle size, *Salmonella* populations had a less than one log decrease from Day 0 to Day 1 in the lowest moisture content category. However between Day 0 and 7, *Salmonella* populations in dust at 10% and 15% moisture levels had declined rapidly by 3 log units (CFU/g), while the 5% moisture dust only supported 1 log CFU/g decline in *Salmonella* population (Fig IV - 7). By day 14, no viable *Salmonella* colonies could be recovered on XLD or VRBG agar plates of both 10% and 15% moisture content dusts, not even in the lowest dilution (10^{-1}). However, up till day 28 post-inoculation, the lowest 5% moisture content dust continued to have viable plate counts up to 10^2 CFU/g, which happened to be the lower limit of detection (LOD) for this experiment using traditional culture procedures. The lowest reportable result (for 1 colony on the plate) was 10^2

CFU/g, therefore a bacterial load of $<10^2$ CFU/g was read as 'no viable count' since no *Salmonella* colonies were recovered on those plates. The LOD for enrichment, which was done after culturable organisms could not be recovered on the selective XLD media, was 1 CFU/g.

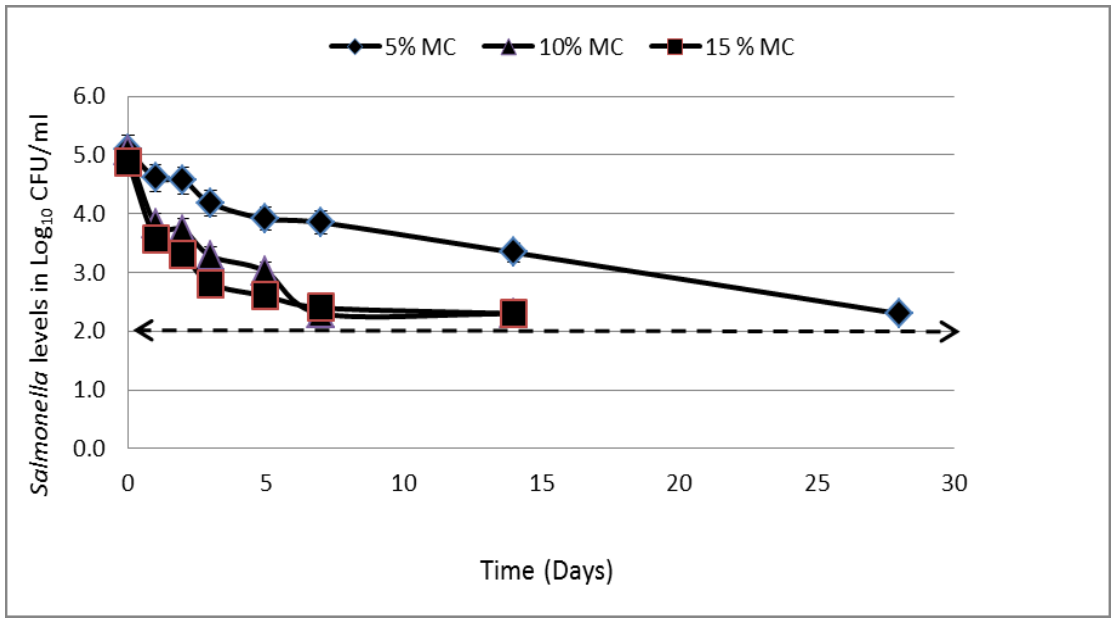


Figure IV - 7: Comparison of *Salmonella* survival in 125 µm manure dust using XLD as recovery media. Dotted lines represent lower limit of detection (10^2 CFU/g). Each point on the graph is an average of three replicates. Error bars display standard error.

Survival Trend in Larger Manure Particles

The 2nd, 3rd and 4th experiment trials which observed the survival of *Salmonella* in manure dust of particle sizes 212 µm, 355 µm and 500 µm respectively, and at 5, 10 and 15% moisture levels used exactly the same process as the first experiment. These experiments displayed similar trends and showed a significant particle-size effect on survival (Table IV - 3). However, inactivation based on moisture content were less-pronounced in these larger particle sizes when compared to the 125 µm particles size (Fig IV - 5), and there were no significant differences in survival among particle sizes 212,

355 and 500 μm (data not shown). Survival patterns were consistent with all experiment trials: there was a sharp decline from Day 0 to Day 1, then a gradual die-off from day 2 up to day 7, or up till the point when plate counts reached the limit of detection. Only 5% moisture content samples in the 125μm particle sizes had viable cell recovery after 28 days. When culturable cells could no longer be recovered on XLD agar, VRBG agar was still able to recover some injured *Salmonella* cells. However, in order to adhere to standard protocols, XLD counts were largely used to analyze survival data.

Table IV - 3: *Salmonella* survival decreased with increase in moisture content. 'ND' (highlighted areas) indicates *Salmonella* cells are non-detectable by plating. Enrichment procedures were carried out after *Salmonella* could no longer be detected by plate counts. Each value is an average of three replicates.

Reduction in *Salmonella* levels [Log(CFU/g)] in 5% moisture content manure particles over 28 days								
Day	**0**	**1**	**2**	**3**	**5**	**7**	**14**	**28**
125µm	0	0.48	0.52	0.91	1.18	1.24	2.07	2.79
212µm	0	1.9	2.04	2.5	2.95	3.42	***ND***	***ND***
355µm	0	1	1.3	1.8	1.91	2.36	***ND***	***ND***
500µm	0	1.52	2.02	2.11	3.14	3.33	***ND***	***ND***

Reduction in *Salmonella* levels [Log(CFU/g)] in 10% moisture content manure particles over 28 days								
Day	**0**	**1**	**2**	**3**	**5**	**7**	**14**	**28**
125µm	0	1.26	1.35	1.8	2.06	2.78	2.69	***ND***
212µm	0	1.73	2.12	2.82	3.41	5.76	***ND***	***ND***
355µm	0	1.01	1.66	1.81	2.11	4.11	***ND***	***ND***
500µm	0	1.15	2.31	2.74	2.98	5.79	***ND***	***ND***

Reduction in *Salmonella* levels [Log(CFU/g)] in 15% moisture content manure particles over 28 days								
Day	**0**	**1**	**2**	**3**	**5**	**7**	**14**	**28**
125µm	0	0.92	1.58	2.07	2.28	2.48	2.81	***ND***
212µm	0	2.18	3.33	3.61	***ND***	***ND***	***ND***	***ND***
355µm	0	1.46	2.16	2.16	***ND***	***ND***	***ND***	***ND***
500µm	0	1.75	2.36	3.01	***ND***	***ND***	***ND***	***ND***

E. *Salmonella* Survival Monitored by Enrichment

After plate counts could no longer detect presence of *Salmonella*, enrichment procedures were carried out. Manure dust of 125 µm and 5% moisture content had the longest detectable survival period as monitored by enrichment. Regular samplings to monitor

surviving *Salmonella* cells were carried out during the ~10-month duration of the experiment. The survival trend was consistent with that obtained for viable plate counts: manure dust with the lowest moisture level supported the longest survival in the tiniest dust particle size. Manure dust of size 125 um dust and 5% moisture survived for up to 291 days, while the 15% moisture level only survived for about 68 days (Fig IV – 8).

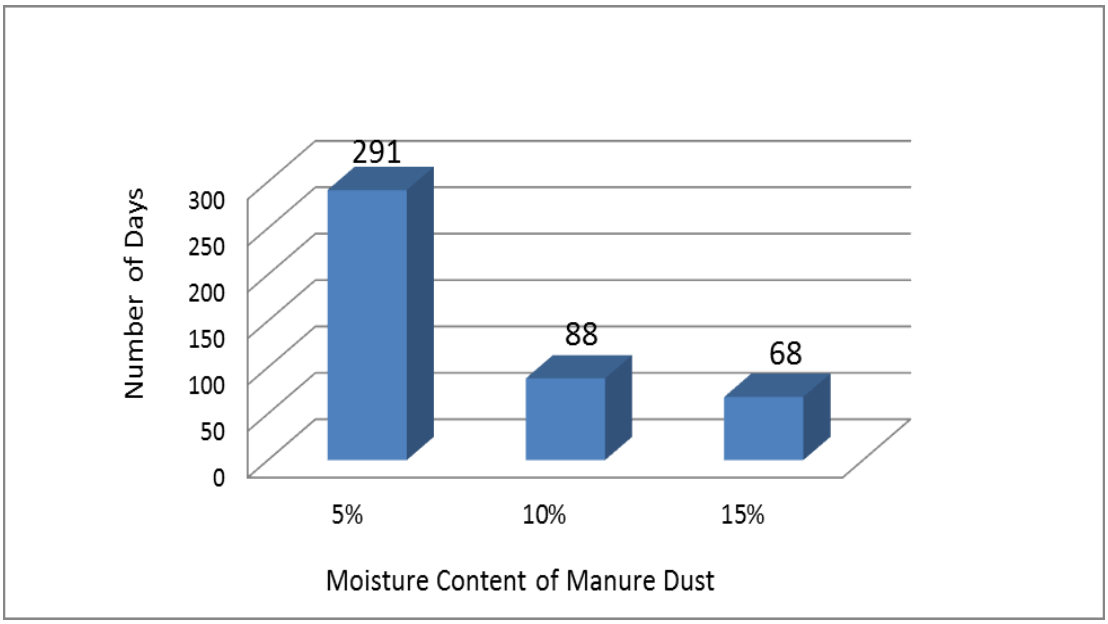


Figure IV - 8: Survival of *Salmonella* in 125µm manure dust based on recovery after enrichment. Enrichment was carried out after *Salmonella* could no longer be detected by plate counts. Limit of detection for enrichment: 1 CFU/g

F. Survival of Indigenous Bacteria and Injury Effect

The only microorganism that seemed to survive the manure's dehydration and extended storage process was presumptive *Bacillus* spp., as identified by morphology and examination under phase-contrast microscope. This bacterium was constantly recovered on BHI agar even after 10 months of storage. As expected, plating of samples on non-selective media helped recovery of injured *Salmonella* cells, and facilitated the growth of

other viable microorganisms present in the dried manure samples. Therefore, BHI Agar plates consistently had higher counts than VRBG or XLD agar plates (Fig IV- 9).

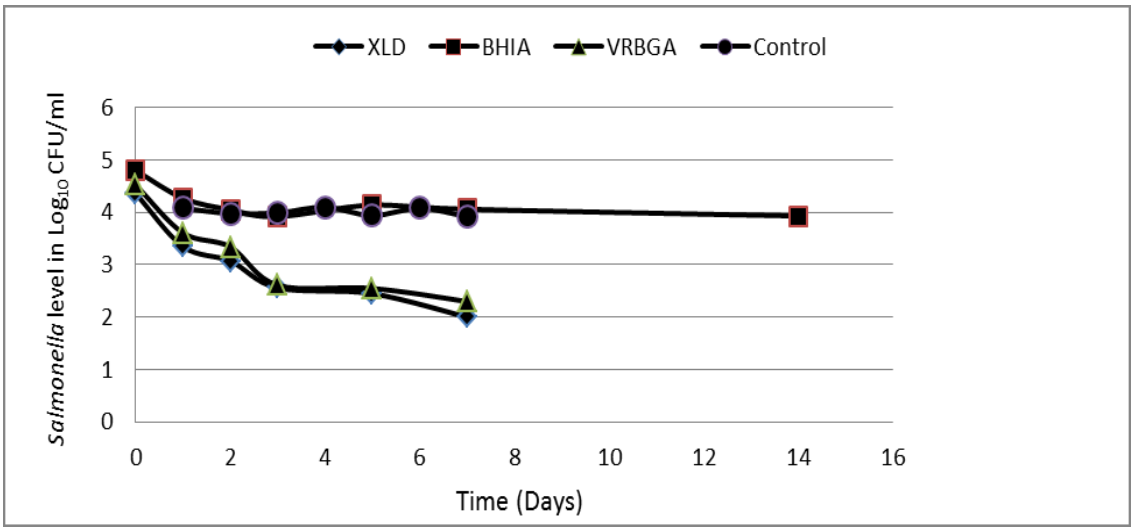


Figure IV - 9: Injury Effect: a representative graph showing difference between XLD, VRBG and BHI agars used for recovery. VRBG Agar recovered a slightly higher population of *Salmonella* cells than XLD agar, but much lower than non-selective BHI Agar. Control samples plated on BHI Agar. Each point is an average of three replicates.

It was apparent that no *Enterobacteriaceae* present in the fresh manure survived the intense initial dehydration process, and as a result, all cells recovered on violet red bile glucose agar (purple/pink colonies surrounded by purple halos of precipitated bile salts), were deemed to be and processed as *Salmonella* cells. Throughout the duration of the experiment, bacterial counts on VRBG agar were constantly slightly higher than those on XLD (see Fig IV - 10 for pictorial illustration). This was attributed to the less-selective nature of VRBG agar which enabled injured cells to recover (the inclusion of dextrose improves recovery of *Enterobacteriaceae*). To confirm this, random colonies were picked periodically from the VRBG agar plates and streaked on XLD agar for confirmation as

Salmonella. Additionally, control samples (un-inoculated dust) plated on VRBG agar plates did not recover any colonies.

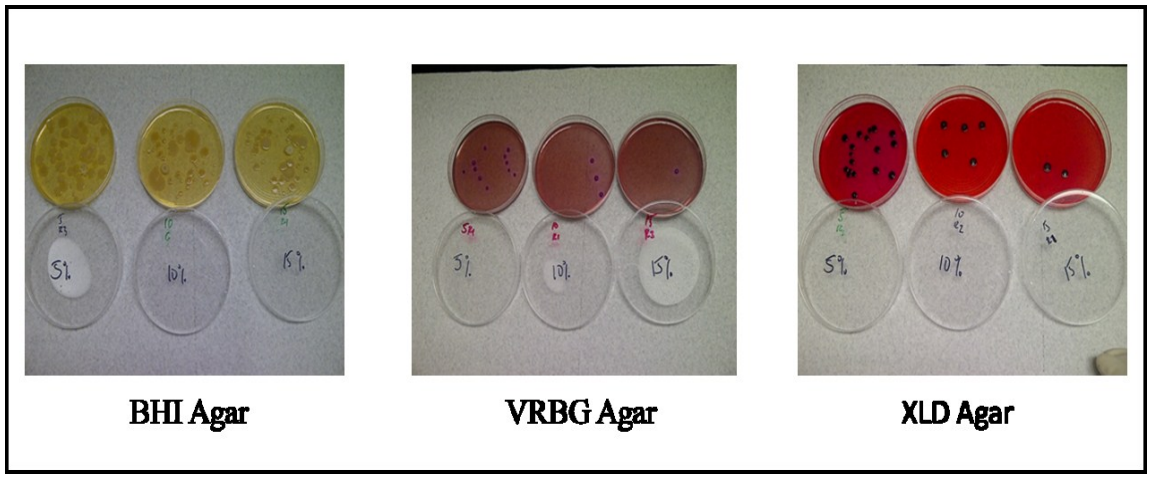


Figure IV - 10: Agar plates used to monitor *Salmonella* survival. The trend of decreasing CFU counts with increase in moisture content was consistent across all experiments irrespective of particle size

4.5 Discussion

This study showed that *Salmonella* survival in turkey manure dust was inversely proportional to particle size and moisture content, and was able to survive in dust particle sizes of 125 μm for up to 291 days. These results do indicate that the drier manure particles become, the more likely is the survival of *Salmonella* under ambient conditions. Manure dust particles with moisture levels of up to 10 – 15% appear to pose a lower contamination risk in terms of aerosol dispersal, as data from these experiments indicate that the amount of moisture in those particles did not foster prolonged survival of *Salmonella* cells beyond 88 days. A more apparent risk was suggested when there was

5% moisture in combination with the smallest particle sizes (125 μm) as evidenced by recovery of the pathogen up to 291 days post-inoculation.

Injury Effects

The assessment of microbial injury in this study confirmed the general knowledge that the use of non-selective media does help to recover stressed cells. For example, on days when viable counts were not recovered on XLD plates, suspect colonies were randomly picked off BHI agar plates, and streaked onto selective XLD media to check whether they were *Salmonella* that had managed to recover from injury. On some occasions, the colonies were found to indeed be *Salmonella*, on other occasions, this was not the case. A comprehensive assessment of injury rate of *Salmonella* based on the different moisture content or particle sizes was not possible due to the background microflora that always showed up on the non-selective BHI media. Generally, however, the observation of microbial injury in this study emphasizes the fact that the physiological state of a bacterium is an essential consideration when studying its response to mitigation strategies designed to combat its spread and survival in the environment.

Water Activity (a_w), Moisture Content and *Salmonella* Survival

After a critical look at the results obtained from experiments in this study, the question that naturally arose was: what makes *Salmonella* cells better able to survive at a lower moisture level of 5% compared to 10 or 15%?

It has been well-documented that *Salmonella* can survive for an extended period in dry materials with low water activities: 0.84 in controlled microcosms (frosted glass rectangles placed in closed chambers) *(35)*, 0.94 on modified a_w agar surfaces *(58)* and

0.75 in chicken manure *(46)*. Other factors such as pH, relative humidity, type of bacterial strain, temperature and time also played influential roles in survival in these conditions. Research has also shown that exposure to low a_w may change normal morphology and physiological behavior of microorganisms like *Salmonella (58)*. However, there seems to be little known about the survival response of *Salmonella* to extremely dry conditions of dehydrated particulate poultry manure (a_w as low as 0.40), or manure with as low as 5% moisture content. Largely, a_w was proportional to both moisture content and particle size in all experimental samples.

There is a marked difference between moisture content and a_w, although both are related to water content within a matrix. Organisms require an aqueous environment that includes "free" water which is not bound in complex structures and is needed for transfer of nutrients and toxic waste products. This 'free water' refers to the a_w or % equilibrium relative humidity (ERH) in the matrix being referred to. The ERH of a substance is the state at which the substance neither gains nor loses moisture. Water activity, in the present study, would mean the amount of water available to the *Salmonella* cells in the manure particles. Moisture content on the other hand, is described as the total amount of water, both bound and free that is available in a material. The relationship between moisture content and ERH is usually described by moisture sorption isotherms graphical representations displayed on a curve. It is possible that the larger dust particle sizes, with larger, crusty surface areas, collected more moisture (adsorption) during rehydration than the smaller dust particles, hence their higher water activity values.

Quantitative studies which have investigated the survival of *Salmonella* in poultry litter under different a_w levels, have indicated that increased levels of a_w in poultry litter were

associated with a higher recovery rate of *Salmonella (35)*. The complete opposite seems to be the case in the present study where *Salmonella* cells are presented in an environment with drastically reduced moisture levels within the same matrix – poultry litter. The major difference between both studies is therefore the water activity level. A study published in 2007 used predictive modeling to assess the growth and death kinetics of *Salmonella* in poultry litter as a function of pH and a_w at a constant temperature. The researchers document that lowering the a_w of poultry litter to ≤0.84 and pH to ≤4 is an effective way of reducing *Salmonella* populations *(75)*. Although, the present study did not vary the pH of dried manure, an opposite scenario to the modeling study described above seemed to play out as *Salmonella* survival increased with decreased a_w. Values between 0.84 - 0.96 is said to represent a common range of poultry litter a_w values from which positive *Salmonella* samples have previously been detected *(46, 73)*. Observations from another study which looked at the effect dry litter could have on *Salmonella* survival in poultry houses, did find that reduced a_w (<0.89) and moisture content (<35%) levels corresponded to reduced *Salmonella* populations *(34)* . While this makes sense at the higher end of the a_w spectrum, an alternative explanation might be required when extreme conditions are encountered. In the case of the present study, lowering moisture content of manure dust increased the chances of *Salmonella* surviving and eventually entering a viable but non-culturable state. Perhaps, when *Salmonella* cells encounter an environment which is far below optimal conditions of growth (optimal a_w is 0.99) *(58, 65)*, the cells begin to minimize their metabolic activity, conserve cell power and utilize one or more of known survival mechanisms including physico-chemical changes in the cell due to selective environmental pressure, entry into dormant state and

formation of protective biofilms *(35)*. At this point, *Salmonella* cells are not able to proliferate because they have moved from their 'growth phase' to survival mode. Viable *Salmonella* cells in this mode could be present at low numbers, but are still able to cause illness should they re-encounter favorable conditions for growth, colonize the right transmission vehicle, e.g. leafy greens, and survive until consumption. A possible explanation for this ability of *Salmonella* to adapt to dry conditions has also been offered by the 'filament formation' theory explained in the literature review section. Filament formation occurs when cells grow without dividing at a_w levels that are suboptimal for growth, and it is possible that *Salmonella* cells at an extremely low a_w level of 0.402 (lowest value measured from the current study) can utilize the filament formation mechanism to support survival. Mattick *et al (65)* also reported that survival of *Salmonella* at low a_w was strain dependent. Although a cocktail of three strains of *Salmonella* were used for inoculation in the present study, no attempt was made to differentiate the individual isolates in terms of survival. If the filament formation mechanism is dependent on RpoS, a stress sigma factor, one of the strains used in the study – the lab attenuated *S. enterica* LT-2 - is unlikely to utilize this mechanism because it is RpoS negative and may have lost a lot of its environmental fitness.

The observation that *Salmonella* cells died off faster at a moisture content of 15% compared to 5% might be explained in two ways. First, it is possible that *Salmonella* cells in moisture conditions slightly below the level permitting growth are able to continue metabolism, thereby producing substances which damage and probably destroy the cells *(46)*. A previous study had demonstrated that a_w slightly below the level required for growth have a pronounced killing effect on *Salmonella* in manure *(46)*. Secondly, the

"water replacement hypothesis" discussed under the literature review section could provide possible rationalization for an underlying survival mechanism.

Particle Size Effect

There were limited inferences that could be drawn from the particle size effect on survival. However, one plausible explanation is that *Salmonella* cells preferably attach to a smaller surface area that can afford them more protection. In other words, the cells are more exposed to ambient conditions when they are present on a matrix with a larger surface area, and are therefore more susceptible to those unfavorable conditions. An alternative reasoning is that the tiny manure particles provide less protection to the *Salmonella* cells, thereby increasing their resistance and nudging them towards earlier dormancy.

An in-depth analysis, such as predictive microbial modeling beyond the scope of this thesis work, might be required to investigate further reasons why *Salmonella* cells behave the way they do in manure dust of very low a_w and moisture content.

Chapter 5: Effect of UV Radiation on Survival of *Salmonella enterica* in Dried Manure (Dust)

5.1 Background

Based on the observation from previous experiments (that *Salmonella* survives in tiny, manure dust particles at lower moisture levels), manure dust with 5% moisture content was picked as the host material for studying UV effects on survival. The basic approach employed was to compare *in vitro* the survival of *Salmonella* in inoculated manure dust dispersed as a thin layer vs. exposure under similar conditions using a thin layer of cells that were directly applied to the test surface. Time (length of exposure) was used as the main variable. The UV inactivation trials were designed as a simple means for initially assessing whether the dried manure particles act as a barrier, protecting *Salmonella* from the damaging effects of UV light. It was important to first understand how these *Salmonella* cells are able to resist natural environmental stresses such as desiccation and solar UV radiation so that subsequent studies of the survival of *Salmonella* in the farm environment could be appropriately interpreted. To our knowledge, there has been no report of studies done on the effect of UV on survival of *Salmonella* in dried manure particles.

5.2 Study Objective

The objective of the second phase of this research study was to investigate the effect of UV radiation on the survival of *Salmonella* in dried manure particles.

5.3 Materials and Methods

Inocula Preparation & Inoculation Procedure

For this experiment, only manure dust particle sizes of 125 μm was used. Inoculum preparation for this study followed a modified procedure from the one described in phase 1 (chapter 4). Two sets of inocula had to be made for this experiment. On the day an experimental trial was scheduled to begin; the steps in Table V-1 were followed to obtain the two sets of inoculum.

Table V-1: Preparation steps for *Salmonella* inocula

Manure Dust Inoculum (category A)	Control Inoculum (category B)
• About 2 g of 5% moisture manure dust was weighed into a sterile container. • After combining all three strains of *Salmonella* into a single tube and pelleting by centrifugation (see chapter 4), the supernatant was decanted and discarded. Working under the biosafety hood, a sterile spatula was used to scoop out some of the resulting pellet, which was then stirred into the 2 g of dust in the sterile container. A few drops of food coloring was added to the pellet to help monitor even distribution, and the combination was mixed thoroughly. • The resulting dust/*Salmonella* cocktail mixture was spread onto a sterile Petri dish and dried under a fume hood for ca. 1 hour. This provided a sufficiently dry inoculum. • The dust/*Salmonella* mixture was combined with the pre-equilibrated 5 g of 5% moisture content manure dust and agitated thoroughly to ensure even distribution. • This produced an inoculum level of ca. $10^7 – 10^9$ CFU/g. (Aim was to obtain a high level of *Salmonella* in dust)	• After combining all three strains of *Salmonella* into a single tube and spinning it down in the centrifuge (as described above), the supernatant was decanted and discarded. • The resulting pellet was suspended in 5 ml of 0.1% peptone water; this provided a cocktail inoculum with a level of ca. $10^9 – 10^{10}$ CFU/ml. A few drops of food coloring were added to help monitor distribution of inoculum on filter paper (see below).

Procedure for UV Exposure

Four categories were created for ease of sampling:

- Category A – 0.1 g dust inoculum + Long Wave UV-A
- Category B – 0.1 ml liquid inoculum (positive control) + Long Wave UV-A
- Category C – 0.1 g dust inoculum; no UV
- Category D – 0.1 ml liquid inoculum; no UV

Seven petri dishes (category A) were labeled as 0 min (control), 2 min, 4 min, 8 min, 16 min, 24 min and 32 min to represent length of exposure. In a similar manner, another set of 7 Petri dishes were labeled for categories B, C and D. A handheld ultraviolet lamp was used as a UV source (Model UVGL – 58, Mineralight™, Upland, California) (Fig V-1). The UV lamp and petri plates were arranged inside a biosafety cabinet in order to contain the UV radiation. A 0.9 cm round Whatman® filter paper (size #42) was inserted into all the plates of each category (the lids of the plates were used as the base into which the filter paper was inserted). The size was selected to completely cover the bottom surface of the lid. This allowed uniform distribution of the inocula and minimized static.

Figure V - 1: Handheld ultraviolet lamp used as a UV source (Model UVGL-58)

For the manure dust samples, 0.1g of inoculated dust was weighed out and transferred to the filter paper in the category A petri dishes and spread so that a thin, evenly spread layer was obtained, thereby minimizing any shadowing effects. A small paint brush was used to distribute the dust across the surface of the filter paper, and the lids were put back on the plates and held at room temperature while awaiting treatment (Figure V-2). The manure-free control (liquid medium) samples were prepared by transferring 0.1 ml of the *Salmonella* cocktail directly onto the filter paper inside each of the petri dishes. An even spread of liquid inoculum was achieved across the central surface of the filter paper without the need for additional spreading. Again, the culture dishes were covered and held at room temperature (22°C) while awaiting treatment.

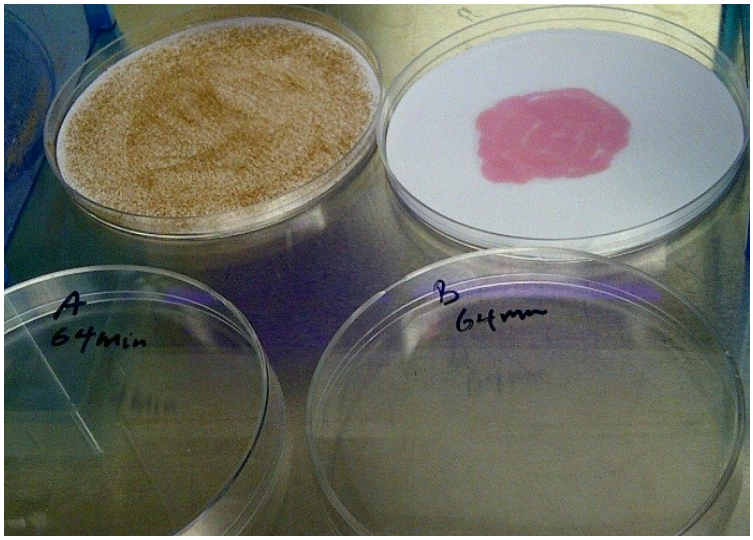


Figure V-2: Spread of *Salmonella* inocula in different matrices – manure dust and liquid - in petri dishes exposed to UV radiation

The UV lamp was mounted such that there was a measured distance of 10 cm between the light source and the petri dishes containing the samples. The wavelength band range of the UV lamp was 254 – 366 nm, which spans across all three subgroups of UV radiation. Out of the two settings – Long Wave (365 nm) and Short Wave (254 nm) – available on the lamp, the Long Wave setting was utilized because it had the most realistic comparison to plants' UV exposure under field conditions (Dr. Sullivan J. H., personal communication).

With a timer to monitor time elapsed, the petri dishes were placed one after the other under the UV source, and positioned for maximum exposure. The lamp was turned on 20 min prior to use to allow for stabilization of the UV output. To begin exposure, the petri dishes labeled 2 min from categories A and B were arranged under the UV source set-up in the biosafety cabinet. The petri dishes in categories C and D which received no UV exposure also remained inside the biosafety cabinet but with the lids in place. The UV lamp was switched on to begin UV exposure on Category A and B dishes after the covers

were removed. After two minutes elapsed, the lamp was turned off and sampling commenced immediately as follows:

- For categories A and C, sterile forceps were used to gently transfer the filter paper and its contents to a stomacher bag containing 25 ml of 0.1% peptone water. The filter paper + contents were then stomached for 1 min.
- For categories B and D, a sterile forceps was used to pick up and transfer the damp filter paper into a stomacher bag containing 25 ml of 0.1% peptone water.

The steps above were repeated for the 4, 8, 16, 24, 32, 48, 64 and 80 min exposure times sample plates in categories A to D. All samples were spiral-plated onto XLD and BHI agars in duplicate using appropriate dilutions.

Enumeration and Statistical Analysis

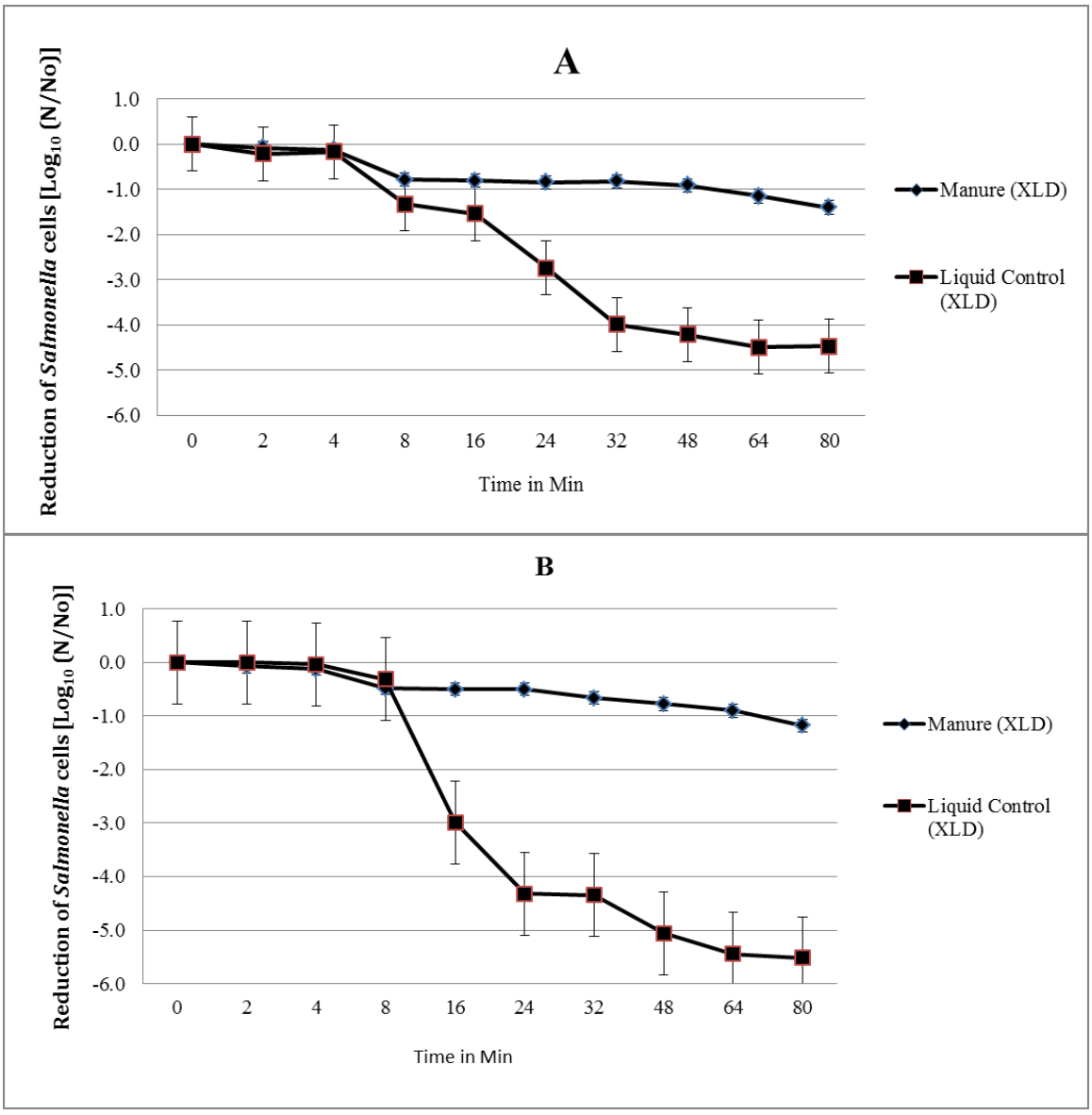
All plates were incubated at 35°C and plate counts were done at 24 h and 48 h using an automated colony counter. The whole experiment was replicated three different times. Population density averages in Log CFU/ml or CFU/g were taken and survival curves generated. Microbial counts were log transformed prior to analysis. Data were subjected to statistical analysis using the Statistical Analysis System (SAS Institute, Cary, N.C.) and analyzed as a two-factor (treatment and time) linear model using the PROC MIXED procedure. Each point value presented in the results section represents the mean of three values (three independent trials). Assumptions of normality and variance homogeneity of the linear model were checked, and variance heterogeneity was corrected using the

variance grouping technique *(74)*. Whenever effects were statistically significant, mean values were compared using Sidak adjusted p-values *(48)* to maintain experiment-wise error ≤0.05. A p-value of <0.05 was considered to be statistically significant.

5.4 Results

Comparisons of *Salmonella* Survival in Manure Dust vs. Control (Liquid) Exposed to UV over Time

The shapes of the survival curves for *Salmonella* in manure and in liquid media were consistent across all three experimental trials. The small inactivation observed in the manure dust samples is shown by the near-linear curve where one complete log decrease is not achieved until after 64 min (Fig V - 3), although further analysis reveals that the first significant ($P < 0.05$) inactivation was achieved after the first 4 min (Fig V - 5). The survival curve of the control samples showed a lag phase of approximately 8 min, followed by an exponential death phase that rapidly continued up until 64 min. After this, the inactivation rate decreased and seemed to even out between 64 and 80 min. Interestingly, the point at which the death phase of the control samples started to level off coincided with the point where manure dust samples began their exponential death phase. This observation is most evident in the first and third trials (Fig V - 3A & V - 3B).


A
Reduction of Salmonella cells [Log10 (N/No)]
1.0
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
-6.0
0
2
4
8
16
24
32
48
64
80
Time in Min
Manure (XLD)
Liquid Control (XLD)
B
Reduction of Salmonella cells [Log10 (N/No)]
1.0
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
-6.0
0
2
4
8
16
24
32
48
64
80
Time in Min
Manure (XLD)
Liquid Control (XLD)

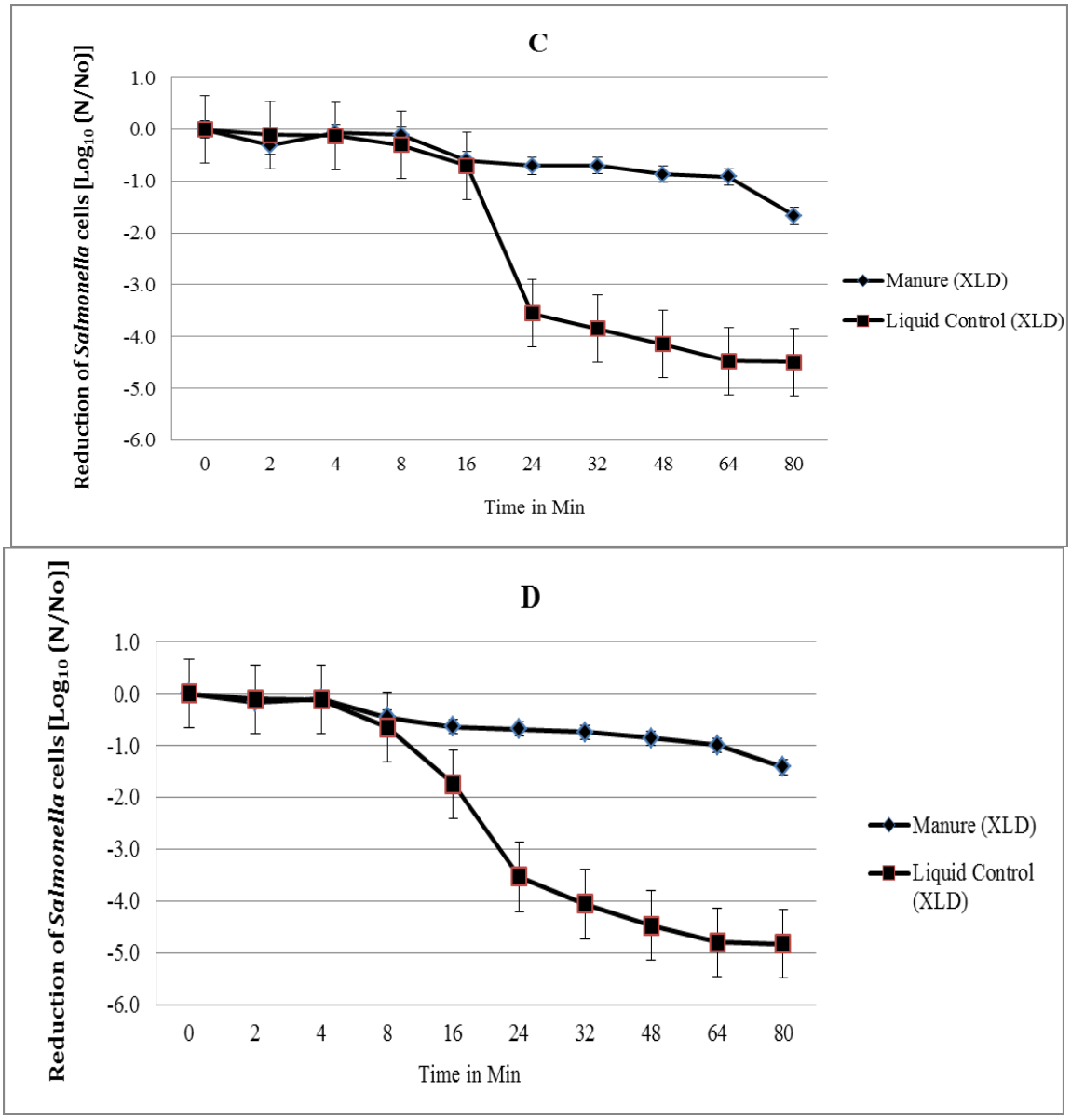


Figure V - 3: Comparison of *Salmonella* survival in manure dust vs. liquid media (control) after UV exposure. Colonies were recovered and counted on XLD agar. Graphs A, B, C show data from experiment trials 1, 2 and 3 respectively; graph D shows data of the mean of the three trials. Error bars display standard error. Data shown has been normalized.

The median survival of *Salmonella* in manure dust particles exposed to UV for 80 min (category A) was approximately 3.4 logs higher than the survival of *Salmonella* from liquid medium (category B): *Salmonella* population in category B declined by approximately 5 log, while the reduction in population in category A averaged out at ~1.5

log. This was in spite of the higher initial inoculum level of the liquid control samples (liquid inoculum of 7.5 x 10^{10} CFU/ml; dust inoculum of 6.75 x 10^{6} CFU/g). For the liquid control samples, a sharp decline in population density was constantly observed between 8 and 24 min, a trend that was not seen in the *Salmonella*-in-dust samples (Fig V - 3). For control samples which were not exposed to UV (categories C and D); there was no significant log reduction of *Salmonella*-in-dust inoculum (Category C) over 80 min (Table V - 2). However, *Salmonella*-in-liquid inoculum (Category D) had at least 1 log reduction over 80 min.

Table V - 2: Survival in UV vs. non-UV samples at selected times showing log reduction comparisons across categories. No significant log reduction in control samples not exposed to UV (Categories C and D)

	Categories			
Time (min)	A (UV, dust)	B (UV, liquid)	C (no UV, dust)	D (no UV, liquid)
0	0	0	0	0
24	0.72	3.54	0.08	0.67
48	0.85	4.47	0.29	0.77
80	1.42	4.93	0.31	1.03

Selective XLD agar was used alongside non-selective BHI agar to recover and plate samples. The difference in recovery rates between these two media helped detect the population of injured cells, and also distinguished between *Salmonella* and background microflora (mostly *Bacillus* spp.). Even after taking background microflora into account, the BHI agar counts had slightly higher CFU counts (0.6 – 1.0 log) for all samples in all categories tested; indicating that some *Salmonella* cells injured during exposure to UV were able to recover on the non-selective BHI media (Fig V - 4).

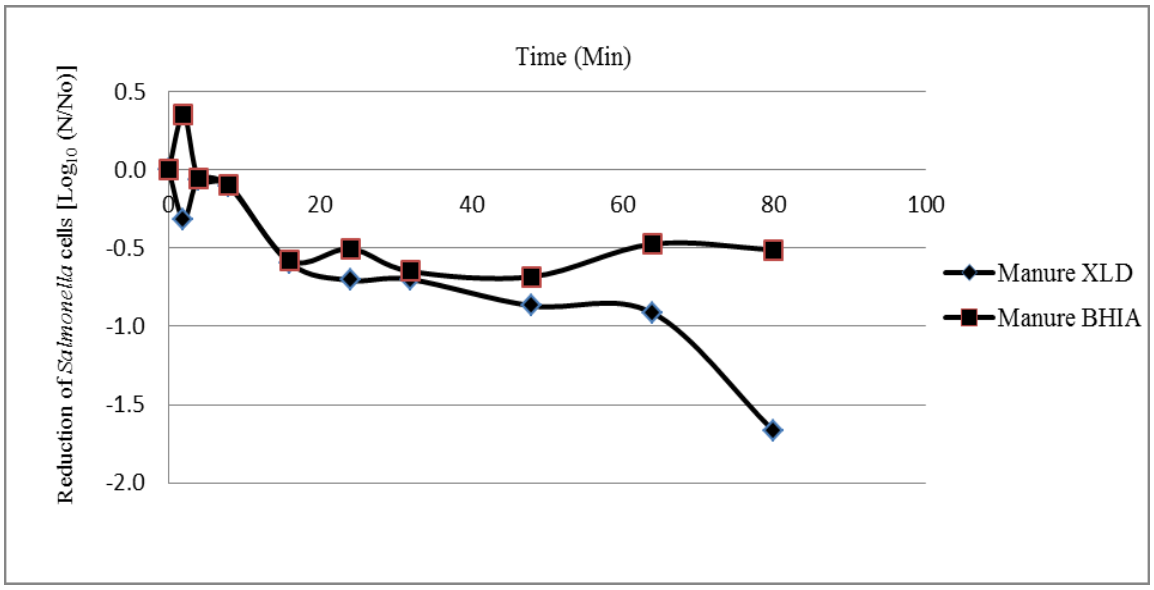


Figure V - 4: Injury effect on *Salmonella* cells shown by the different recovery rates on non-selective BHI media vs. selective XLD media. Graph displays normalized data from a representative sample of trials.

The main microflora which seemed to have survived the 80-min UV exposure was *Bacillus* spp., as presumptively identified by morphology on BHI agar plates and phase contrast microscopy.

Analysis of variance tests showed that differences in survival between *Salmonella*-in-dust inoculum and *Salmonella*-in-liquid inoculum only became significant ($P < 0.05$) after 24 min (Fig V – 5A), while the first significant ($P < 0.05$) decrease in population did not occur until after 48 min (Fig V - 5B).

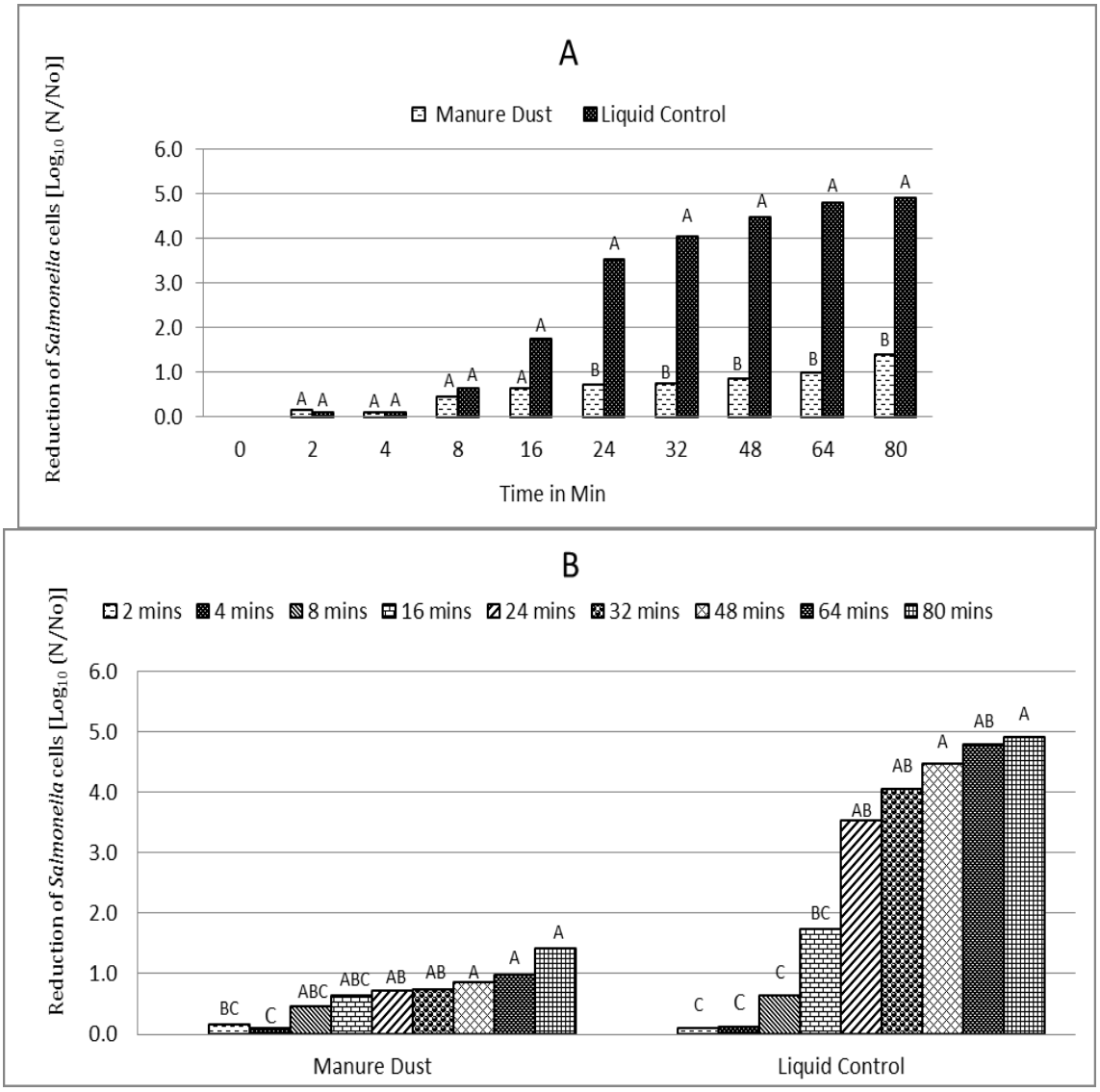


Figure V - 5: Comparison of *Salmonella* inactivation under UV in manure dust vs. liquid control categories. Graph A compares survival based on treatment within each time. Graph B compares length of survival time within each treatment. Within each category, different letters above bars indicate significant differences (P < 0.05) in mean populations. Data shown has been normalized.

5.5 Discussion

Analysis showed that the presence of manure particles significantly ($P < 0.05$) protected *Salmonella* from UV exposure. *Salmonella* cells exposed to UV in a liquid medium showed a ~5 log decline in 80 min compared to the ~1.5 log decrease of *Salmonella* cells

in the manure dust matrix (Fig V - 5). The difference in the inactivation rates between the manure dust negative control samples and the liquid negative control samples further establishes the resistance which *Salmonella* cells in the manure particles could have even in the absence of UV light (Table V – 2). The observation that the inactivation of *Salmonella* in the liquid control samples was particularly pronounced between 8 and 24 min (Fig V - 3), leaves no doubt that the direct exposure of the *Salmonella* cells to UV radiation had significant impact on their survival over time. Although, the reason for this pattern remains to be elucidated, it is possible that substantial damage to the DNA of the *Salmonella* cells occurred at some point between 8 and 24 min during exposure to UV under the conditions in which this experiment was carried out. And it appears that this damage does not occur in the manure dust samples until after 48 min as suggested by the shape of the survivor curve and statistical analysis (Fig V – 3 & V – 5B). Another interesting observation was that, even though there was a less than 2 log die-off in the *Salmonella*-in-dust samples over a total time of 80 min, the first significant ($P < 0.05$) decrease in population did not occur until after 48 min (Fig V - 5B). It is likely that, prior to this time, *Salmonella* cells were well-protected within the manure particle matrix, and were biologically able to overcome the stress caused by the UV rays. Stressed cells under both test conditions were able to recover from injury as demonstrated by the difference in inactivation rates between XLD and BHI media (Fig V - 4). This lends credence to the idea that injured microorganisms could present a potential threat since they may undergo reparation under suitable conditions.

UV Effects on Survival of Organisms on Plant Phyllosphere

Based mainly on biological effects, the daily influx of solar UV radiation (UVR) is classified into three subgroups: UV-A, consisting of light with a wavelength between 400 and 320 nm; UV-B, with wavelengths between 320 and 290 nm; and UV-C, with wavelengths between 290 and 200 nm *(66)* (Fig V – 6). Although slight variations in these three division points exist among other groups of researchers *(17)*, physicists and photo-biologists generally break the UV light spectrum into these three wavelength bands. The subgroup that is most inhibitory to organisms is the high-energy UV-B wavelengths because they can cause direct DNA damage by inciting formation of lesions in cellular DNA *(52)*. There is little or no practical biological significance for wavelengths below 180 nm since the atmosphere readily absorbs them *(17)*. Generally, the shorter the wavelength of radiation energy, the more biologically destructive the energy source can be.

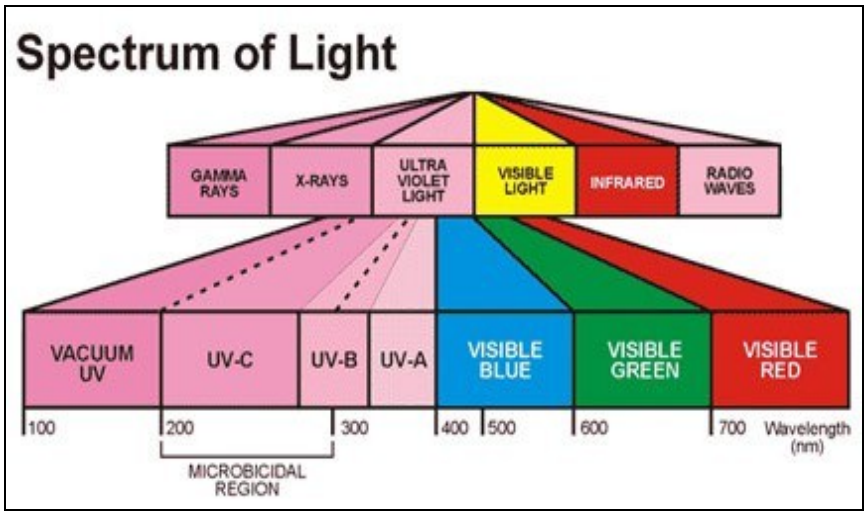


Figure V - 6: Microbiocidal region of UV radiation. Ultraviolet light (UV) is at the invisible, violet end of the light spectrum *(5)*

Studies have been done on the sensitivity of *Salmonella* to these UV wavelengths and their resistance to UV exposure *(11)*. Plant leaf surfaces, also known as phyllosphere generally support the growth of a diverse flora of bacteria and fungi. However, the ecological success of these microorganisms would depend on a number of factors, one of the most important being their ability to cope with exposure to solar UVR *(52)*. One of the ways organisms can avoid UV penetration in the phyllosphere is by colonization of sites protected from radiation such as the interior sites of plant leaves or the base of trichomes which are external, physically shaded locations *(52)*. Because UV light is generally non-penetrating in a dynamic air-stream, microorganisms beneath dust particles might not be affected by the UV irradiation *(17)*. This could help explain the minimal death of *Salmonella* cells in manure dust particles observed in the current study. Biological effects arising from UV radiation is said to vary with wavelength, exposure level, as well as duration of exposure *(17)*. Many studies have established that exposure of bacterial cells to UV radiation can induce direct DNA damage *(52)*, cause RNA and protein alterations, depolarize cell membrane as well increase the permeability of the membrane *(11, 18)*. The ability of the organism to efficiently repair DNA damage as it occurs could predict their ability to survive in the phyllosphere or in other matrices which are constantly exposed to UV radiation.

Comparability of UV Lamp and solar UV Normally Received by Plants

The UV light energy source used in this experiment – long wavelength of 365nm, favorably compares with the UV radiation plants receive from sunlight. This is because

the daily influx of solar UVR generally includes UV-A and UV-B wavelengths *(52)* (Fig V - 7).

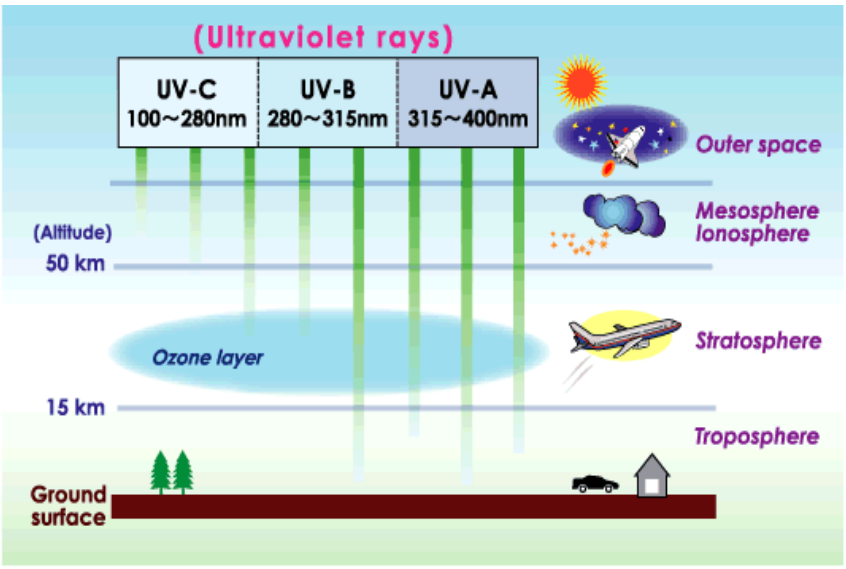
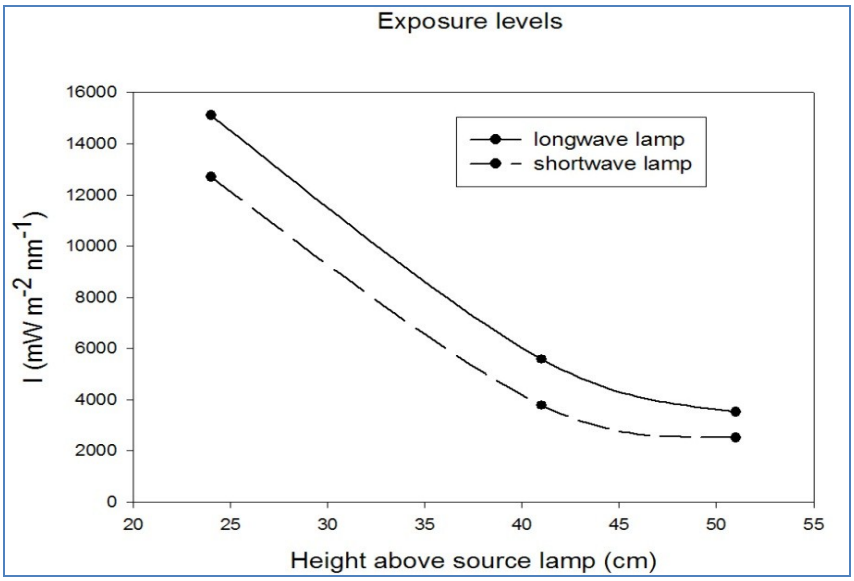


Figure V -7: Distribution of UV radiation to the earth *(6)*

A regular germicidal lamp like the one used in this experiment, is said to emit energy in an order of magnitude less than that of solar radiation *(18)*. For example, irradiance from a 15-Watt germicidal lamp emitting radiation at 254 nm is only 10^{-4} of the energy coming from the sun at midday *(18)*. In the present study, extrapolation from the graph in Fig. V - 8A approximates that the amount of UV energy emitted by the long wave lamp at 365 nm and received by the samples in the petri dishes is only about 15,000 mW per cm^2.

A.



B.

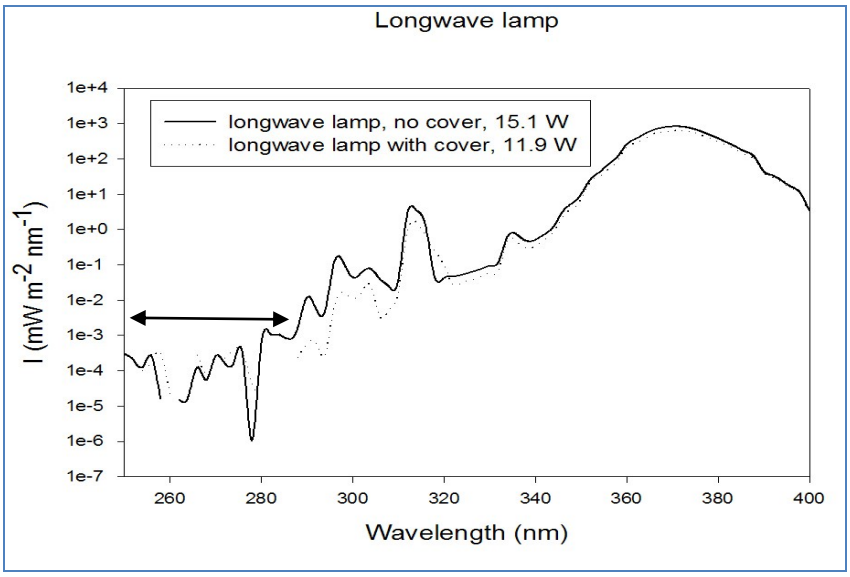


Figure V - 8 : UV lamp irradiance measurement graphs: Graph A measures exposure levels (UV intensity (x-axis) and distance of UV lamp from sample (y-axis)), while Graph B measures longwave lamp output (UV intensity (x-axis) and wavelength (y-axis)). Fig. B also shows the difference in the amount of UV radiation received by manure particles in petri dishes, with and without petri-dish cover in place. Arrow indicates that the shorter wave region which actually kills bacteria cells is mostly blocked by the petri-dish cover.

As shown in Fig (V - 8B), the shortwave region which actually destroys bacteria cells is mostly blocked by the petri-dish cover. This explains the observed difference between samples exposed to UV and the samples which remained covered in the petri dishes. Although, interpretation of results from this experiment is constrained by the inability of any UV lamp to precisely mimic the solar spectrum, unambiguous inferences can still be made based on the experimental design as well as observations from documented studies. NSF International, an independent organization that standardizes sanitation and food safety requirements conservatively established a UV dose of 40 mJ/cm^2 as the minimum UV dose required to inactivate most bacterial pathogens *(1)*. Given that the degree of UV inactivation of pathogens is directly proportional to the UV dose applied, it can be inferred that manure dust particles will provide protection to *Salmonella* cells present in the particles depending on the UV intensity and exposure time (UV dose is the product of UV light intensity and exposure time and is expressed in mJ/cm^2).

In making assessments of the impact of manure particles in the survival of *Salmonella*, it is also important to bear in mind that different climate regions receive exposure to UV radiation from the sun in varied amounts, mostly depending on seasonal conditions and changes. For example, the amount of sunlight received in Arizona, a dry climate with very little rainfall, would be considerably higher than the amount received in Alaska, a much colder and wetter region. Reports from previous studies indicate that the survival of *Salmonella* in animal manure applied to soil is dependent on climatic, and subsequently soil conditions *(76)*. This observation can be assumed to apply to the present study where *Salmonella* survival could depend on the host matrix (manure particles) and the length of

UV exposure. The possibility and extent of survival of *Salmonella* on the phyllosphere of plants exposed to UV is further explored in the final phase of this research (next chapter).

Chapter 6: Survival of *Salmonella* in Manure Dust on Baby-spinach Leaves (under greenhouse conditions)

6.1 Background

The third phase of this research project was an assessment of the survival of *Salmonella* in manure dust on spinach leaves. Evidence gathered from the two previous phases of this research shaped the design of the current study. It would have been ideal to do the trials in the field using airborne transmission of *Salmonella* contaminated manure dust as a vehicle. However, because of the need to ensure a high inoculum level to study survival and the difficulties in getting permission to do such research with a known human pathogen, it was decided to simulate field conditions using growth chambers. Manure dust (125 μm) with a moisture level of 5% was used in this study, based on the previous observations that the smallest and driest manure particles best supported *Salmonella* survival. The effect of UV light on the survival of *Salmonella* in manure dust particles was factored into this design of the experiment. Also, because survival of bacteria on plant surfaces has been observed to be variable and results in a heterogeneous distribution, with the abaxial side of the leaf harboring more organisms *(52)*, this variable was tested in the present study.

6.2 Study Objectives

This study had two primary objectives:

1. To evaluate the survival capabilities of *Salmonella* in dry manure particles (of a size capable of being airborne) on spinach leaves.

2. To investigate the effect of UV radiation on the survival of *Salmonella* in dried manure particles on spinach leaves.

6.3 Materials and Methods

Inoculum Preparation & Inoculation Procedure

125 μm manure dust particles which had been pre-hydrated to 5% moisture content were used throughout this study. Inoculum preparation followed the same procedure as described in phase 1, with the modifications described below.

Procedure for Manure Adaptation of *Salmonella* Strains

The *Salmonella* cells were adapted to manure dust by growing in manure dust slurry using a modified procedure originally described by Sharma *et al (86)*. This was done to create a potentially protective menstruum for the cells, thereby more closely simulating natural contamination conditions. A 1:5 manure dust: sterile water slurry was made in six 50 ml centrifuge tubes, and pellets were obtained by centrifugation (3, 000 × *g* for 10 min at 5°C). The supernatant was then harvested and the three strains of *Salmonella* used in this study were separately inoculated into 10 ml of the manure dust supernatant. Incubation with shaking (37°C for 48 h at 120 rpm) followed, after which the cultures were centrifuged at 3, 000 × *g* for 10 min at 5°C. The resulting pellets of each *Salmonella* strain were washed three times and re-suspended in 5 ml of sterile 0.1% peptone water. Equal volume of each culture strain was then combined and re-centrifuged to produce a three-strain cocktail pellet. The initial *Salmonella* concentration in both the manure and liquid inocula was determined by plating appropriate dilutions in duplicate on XLD and BHIA agar, and averaged at 6.07×10^7 CFU/g and 2.51×10^9 CFU/ml respectively.

Preparation of Manure Dust Inoculum

In order to minimize addition of moisture into the manure dust which had been pre-hydrated to 5% moisture content, ca. 2 g of manure dust was weighed into a sterile container and inoculated with the *Salmonella* cocktail pellet. A few drops of food coloring was added to the pellet to help monitor even distribution within the manure dust. The resulting dust/*Salmonella* cocktail mixture was spread onto a sterile Petri dish and dried under a fume hood for 1 h. This provided a dry enough bacterial cocktail inoculum which was then combined with the pre-equilibrated 5 g of 5% moisture content manure dust and agitated thoroughly to ensure even distribution. For inoculation of control samples, sterilized distilled water was used to re-suspend and dilute the cocktail culture, and therefore served as the delivery vehicle to the spinach plants.

Spinach Cultivation: Seeds of the hybrid semi-savoy spinach (*Spinacia oleracea*), Santorini (Siegers Seed Co., Holland, MI) were germinated and grown in commercial soil (Sunshine Mix LC-1, Sun Gro Horticulture, Canada) at the Research Greenhouse Complex at the University of Maryland, College Park. After 21 days of growth, the spinach plants were transferred to a controlled environment growth chamber (CMP 4030, Conviron, Winnipeg, Manitoba, Canada) located at a USDA facility where inoculation was later carried out. The conditions of the growth chamber were: 70 to 72% relative humidity, light intensity of 1.5×10^{-1} microeinsteins/m2/s (14 h light, 10 h dark), and temperature of 25 ±1°C. Throughout the duration of the experiment, the plants were watered regularly (at least once a week) and fertilizer (Jack's Classic All Purpose 20–20–20 fertilizer - J.R. Peters, Inc., Allentown, PA) was applied as needed.

Spinach Leaf Inoculation and Sampling: For ease of sampling, all plants were grouped into five categories and each group was inoculated as shown below. Spinach leaves were inoculated 21 days post-planting when the leaves were relatively large enough to obtain sufficient amounts of sample. All inoculation procedures were done under a fume hood in order to contain the dust aerosol.

Category A

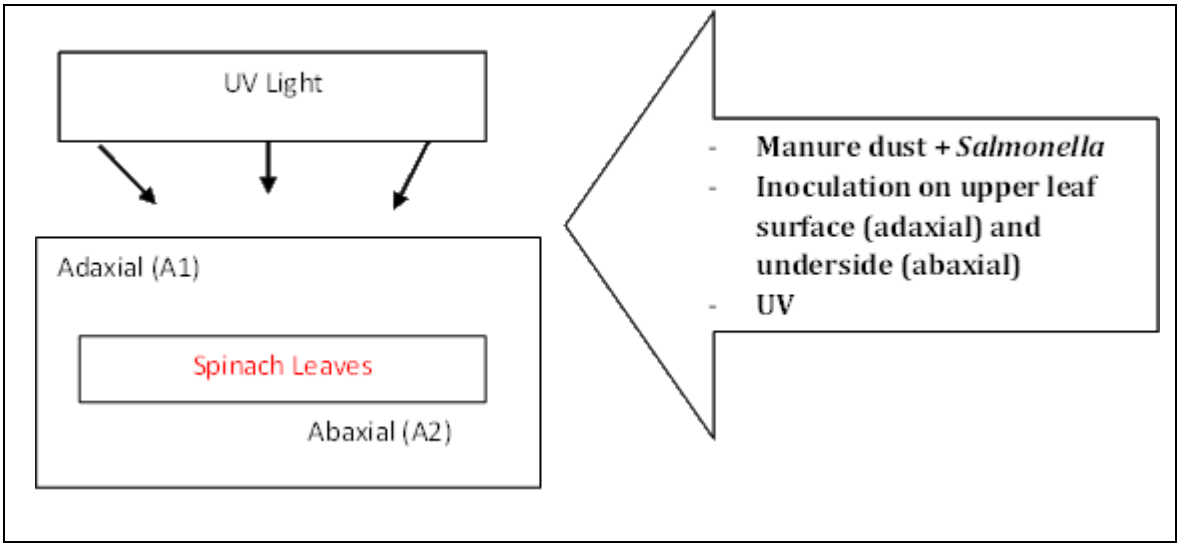


Figure VI - 1 : Diagrammatic representation of inoculation set-up for sample category A: spinach plants grown under UV spectrum

Procedure for inoculating Category A**:** Category A1 leaves were inoculated using a small paint brush dipped into the inoculated manure dust and gently moved back and forth across the upper side of each leaf on each plant (4 - 5 leaves per plant). The sweeping motion was repeated across each leaf until the entire surface was covered with a thin layer of dust. The undersides of category A2 spinach leaves were also thinly coated with inoculated manure dust in a similar manner.

Category B

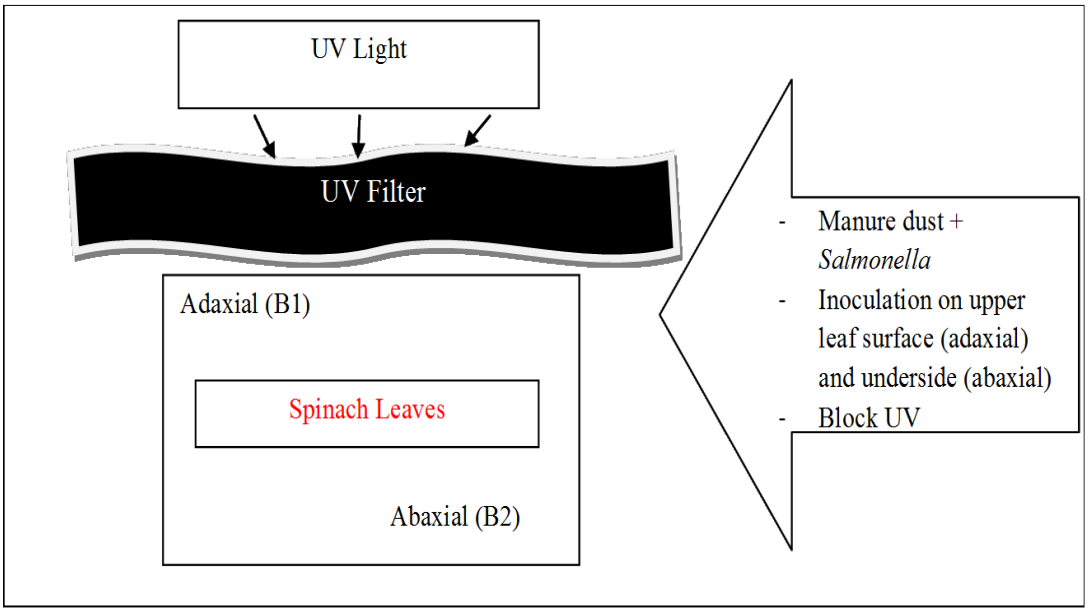


Figure VI - 2: Diagrammatic representation of inoculation set-up for sample category B: plants grown With UV Light Blocked

Procedure for Inoculating Category B**:** both sides of leaves were inoculated as described in Category A above.

Category C

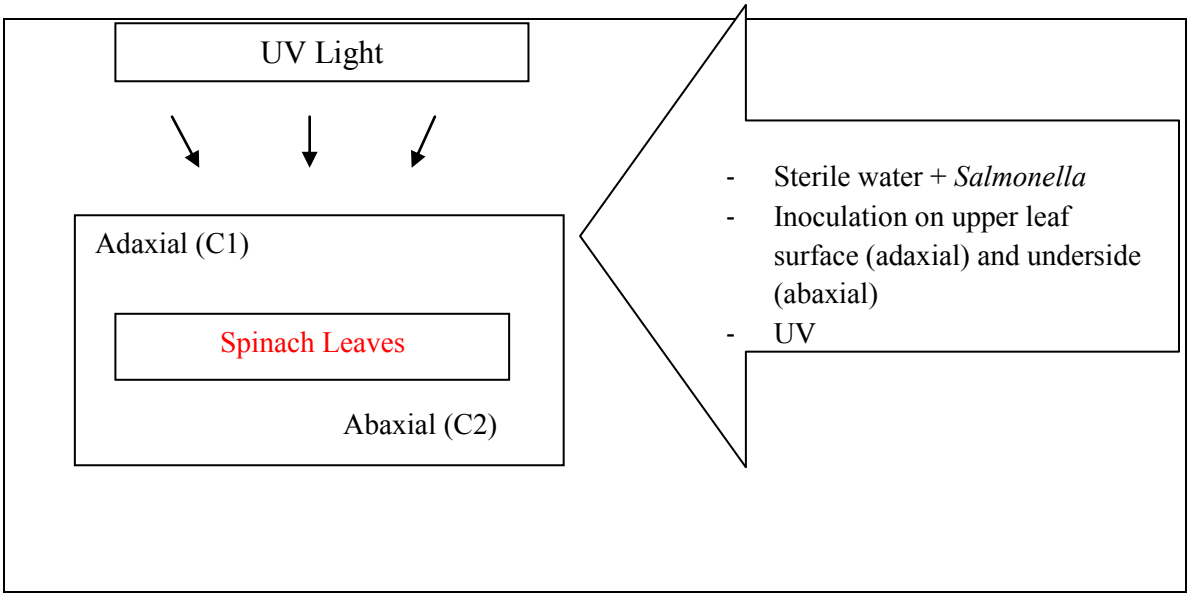


Figure VI - 3 : Diagrammatic representation of inoculation set-up for sample category C: positive control samples

Procedure for Inoculating Category C**:** Spinach leaves in category C1 were inoculated by spraying individual leaves with a fine mist of the *Salmonella* inoculum. This process was repeated for the abaxial side (category C2) as well, and leaves were left to air dry for about 1hr before sampling commenced.

Category D

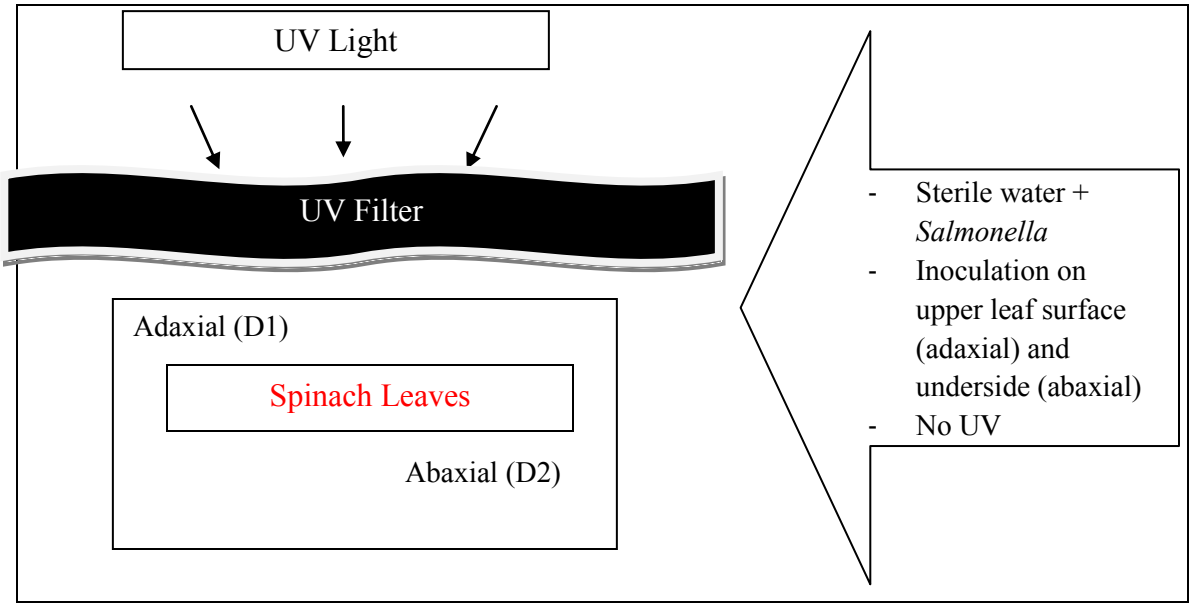


Figure VI - 4: Diagrammatic representation of inoculation set-up for sample category D: positive control samples with UV light blocked

Category E

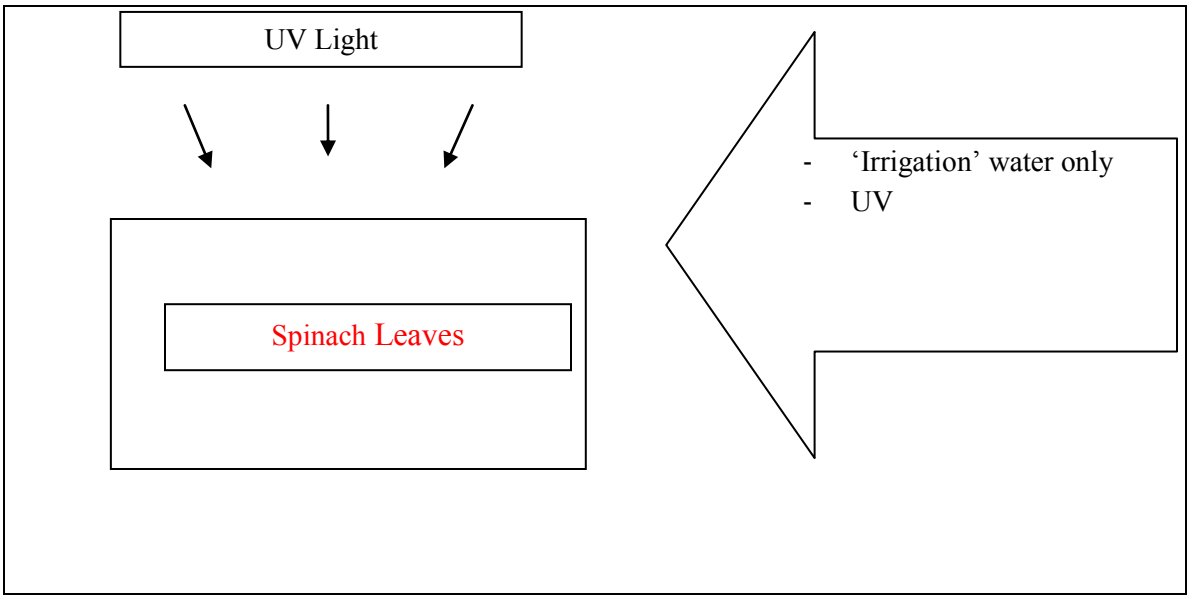


Figure VI - 5: Diagrammatic representation of inoculation set-up for sample category E: negative control samples

A UV filter sheet (UV Process Supply Inc., Chicago, IL) was placed over the rack where plants in categories B and D were growing.

Sampling of Spinach Leaves: At designated sampling times of 0, 1, 3, 5, 7, 14 and 21 days post-inoculation, plants were harvested in triplicate from each category using sterile methods: three plants from each category were randomly picked, and only the upper portion of each plant which bears the leaves (shoot) was clipped off directly into a sterile stomacher bag using sterile scissors. The scissors was sterilized with 80% alcohol in between every sample collection. All samples were transported to the lab on ice and processed within 24 h. Twenty five ml of sterile 0.1% peptone water was measured into each of the stomacher bags and each sample was homogenized by stomaching for 1 min (Seward Stomacher Model 400C, Thermo Fisher Scientific). Appropriate dilutions of the homogenate solution of each sample were spiral plated in duplicates onto XLD and BHI agar plates. Plates were incubated at 35°C for 48 h and enumerated using an automated colony counter.

Enrichment procedures: Samples were subjected to an enrichment protocol to determine the presence or absence of *Salmonella* when initial values fell below the detection limit (see chapter 5).

Statistical Analysis

Population density averages were measured in terms of Log CFU/shoot and survival curves were generated. Microbial counts from two separate experiment trials were log transformed [Log_{10} (N/N_o)] prior to analysis. Data were subjected to statistical analysis using the Statistical Analysis System (SAS Institute, Cary, N.C.) and analyzed as a two-factor (treatment and time) linear model using the PROC MIXED procedure. Assumptions of normality and variance homogeneity of the linear model were checked, and variance heterogeneity was corrected using the variance grouping technique *(74)*. Whenever effects were statistically significant, mean values were compared using Sidak adjusted p-values *(48)* to maintain experiment-wise error ≤ 0.05. A p-value of < 0.05 was considered to be statistically significant.

6.4 Results

Site of Deposition on Leaves: Effects on Survival

Linear regression trendlines show that *Salmonella* survived preferentially ($P < 0.05$) on the abaxial (underside) surface of spinach leaves, irrespective of the mode of inoculation (manure dust or liquid). Initial inoculum levels of *Salmonella* on both the adaxial and abaxial surfaces of spinach leaves with the manure dust treatment were about the same (3.5 to 4 log CFU/shoot). However, survival on the abaxial leaf surface was significantly ($P < 0.05$) higher for the manure dust samples (Fig. VI – 6). By day 14 post-inoculation, *Salmonella* cell numbers were significantly lower on leaves dusted on the adaxial side than on leaves dusted on the abaxial side (33% versus 0% viable colonies recovery on XLD respectively), although enrichment indicated *Salmonella* was still present on leaves irrespective of location (raw data in Appendix V). Thus, the influence of deposition site of manure dust contaminated with *Salmonella* on the spinach leaves became more evident with time.

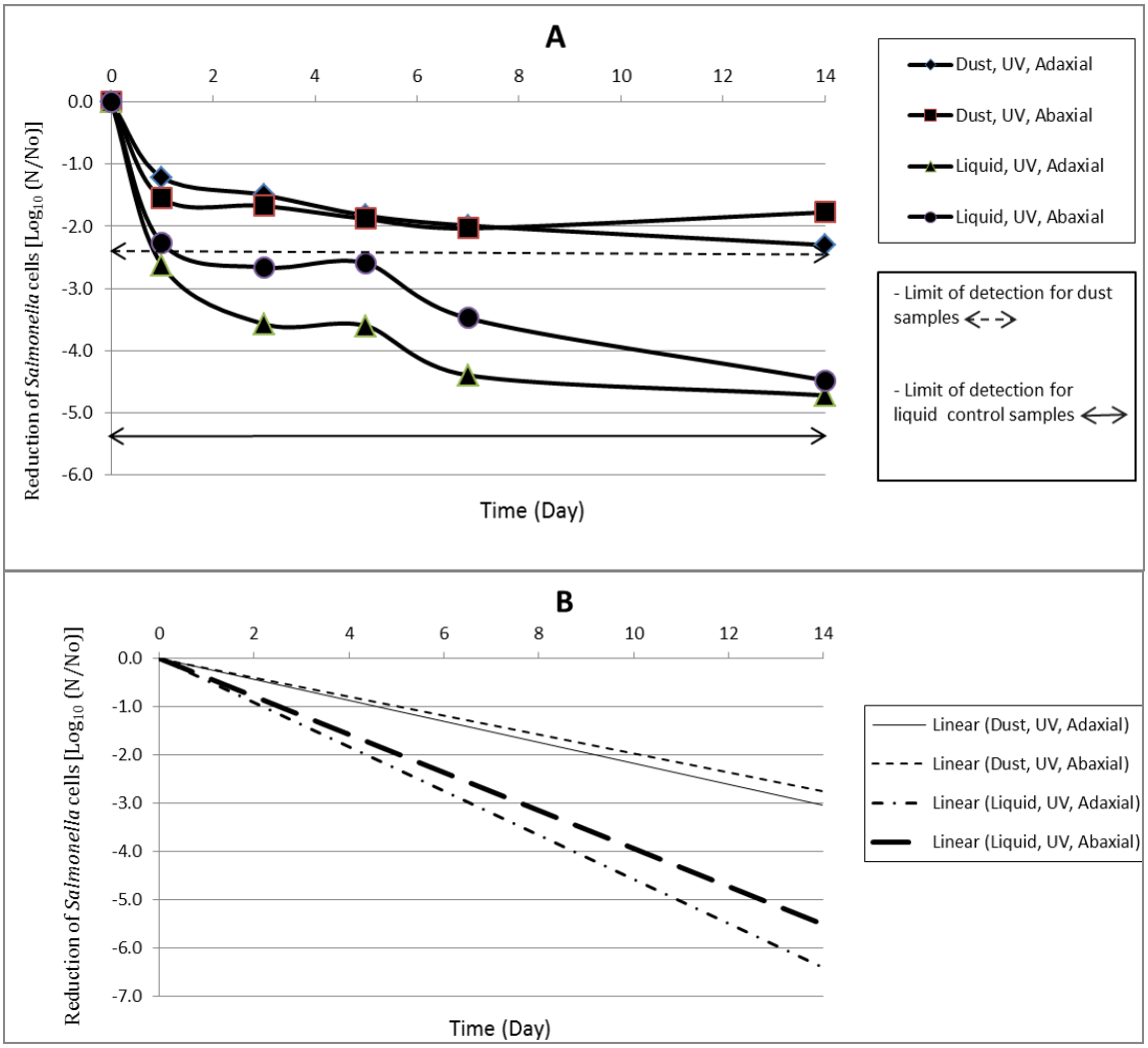


Figure VI - 6: Comparison of *Salmonella* survival on spinach leaves based on site of deposition. Graph A shows the survival of *Salmonella* on the adaxial and abaxial leaf surfaces grown under UV. Two separate limits of detection are shown only to reflect the difference in initial inoculum levels between the dust and liquid control samples. Graph B shows the linear regression of the same data set as Graph A. Data shown has been normalized.

UV Effects on Survival

The adverse effect of UV on survival of *Salmonella* was lowest on the adaxial surface of the spinach leaves inoculated with manure dust, as compared to the liquid control samples in the same category (Fig VI - 7). *Salmonella* levels, although higher in the liquid control samples, had a faster inactivation rate when compared to the manure dust samples.

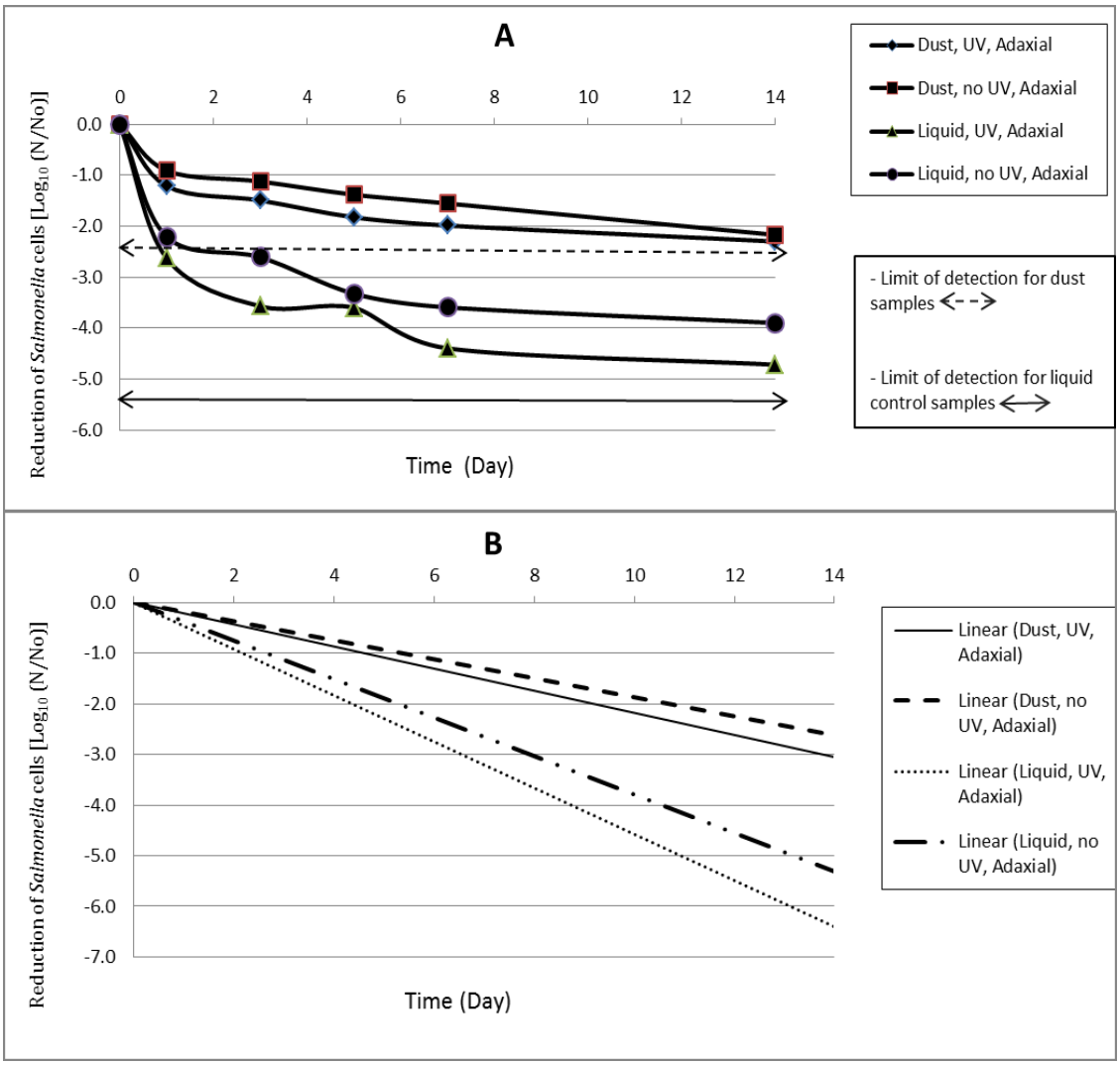


Figure VI - 7 : Comparison of *Salmonella* survival on spinach leaves based on UV effects. Graph A shows the survival of *Salmonella* on the adaxial leaf surfaces in the manure dust treatment and liquid control samples, with and without UV effects. Two separate limits of detection are shown only to reflect the difference in initial inoculum levels between the dust and liquid control samples. Graph B shows the linear regression lines of the same data set as Graph A. Data shown has been normalized.

By day 7, *Salmonella* cells in manure dust on spinach leaves had achieved a 2 log reduction (Table VI - 1), while the comparable control had declined in *Salmonella* population by approximately 5 logs.

Table (VI - 1): Average Log Reduction of *Salmonella* cells in [Log_{10} (N/N_o)] across all sampling categories. Each data point is a mean of two separate trials (3 replicates per trial). ND indicates *Salmonella* cells are non-detectable by plating.

Average Log Reductions of *Salmonella* [Log_{10} (N/N_o)]									
	Sample Code	**A1**	**A2**	**B1**	**B2**	**C1**	**C2**	**D1**	**D2**
Day	**Parameters**	**Adaxial, UV, Dust**	**Abaxial, UV, Dust**	**Adaxial, no UV, Dust**	**Abaxial, no UV, Dust**	**Adaxial, UV, liquid**	**Abaxial, UV, liquid**	**Adaxial, no UV, liquid**	**Abaxial, no UV, liquid**
0		0	0	0	0	0	0	0	0
1		1.21	1.55	0.91	1.00	2.63	2.27	2.21	1.33
3		1.50	1.67	1.12	1.15	3.57	2.66	2.61	2.65
5		1.83	1.88	1.38	0.97	3.60	2.59	3.32	3.35
7		1.99	2.03	1.55	1.64	4.39	3.48	3.59	3.12
14		ND	1.77	2.17	1.48	4.72	4.47	3.90	3.50

The effect of UV on the survival of *Salmonella* in the manure dust particles on the spinach leaves became more prominent after two weeks post-inoculation. By day 14, 50% of adaxial spinach samples not exposed to UV tested positive (viable counts) for *Salmonella*, while none of the adaxial spinach samples exposed to UV recovered viable counts, although all were positive via enrichment. See Appendix V for details on enrichment results.

6.5 Discussion

This study demonstrates that *Salmonella* has a slower rate of inactivation on the leaves of spinach plants when present in a manure dust particulate medium than when introduced to leaves via a liquid medium. The influence of the site of deposition of *Salmonella* on spinach leaves also played a significant role in survival: in both the manure dust treatment and controls used in this experiment, *Salmonella* survived preferentially ($P < 0.05$) and longer on the abaxial (underside) surface of spinach leaves than it did on the adaxial surface, although the manure dust treatment has a greater impact. These results are in agreement with studies which have investigated deposition site as an influential factor in pathogen survival both on the field and in environmental chambers *(33, 105)*. Investigations into the effect of UVR on the phyllosphere bacterial community of leaves (field-grown peanut plants, spinach and lettuce) *(33, 52)* also agree that preferential colonization of the abaxial leaf surface was an important survival strategy of pathogens.

Linear regression lines show that both UV effects and leaf deposition sites are important factors that enhanced the greater survival of *Salmonella* in the manure dust. In Figure (VI – 6) which compared survival based on site of deposition on leaves, *Salmonella* in dust present on the abaxial side of the leaves had the least inactivation rate. A similar trend was observed for the controls. However, when comparisons were made based on UV effect on survival, *Salmonella* in manure dust residing on the adaxial part of the leaves and not exposed to UV, had the slowest inactivation rate. Reconciling this observation with the UV studies from phase 2 suggests that the susceptibility of *Salmonella* to UV could be moderated by the part of the leaf which it colonizes. Zone of contamination on

leaf did not seem to play a significant role in spinach plants not exposed to UV. Therefore, UV effects were not analyzed in plants inoculated on the abaxial side and blocked from UV.

Previous studies have shown that *Salmonella* cells present in cattle manure were undetectable after 19 days at 37°C *(46)*, but survived for up to 231 days when contaminated manure was applied to soil *(51)*. Comparing this observation to the present study would suggest that it is easier for *Salmonella* to survive within a soil-manure matrix than in a manure matrix alone. In the current study however, *Salmonella* did survive for up till 21 days (detected by enrichment) on 75% of all spinach leaf samples tested. Initial *Salmonella* contamination levels on spinach leaves seemed to play a role in survival. In the study done by Islam *et al* mentioned above, initial bacterial inoculation dose played significant role in length of *Salmonella* survival. Other similar studies also observed that higher levels of contamination generally result in longer survival *(9, 33)*. This observation provides support for the occurrence in the current study where higher inoculum levels of *Salmonella* in liquid controls had longer survival of viable *Salmonella* cells (see Appendix V for raw data) compared to manure dust inoculum. Although, the initial manure dust inoculum level on the spinach leaves was comparably low – 10^4 CFU/g, it was apparent that *Salmonella* cells died off faster when present on spinach leaves in the liquid medium controls (initial levels of ~10^7 CFU/g) than when present in manure dust. It is worth noting that the relatively small spinach leaves used in this study did not allow for very much manure dust to settle on the leaves. The amount of dust particles that adhered to the leaves (4 - 5) of one spinach plant at any point in time ranged between 0.1 to 0.6 g. Perhaps, the results would have been a little different if a large-leaf

spinach variety was used, i.e., higher *Salmonella* levels might have supported survival at levels similar to controls. It is also possible that *Salmonella* would not have been detected in liquid control samples if initial cell numbers had been lower or at least, comparable to that of the test group (manure dust).

6.6 Discussion of Integrated Results from Phases 1, 2 and 3

Observation from UV effects on survival in the spinach inoculation study matches up with results from experiments done in phase 2 of this research. Faster inactivation of *Salmonella* cells exposed to UV in liquid medium versus manure dust medium was consistent in both studies. However, survival of *Salmonella* in manure dust on spinach leaves did not correlate with the survival pattern observed in the first phase of this research. The length of survival of viable and culturable *Salmonella* in the manure dust on spinach leaves was less than that observed in manure dust alone. By day 14 of the spinach inoculation experiments, viable counts were recovered from only 50% of spinach samples not exposed to UV light. Comparably, viable counts were still being recovered 28 days post inoculation in all samples of the 5%, 125µm manure dust. Considering the conditions *Salmonella* in each phase was subjected to, these differences are not totally unexpected, and several factors could have been responsible for this. In phase 1, manure dust was inoculated and stored away from harsh light at ambient temperature, in order to evaluate the moisture content effect on survival. Once these *Salmonella* cells were subjected to environmental influences, their survival rate noticeably decreased. One plausible explanation is that bacteria cells residing in microcosms protected from harsh environmental conditions have a better chance of survival than unexposed cells *(69)*. Moreso, phase one cells entered dormant state after exposure to desiccation stress, while

phase 3 cells were physiologically active. In phase 3 experiments, *Salmonella* cells seemed to encounter more physiological stresses besides UV radiation. This observation is in agreement with opinions from literature that shorter survival times of pathogenic microorganisms on crops (versus those for water or soil) reflect an increased exposure to sunlight and desiccation for these pathogens on crop surfaces *(90)*. Also, pathogens are known to have to put up with competition from indigenous bacteria on plant phyllosphere *(23)*, and this ultimately affects their chances of survival. Several possible mechanisms that could enable *Salmonella* to tolerate low moisture levels, low water activity and UV exposure have been expounded in the literature review section of this thesis.

It remains to be seen whether this pattern of survival translates unto field-grown spinach where plants are exposed to more complex environmental factors. Research has shown that pathogens applied to soils, compost or manure slurry survives for longer periods than those applied directly to plants via irrigation water *(9, 51)*.

The 'liquid medium' used as control in this experiment could serve as a good prototype for contaminated irrigation water used on leafy green fields. During the evaluation period, the reduction in *Salmonella* cell numbers in the manure dust medium, compared to the contaminated irrigation water prototype, highlights the importance of time lapse in evaluating contamination risks. If *Salmonella*-contaminated manure dust particles from a poultry facility are dispersed to nearby produce (e.g. leafy green) fields a few days before harvest, the risks of contamination could become rather significant. This is because *Salmonella* could survive in significant numbers for up to 14 days after initial contamination occurs. Also, low contamination levels do not preclude the possibility that

these dormant *Salmonella* cells can proliferate once optimal conditions are met on, either during or after harvest.

A lot of consideration has been given to irrigation water, soil, insects and wild animals as dissemination vehicles of pathogens in agricultural fields. This study brings to light an additional vehicle of transmission which could potentially exist in farm settings, especially in areas where poultry facilities are interspersed with produce fields. This study has also provided a good background which can be used in further research about the role manure particles play in the sustained contamination of leafy greens.

Chapter 7: Summary, Conclusions and Recommendations for Future Research

7.1 General Findings of Study

In summary, findings from this research project demonstrated that dried manure dust particles can act as a potential vehicle for *Salmonella* contamination of spinach leaves. Influencing this pathogen's survival are the moisture content and particle size of the manure dust, the UV exposure received by the leaf, as well as the site of deposition of *Salmonella* cells on the leaves.

7.2 Conclusions

- Manure dust particles at low moisture levels of 5% could act as a protective matrix to *Salmonella* cells. This ability dwindles significantly when moisture levels go up to 15%.
- Low moisture (5%) manure dust particles, especially when of a size capable of being airborne (125 μm), can support the survival of *Salmonella* for as long as 291 days under ambient conditions.
- Under UV light, particulate manure dust can reduce the inactivation rate of *Salmonella* on spinach leaves.

This research supports the hypothesis that the dust generated from poultry manure can cause significant contamination risks in agricultural settings, especially in arid areas. This

information could act as a basis for future experiments which could investigate in greater depth, the role of manure dust particles in pre-harvest settings. This is the first study to demonstrate that manure dust particles can significantly extend the survival of *Salmonella* cells exposed to UV light under ambient conditions.

7.3 Significance and Impact of Study

Largely, the trends observed from the first two phases of this study seemed to integrate well into the final phase, all of which can be incorporated into a final conclusion. If the manure dust matrix can shield *Salmonella* from UV light and its damaging effects, then the chance of survival of this pathogen increases. Ultimately, the risk of subsequent handling processes and eventual consumption also increases. For example, in cases where freshly harvested (and possibly dust-coated) vegetables do not undergo rigorous washing before consumption, contamination risk can be transmitted from the farm to the final consumer. This is because in such cases, the use of chlorine and other disinfectants which may have helped mitigate bacteria contamination levels on the vegetable leaves is generally absent. A 'Farmer's Market featuring produce sold directly by farmers to consumers is an example of this scenario. Based on reviews from literature which had suggested internalization of bacteria into leave tissues, it is possible that, even when proper washing procedures are carried out, a significant risk still exists for the end user who consumes these items without cooking. There is also the risk that dormant *Salmonella* cells present in manure dust could re-contaminate the environment once conditions become conducive: e.g. optimal relative humidity or water activity levels.

One of the objectives of this study was to investigate the risk which unintended aerosolization of manure particles could present to leafy greens on produce fields. If manure dust particles contaminated with *Salmonella* were dispersed to produce (e.g. leafy green) fields a few days before harvest, the risks of contamination become quite significant. Furthermore, low contamination levels do not preclude the possibility that these dormant *Salmonella* cells can proliferate once optimal conditions are met either during or after harvest. Regions such as Arizona or Texas where weather conditions are generally dry for the greater part of the year might pay more attention to this mode of contamination.

7.4 Recommendations for future research

Many gaps still exist in investigating the role particulate manure dust could play in pre-harvest settings as an aerosol contaminant. Because this is a first study of its kind, several areas for further research have been identified.

- First, future work specifically designed to simulate airborne transmission of *Salmonella* to growing spinach leaves using contaminated manure dust as a vehicle should be carried out so that the interactive effects of other environmental factors such as relative humidity and temperature could be properly evaluated over time. Inferences that were drawn from this research project are limited to laboratory as well as environmental growth chamber conditions. The likelihood that the incidence of *Salmonella* in real manure dust would be associated with an aerosol event, as well as the duration of survival after the event, should be explored.

- Secondly, field studies in areas where wind activities are significant could be carried out in order to evaluate the effect, if any, of wind speed in contamination. Since a number of studies have demonstrated internalization of bacteria into plant tissues, with a few suggesting internalization via leaf stomata, it would be beneficial to investigate the possibility that small manure particles can prolong the survival of pathogens on leaves, and consequently, encourage internalization into the tissue of spinach plants via stomata or trichomes. It would also be helpful to find out if dormant *Salmonella* cells in desiccated manure are capable of sustained attachment to leaves.
- In terms of epidemiology and public health, it would be useful to investigate which of the *Salmonella* strains used in this study demonstrated the greatest resistance to low moisture levels, UV stress and/or competition with leaf indigenous microflora. Is survival of *Salmonella* in manure dust matrix strain-dependent or specific to particular geographical locations? Does *Salmonella* Enteritidis or other serotype commonly found associated with poultry, for example, have an edge in UV resistance over the other serotypes? Are there any specific adaptive mechanisms these strains use to overcome low moisture conditions? Further study involving molecular microbiology and genetics would provide useful insights into these inquiries.
- For comparison sake, it might be worthwhile to find out if dust particles generated from other types of farmyard animal manure have similar survival patterns as turkey manure dust. Also, inquiries could be made into whether or not other pathogens associated with poultry manure e.g., *Campylobacter* behave in a

similar manner to *Salmonella* under similar settings. Investigations in this regard might also provide an insight into the factors which would allow *Salmonella* to be present in poultry manure and bedding mixtures.

- Electron microscope studies would help determine if *Salmonella* adheres only to the surface or can be found in the interior of the dust particles. In other words, do the dust particles actually encase the *Salmonella* cells?
- Finally, in order to further assess the potential of manure dust particles as a pre-harvest contamination risk of general produce, studies must be done to investigate survival of *Salmonella* (and other suitable pathogens) on the surface of other selected produce. Items such as cantaloupe (particularly interesting due to abundant cracks and crevices in its rind), lettuce or sprouts, which have previously been associated with produce outbreaks, would do well to serve as case studies.

Appendices

Appendix I: Microbiological analysis of the freshly collected manure and presumptive identification of some bacteria commonly found in poultry litter

Fresh Turkey Manure – Pre-dehydration			
ARRAY OF BACKGROUND MICROFLORA (presumptively identified)	pH	MOISTURE CONTENT	WATER ACTIVITY
• ***E. coli (fecal indicator)*** • ***Bacillus*** • ***Enterobacter*** • ***Proteus*** • ***Staphylococci*** • ***Klebsiella*** • ***Shigella*** • ***Listeria***	6.46	49%	0.97
Turkey Manure 'Dust' – Post-dehydration			
ARRAY OF BACKGROUND MICROFLORA	pH	MOISTURE CONTENT	WATER ACTIVITY
Drying reduced microflora to mainly *Bacillus* spp. (soil saprophytes)	6.97	~ 1.9%	0.38

Appendix II: Calculations used for Analytical Drying of turkey manure

Weight (wt.)	Sample 1 (g)	Sample 2 (g)	Sample 3 (g)
Drying of Fresh/wet Manure			
Pan	8.50	8.97	13.57
Manure wet	20.45	20.53	20.52
Manure + pan wt.	28.95	29.50	34.09
Manure + pan, post 24 h drying	18.14	19.80	24.30
- minus pan wt.	8.50	8.97	13.57
24 h-dried manure	9.64	10.83	10.73
Average weight 24 h dried manure	10.40g		
Drying of Dried Manure			
Manure + pan post further 48 h drying	17.95	19.65	24.05
--Minus pan wt.	8.50	8.97	13.57
72 h dried manure	9.45	10.68	10.48
Average weight 72 h dried manure	10.20g		

Appendix III: Moisture Content (of manure dust) Calculation Examples

$$MC = \frac{\text{wet weight} - \text{dry weight}}{\text{Wet weight}} \times 100 \qquad \text{(wet basis calculation)}$$

For three samples:

$$MC_1 = \frac{20.45g - 9.64g}{20.45g} \times 100$$

$$= 52.86\%$$

$$MC_2 = 46.57\%$$

$$MC_3 = 47.71\%$$

Average MC of fresh litter = 52.86 + 46.57 + 47.71/3

$$= 49.05\%$$

$$\text{Average MC of dried litter} = \frac{\text{Avg wt. 24hr dried litter} - \text{Avg wt. 72hr dried litter}}{\text{Avg wt. 24hr dried litter}} \times 100$$

$$\text{Average MC of dried litter} = \frac{10.40g - 10.203g}{10.40g} \times 100$$

$$= 1.895\% \text{ (approx. 1.9\% MC)}$$

Appendix IV: Sample recording table for Phase 1 Experiments

5% Moisture Content Colony Counts (CFU/ml)					
			XLD	BHIA	VRBGA
Control					
10^{-1}	a		0	109	0
	b		0	111	0
Rep 1					
10^{-1}	a		583	588	599
	b		622	610	574
10^{-3}	a		13	9	10
	b		5	16	4
10^{-5}	a		0	0	0
	b		0	0	0
Rep 2					
10^{-1}	a		642	654	590
	b		673	672	628
10^{-3}	a		12	13	11
	b		12	13	12
10^{-5}	a		0	1	0
	b		0	0	0
Rep 3					
10^{-1}	a		602	674	587
	b		551	646	611
10^{-3}	a		7	7	9
	b		12	8	12
10^{-5}	a		0	0	0
	b		0	0	0

Appendix V: Raw data [(CFU/ml) counts and enrichment results) for Phase 3 experiments

+ indicates positive by enrichment; - indicates negative by enrichment

Spinach Inoculation Experiment Trial 1									
	Sample Code	**A1**	**A2**	**B1**	**B2**	**C1**	**C2**	**D1**	**D2**
	Parameters	**Adaxial, UV, Dust**	**Abaxial, UV, Dust**	**Adaxial, ~~UV~~, Dust**	**Abaxial, ~~UV~~, Dust**	**Adaxial, UV, liquid**	**Abaxial, UV, liquid**	**Adaxial, ~~UV~~, liquid**	**Abaxial ~~UV~~, liquid**
	Rep 1	4.35E+04	3.38E+04	4.84E+04	4.68E+04	3.48E+07	1.46E+07	4.27E+07	4.34E+07
Day 0	Rep 2	4.68E+04	2.73E+04	5.36E+04	1.69E+04	5.10E+07	2.51E+07	5.28E+07	4.74E+07
	Rep 3	2.11E+04	3.20E+04	3.72E+04	3.12E+04	4.50E+07	5.27E+06	2.74E+07	5.34E+07
	Rep 1	4.94E+03	7.80E+02	3.90E+03	5.20E+03	2.94E+04	1.72E+04	6.89E+05	1.03E+06
Day 1	Rep 2	1.04E+03	5.20E+02	2.60E+03	7.80E+02	5.19E+04	1.25E+04	1.97E+05	7.09E+06
	Rep 3	1.53E+03	5.85E+02	4.94E+03	5.20E+02	2.16E+04	2.29E+04	9.88E+04	1.37E+06
	Rep 1	1.04E+03	1.30E+03	4.94E+03	2.86E+03	7.35E+03	2.07E+04	9.95E+04	3.87E+04
Day 3	Rep 2	1.30E+03	5.20E+02	3.90E+03	2.60E+03	5.46E+03	3.12E+04	3.71E+04	4.62E+04
	Rep 3	1.82E+03	5.20E+02	2.86E+03	7.80E+02	1.22E+04	3.04E+03	1.75E+04	3.15E+04
	Rep 1	7.80E+02	5.20E+02	2.60E+03	3.64E+03	7.54E+02	2.47E+04	2.24E+04	2.18E+04
Day 5	Rep 2	5.20E+02	5.20E+02	2.86E+03	2.08E+03	3.64E+03	3.12E+03	2.51E+04	1.03E+04
	Rep 3	5.20E+02	2.60E+02	7.80E+02	5.20E+03	3.67E+03	4.21E+04	3.80E+04	9.56E+03
	Rep 1	2.60E+02	4.55E+02	9.83E+02	2.64E+02	9.10E+02	9.10E+03	6.24E+03	1.46E+04
Day 7	Rep 2	5.20E+02	5.20E+02	3.51E+03	4.58E+02	2.73E+03	8.84E+03	5.98E+03	3.25E+04
	Rep 3	5.20E+02	4.55E+02	7.80E+02	5.20E+03	1.37E+03	1.51E+03	4.84E+03	2.31E+04
	Rep 1	+	5.20E+02	3.60E+02	2.08E+03	5.20E+02	5.27E+02	8.45E+03	1.58E+04
Day 14	Rep 2	+	5.20E+02	2.70E+02	7.80E+02	1.16E+03	2.73E+02	3.12E+03	1.98E+04
	Rep 3	+	+	+	2.61E+02	+	7.22E+02	3.90E+03	9.62E+03
	Rep 1	+	+	+	+	2.00E+02	+	+	5.20E+02
Day 21	Rep 2	+	+	+	+	+	+	2.60E+02	+
	Rep 3	-	+	+	+	+	+	+	+
	Rep 4	-	+	-	+	-	-	+	+

Spinach Inoculation Experiment Trial 2									
	Sample Code	**A1**	**A2**	**B1**	**B2**	**C1**	**C2**	**D1**	**D2**
	Parameters	**Adaxial, UV, Dust**	**Abaxial, UV, Dust**	**Adaxial, ~~UV~~, Dust**	**Abaxial, ~~UV~~, Dust**	**Adaxial, UV, liquid**	**Abaxial, UV, liquid**	**Adaxial, ~~UV~~, liquid**	**Abaxial ~~UV~~, liquid**
	Rep 1	3.58E+04	4.45E+04	3.22E+04	3.30E+04	5.51E+06	2.72E+06	3.95E+06	9.23E+06
Day 0	Rep 2	2.03E+04	3.12E+04	1.56E+04	2.37E+04	2.65E+06	9.03E+05	5.26E+06	1.92E+05
	Rep 3	9.62E+03	2.96E+04	3.69E+04	3.12E+04	5.79E+06	8.32E+06	4.67E+06	2.02E+06
	Rep 1	1.56E+03	2.08E+03	6.24E+03	4.16E+03	4.16E+04	5.20E+04	1.04E+04	1.82E+05
Day 1	Rep 2	1.04E+03	1.04E+03	4.16E+03	3.64E+03	3.12E+04	1.61E+05	2.08E+04	9.88E+04
	Rep 3	1.04E+03	1.04E+03	5.20E+03	5.20E+03	2.60E+04	7.80E+04	3.64E+04	9.36E+04
	Rep 1	7.20E+02	7.80E+02	2.60E+03	2.60E+03	4.16E+03	2.13E+04	1.09E+04	1.20E+04
Day 3	Rep 2	4.84E+02	5.20E+02	2.08E+03	3.64E+03	1.56E+02	2.50E+04	5.20E+04	3.47E+04
	Rep 3	5.94E+02	6.28E+02	1.04E+03	5.53E+02	1.04E+03	9.88E+02	4.16E+03	2.24E+04
	Rep 1	4.33E+02	3.55E+02	1.43E+03	4.23E+03	2.31E+03	4.40E+04	7.89E+02	4.10E+03
Day 5	Rep 2	2.52E+02	4.54E+02	1.05E+03	3.34E+03	7.69E+03	2.43E+03	1.22E+03	1.68E+03
	Rep 3	2.13E+02	5.11E+02	7.80E+02	1.34E+03	4.43E+03	4.21E+03	2.53E+03	2.23E+03
	Rep 1	2.00E+02	ND	2.50E+02	2.80E+02	2.00E+02	1.04E+03	5.20E+02	4.87E+03
Day 7	Rep 2	ND	2.00E+02	3.00E+02	2.00E+02	ND	5.20E+02	4.60E+03	4.54E+03
	Rep 3	ND	2.00E+02	1.20E+03	2.50E+02	ND	1.55E+03	1.50E+03	4.16E+03
	Rep 1	+	+	2.00E+02	+	+	4.33E+03	2.00E+02	2.50E+02
Day 14	Rep 2	+	+	+	+	-	-	+	+
	Rep 3	+	+	+	+	-	-	+	+
	Rep 1	+	+	+	+	+	+	-	+
Day 21	Rep 2	-	+	+	+	+	5.00E+02	-	+
	Rep 3	-	-	+	+	-	+	-	+

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