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Quantitative and qualitative analysis of airborne Listeria monocytogenes on ready-to-eat meats

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Quantitative and qualitative analysis of airborne *Listeria monocytogenes* on ready-to-eat meats

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Chapter 1: Introduction

Thesis Organization

This thesis is organized into three chapters: the literature review, a manuscript and concluding remarks. The literature review cover information on bioaerosols and *Listeria monocytogenes.* The bioaerosols sections including: sources in food-production facilities, control measures, recovery and analysis, and challenges of bioaerosol research. The *L. monocytogenes* section covers: listeriosis, *L. monocytogenes* in ready-to-eat (RTE) foods, and bioaerosols. This review is designed to provide an explanation of the problem of *L, monocytogenes* bioaerosols and why more research is needed. The manuscript is for a research project design to assess the risk airborne *L. monocytogenes* to RTE meats. The risk to the RTE products was assessed using two qualitative methods and direct plating onto a chromogenic media selective and differential for *L. monocytogenes.* The research was designed to see if the risk was dose dependent, differed based on product type and if it changed after 28 days of cold storage. The final chapter is concluding remarks in regard to research and the recommendations for future work.

Abstract

Bioaerosols are living organisms or substances from living organisms suspended in the air. They are responsible for allergies, sick building syndrome, and spread of bacterial and viral disease. Bioaerosols of food-production environments are of growing interest because of the potential for product contamination. Many studies have analyzed the air of meat and dairy production plants, while very few have focused on RTE foods. RTE foods are of concern because of the potential contamination with *Listeria monocytogenes.*

Listeria monocytogenes is a food borne pathogen, which is ubiquitous in the environment. *L. monocytogenes* is capable of survival and growth in many conditions meant to control bacteria. The unique growth characteristics combined with the ability for food contamination by a bioaerosol are a cause for concern. In addition to its unique survival abilities, this pathogen affects individuals with compromised or weakened immune system, such as pregnant women, the elderly and young. In these groups the high mortality rate of 20-40%.

The purpose of this work is to elucidate the problem of *L, monocytogenes* bioaerosols in food production and assess the risk of airborne *L. monocytogenes* to RTE meats in an experimental setting. To simulate an airborne-like condition L. *monocytogenes* attached to dry sterile sand and was dusted onto meat products. The RTE meat products were exposed at three levels (1.0, 5.0 and 10.0g). Half the samples were evaluated at day zero and the other half at day 28. All products were direct plated on chromogenic *L. monocytogenes* media, and then enriched in University of Vermont (UVM) broth and 4-Morpholinepropanesulfonic acid buffered *Listeria* enrichment broth (MOPS-BLEB). Following the enrichment the samples were evaluated for the presence of *L. monocytogenes* by plating onto Modified Oxford

(MOX) media and with a commercially available Polymerase Chain Reaction (PCR) system. It was found the contamination of RTE meats by airborne *L ,monocytogenes* is not dose dependent and cold storage had no effect on the risk . There was, however, a difference in the risk based on the RTE meat product type. These results are by not mean conclusive and more research is needed to better determine what is the true risk to RTE meats from *L. monocytogenes* bioaersosols and how the problem can be prevented or controlled during production.

Chapter 2: Literature Review

Introduction

Listeria monocytogenes emerged in the 1980's as a source of food-borne disease. Research in the last twenty years has focused on *L. monocytogenes's* pathogenicity, immune interactions, and survival mechanisms *(24)*. The study of bioaerosols began when Pasteur showed air contamination of sterile solutions *(41)*. Bioaerosols are implicated in problems ranging from the spread of contagious disease to allergies *(10)*. This review discusses bioaerosols and *L. monocytogenes* separately and also illustrates how *L. monocytogenes* bioaerosols are a problem in food-processing facilities. The first of three major sections covers bioaerosol definitions, sources and the dispersal of bioaerosols, control methods, air quality standards for food production, recovery and analysis methods, and finally composition of bioaerosols in different food-production environments. The second major section discusses *L. monocytogenes* with a focus on listeriosis and pathogen survival, and *Listeria monocytogenes* in ready-to-eat foods, and the final section *L. monocytogenes* bioaerosols.

Bioaerosols

Bioaerosols are airborne microbes, liquids, or particles released from a living organism *(23)*. These particles or molecules are considered airborne if attached to a vector, or an aerosol if suspended in a droplet of water. *(41)*. The *Bioaerosols Handbook* states bioaerosols are aerosolized particles with activities that affect living things. The size range is 0.5 to 100 µm for the particles suspended in air *(7)*. Bioaerosols are composed of a variety of substances: bacteria, fungi, viruses, endotoxins, allergens, and pollutants *(33)*.

Many innocuous and ordinary activities in food-processing environments generate bioaerosols. Cutting, grinding, washing, spraying, and the cleaning of equipment create bioaerosols *(34)*. The flapping of bird wings and the removal of cattle hides are also recognized sources *(25, 35)* . Even workers are a cause, as employees respire and scoff off skin cells *(34)*. A study reported a person sheds 3.3×10^3 to 7.2×10^3 of microbes/minute, and the microbes shed are able to persist *(1)*.

The dispersal of bioaerosols has similar innocuous causes. Climate and weather affect suspended particle behavior. Higher temperature and relative humidity generally aid in the dispersal *(33)*. Movement of workers, open doors, and facility design diffuse bioaerosols *(34)*. Awareness of the factors contributing to production and distribution of bioaerosols is vital to control of the problem.

Control Methods

Bioaerosols are controlled by several methods or combinations of methods. This section outlines four control measures used in the food industry: facility design, good manufacturing practices (GMPs), air cleaning systems, and clean rooms.

Facility design is vital for the control of bioaerosols. The building layout requires considerable thought to identify processes that generate bioaerosols and the potential effects on the food-product. Powitz's examples included not placing sinks or water sources near the food processing areas and to have a distinct separation of the cooked or finished product from the raw *(34)*. Facility design also includes control of the indoor climate through proper ventilation. Effective ventilation was been shown to reduce diseases related to poor air quality and the rate at which microbes are distributed in the air *(41)*.

GMPs give the producer the ability to control specific bioaerosols problems. GMPs are written by the processor and function by recognizing the sources of bioaerosols, their dispersal, and making appropriate changes *(40)*. For example, if the spraying of carcasses has been identified as a source of bioaerosols, a GMP could advise the workers to limit this activity to a specialized area with proper ventilation.

The use of air cleaning systems lowers the number of airborne microbes. One study found a germicidal air purification console unit in a meat-processing area reduced the overall concentration of airborne bacteria, and with more consoles the greater the reduction *(12)*. Another study used a reactive oxygen species (ROS)-generating system to control the airborne microbes in a similar environment. ROS are oxidizing agents that disrupt the cell wall and inactivate microbes. This study showed a significant reduction of *Serretia marcesans* and lactic acid bacteria in two hours with a ROS level of 0.0389 ppm. This level was safe for workers because the 8-h worker permissible limit is 0.1 ppm *(31)*.

Clean rooms, often used in pharmaceutical and biotech industries, are another method of controlling bioaerosols. Clean rooms offer a precisely controlled environment protected from contamination. There are national or international standards which clean rooms must conform to be certified. There are several classes for clean rooms based on number of particles 0.5µm or larger per cubic meter of air. A clean room's ventilation is slightly pressured to prevent inflow of contamination and the air is filtered. Particle tight door and windows prevent contamination from adjacent areas *(6)*.

Air Quality Standards for Food Production

Despite growing concern and information regarding the potential of food contamination by bioaerosols, the United States government has no air quality standards in

food-processing environments. The food industry is regulated on a federal level by the Food and Drug Administration (FDA), Environmental Protection Agency (EPA), and the U.S. Department of Agriculture (USDA). Local and state agencies regulate the food industry as well. Given the abundance of government oversight, there are little or no limits on the amount of microorganisms allowable in the air. Most processors follow the American Society of Heating, Refrigeration and Air-Conditioning Engineers (ASHRAE) standard for the commercial and residential buildings. This standard is based on occupancy, and provides the minimum ventilation required but places no limits on the number of bacteria or fungi in air *(23)*.

Recovery and Analysis Methods

This section focuses on the recovery and analysis of bioaerosols. The two traditional methods for sampling bioaerosols use a solid or liquid medium followed by culturing the microbes. Emerging is the use of these recovery methods in combination of non-culture based analysis. The two types of recovery methods and their analysis will be discussed, however this list not comprehensive.

Solid media collection methods

Impaction devices are commonly used solid media bioaerosols sampling devices. A vacuum source pulls air though the sampler. The stream of air is directed onto a surface where the microorganisms collide and become attached *(7, 22)*. Microbes can be impacted on to agar plates. The medium can be selective or differential for recovery of a specific microbe. The agar plates are incubated and analyzed for growth. A glass slide can also be used as an attachment surface for analysis via microscopy. Both agar plates and slides can be used for enumeration *(7)*.

Another type of air samplers with solid media is a sedimentation agar plate or glass slide. Sedimentation operates on the simple principle of bioaerosols fallout from the air, that then land on to exposure surface. The agar media can again be selective or differential if desired. The glass slides are analyzed using microscopy and agar plate culture for growth. This is the simplest method, but the results do not correlate with other methods. This is believed because the bioaerosol fallout is highly influenced by air currents *(20)*.

Filtration is also a solid media collection method. It is alternative to impaction particularly in areas with high concentrations of bioaerosols. Desiccation of the cells makes this method inappropriate for long sampling periods. Samples are analyzed by direct microscopy or culture *(28)*.

Liquid media collection methods

Impingers operate on the same principle as impactors, but have a liquid collection media. The media used maybe anything, but is usually supportive to the recovered microbes. A liquid is less stressful to cells than agar impaction and typically has higher recovery rates *(19)*. Analysis of samples from impingement is more flexible than an impactor. Impinger samples can be analyzed by growth culture, microscopy, biochemical assay, immunoassay, and polymerase chain reaction (PCR) *(43)*.

Non-culture based methods

Non-culture based methods are available for the assessment of bioaerosols. Vanhee et al. used an impaction sampler and analyzed the samples using both traditionally culture methods and solid phase cytometry (SPC). In SPC, cells are labeled with a fluorescent dye and counted using a flow cytometry. This method differentiates between living (metabolically active) and dead cells. The results from SPC are available within hours,

however, it more demanding technically, and does not report the type of microorganisms. SPC had a higher recovery of bacteria and yeast when compared to culture methods *(46)*. Rinsoz et al. compared real-time PCR to epifluorescence microscopy and culture methods. The researchers found culture methods highly underestimated the numbers of bacteria when compared to the other two methods. The bacterial counted obtained using epifluorescence were highly associated with the DNA concentrations from the real-time PCR *(38)*. Both of these studies showed the limitation associated with culture-based bioaerosols recovery analysis methods.

Types and Populations of Bioaerosols in Food Production Environments

The bioaerosol composition of any environment is highly variable. In food-production facilities, it has been shown the concentration and composition of bioaerosols in the environment can change daily and room to room *(11, 20, 41, 47)*. This section focuses on bioaerosols of dairy and meat and ready- to-eat (RTE)-processing environments in terms of type of organisms and quantities.

Most of the bioaerosol research of processing facilities has been done in dairy production because most products are RTE and the potential for post-pasteurization contamination. A review by Kang and Frank reported a wide variety of microbes recovered from dairy processing facilities. Some of the isolated species included *Pseudomonas, Chaldosporium, Fusarium, Aspergillus, Micrococcus, Staphylococcus, Candida* and *Klebsiella (20)*. Ren and Frank investigated the viable particle counts in different areas of four milk-processing facilities. The average log viable count per 100 liters of air were 2.03 ± 0.46 in the raw milk storage, 2.26 ± 0.57 in the processing area, and 2.41 ± 0.70 in the filling

area *(37)*. Similar viable counts were found in a commercial ice cream plant. The log mean count per 100 liters of air in the pasteurized milk storage was 2.31 ± 0.46, and 2.26 ± 0.47 in the filling area *(36)*.

Bioaerosols of meat-processing facilities have high quantities of suspended bacteria, including pathogens. The total viable counts (TVC) of the deboning rooms of four red meat abattoirs ranged from 1.3 $x10^2$ to 1.0 $x10^3$ CFUs/m^3. The staphylococcal counts of the air ranged from 8.0 to 3,000.0 CFUs/m^3 *(42)*. Pearce et al.\ analyzed the air quality in a pork slaughter plant after 11 hours of production *(32)*. The researchers found a mean log value of 3.52 for the aerobic mesophilic bacteria recovered in the wet rooms (where the carcasses are bled, scalded, dehaired, and polished). The clean room where the carcasses were debunged and eviscerated had a mean log value of 3.34. The samples were analyzed for *Escherichia coli* and *Salmonella* species. *E. coli* was only detected in the evisceration area, and *Salmonella* species was detected in dehairing and evisceration sampling locations *(32)*. Lues et al reported the composition of bioaerosols in a chicken abattoir. The researchers found coliforms, fungi, *B. cereus, S. aureus, E. coli, P. aeruginosa, L. monocytogenes* and *Salmonella* species. The microbial counts were highest in the receiving-killing and defeathering areas *(25)*.

The bioaerosols of RTE food-processing environments are less well studied. An article by Byrne et al. assessed the microbial quality of air in a pork processing plant; where a RTE pork-burgers were made *(5)*. Sampling points were in the raw, cooking, post-cooking, blast chiller, and packaging areas. The researchers sampled for TVC, total coliform count (TCC) and total counts of *Staphlycoccus aureus, Listeria* and *Salmonella* species. The mean TVC count ranged from 5±1 cfu m^{-3} in the packaging area to 133±92 cfu m^{-3} in the cooking

area. The high level in the cooking area was attributed to a greater number of workers. These values were much lower than reported in previous studies. The mean TCC values were also low. The highest mean value was 8 cfu m^{-3} in the blast chiller. The mean *S. aureus* levels ranged from 1 cfu m^{-3}(packaging area) to 8 cfu m^{-3} (blast chiller). No *Listeria* or *Salmonella* species were detected. The authors noted the production facility had a number of systems for prevent microbial contamination, including a physical separation of the raw, cooking and post-processing areas *(5)*.

Challenges of bioaerosols research

There are three major challenges involved in bioaerosols research: lack of technology, the stress on cells in bioaerosols, and lack of standards. The technology for investigating bioaerosols in progressing but limitations still exist *(41)* For example, real-time quantitative PCR yields higher recovery over other methods and gives more accurate information of the true number of bacteria in the air, but does not have the ability to differentiate between species of bacteria when using universal 16s RNA primers *(38)*. Knowledge of species present is crucial to determine the threat level. Being suspended in bioaerosols is stressful to cells, and this stress can lead to a viable but not culturable (VNC) state. To date most methods of bioaerosols research are based on culture methods, and cells in VNC state are undetected and the numbers are underestimated *(41)*. The lack of information on these fronts makes establishing standards difficult. There are not Threshold Limit Values (TLVs) for air. To establish a TLV standard five components are needed: scientific basis of the standard, sampling method, analytical method, sampling strategy, and limit value. There is not enough information to the five components to construct a TLV for bioaerosols *(26)*. This concludes the general discussion of bioaerosols; the next section focuses on *Listeria monocytogenes.*

Listeria monocytogenes

Listeria monocytogenes is the causative agent of listeriosis *(39).* In the 1980s the disease gained interest due to a rise in the number of human cases. Outbreaks in Europe and North America were linked to consumption of contaminated milk, soft cheeses, and coleslaw *(24)*. This section discusses basic information on *L. monocytogenes,* its life a pathogen, the control of *L. monocytogenes* in food production and *L, monocytogenes* in RTE meats.

Basic information

Listeia monocytogenes is a gram positive, non-spore forming, short rod which often occurrs in singly or short chains. It is facultive anaerobe and is motile at 20 to 25˚C, but not 37˚C.The genus *Listeria* has six species, two of which are pathogenic: *monocytogenes* and *ivanoii*. Nonpathogenic species are: *innocua, welshimeri, seeligeri, and grayi.* The species can be classified into different serovars based on the listerial somatic (O-factor) and flagellar (H-factor) antigen serological reactions. *Listeria monocytogenes* serovars most commonly associated with clinical cases are 4b, 1/2a, 1/2b and 1/2c *(24).*

Listeria monocytogenes can be differentiated from the other species of *Listeria* based on its biochemical signature. *L. monocytogenes* uses xylose, rhamnose, and has beta hemolysis on blood agar. The CAMP (Christie-Atkins-Munch-Petersen) test is traditionally considered a definitive test. An isolate can be considered a presumptive *L. monocytogenes* given a positive result of the CAMP test with either *S. aureus* or *R.equi (18).*

L. monocytogenes is able to survive a wide range of environmental factors especially those used to control other microbes. Temperature, salinity, pH, oxygen availability, and organic acids are some of the measure commonly used in food production to control

microbial growth. This pathogen is able to survive and grow at temperatures of 4 to 45˚C. It can survive salt water solutions of 13-16% and pH ranges of 4.2 to 9.5 *(3)*. As a facultative anaerobe, it is capable of growth in vacuum packaged foods *(18)*. The minimal inhibitory concentration (MIC) for some common growth inhibitors are : Lactic acid (3.8-4.6 mM), sodium lactate(800-1000 mM), acetic acid (20 mM), citric acid (3mM), and sodium nitrate(8.4-14.2 µM) *(3)*. The next section focuses on how *L, monocytogenes* functions as a human pathogen including: how's transmitted, the infection process and symptoms of listeriosis in humans.

Life as a pathogen

Listeriosis is a food-borne disease, related to consumption of *L. monocytogenes* contaminated foods. Evidence of transmission of the disease via foods emerged in the 1980s. Before it was an obscure disease usually associated with animals *(24)*. The 1985 Jalisco cheese outbreak established the serotype of *L. monocytogenes* in listeriosis patients matched the serotypes from the cheese *(27)*. Transmission of the disease continues to be primarily related to consumption of contamination foods including: processed meats, cheeses, and raw dairy products *(3)*.

The infection process of *L. monocytogenes* is adapted to evade the host's immune defenses. The bacterial cells enter body on contaminated foods and in the stomach use acid adaptation mechanisms to survive and move with the food to the small intestine. The bile salts in the small intestine further stress the cells, but *L. monocytogenes* secretes two proteins. These proteins have bile salt hydrolase and bile acid dehydrotase activites which decreases the potency of the bile salts. In the small intestine the pathogen invades the host's epithelial cells *(24)*. The entry into the mammalian cells is primarily mediated by the proteins:

internalin and InlB. These proteins interact with host cell-surface components and result in the uptake of *L. monocytogenes* into a vacuole *(8)*. Inside the vacuole, the pathogen escapes using listeriolysin and two different ezymes of phospholipase C *(30)*. To prevent being killed in the vacuole, *L. monocytogenes* produces cytoplasmic superoxide dismutase (MnSOD) and the enzyme PgdA *(9)*. Free from the vacuole *L. monocytogenes* uses the protein ActA, to induce actin-based movement and allows the movement of the bacteria into adjacent host cells. This allows the pathogen to avoid the host's humoral immunity. Entry into macrophage allow for the dissemination to the liver and spleen *(30)*.

Listeriosis manifests with different symptoms depending on the host's predisposing conditions and has a high mortality rate. In pregnant women, the clinical presentation is preterm delivery, stillbirth, and abortion. Newborns have sepsis, pneumonia, and meningitis. Immunocompromised or elderly adults will present with sepsis, meningitis, and focal infections. Healthy adults who consume a large dose will experience diarrhea and fever *(39)*. The most at risk groups are pregnant women, neonates, newborns, the elderly and the immunocompromised. The mortality rate of listeriosis is 20-40% *(24)*. Given the seriousness of listeriosis and that most people acquire the disease from contaminated foods the most logical means of control is to eliminate it from food products.

Control of *L. monocytogenes* in food production

The FDA had a zero-tolerance policy for *L, monocytogenes* in RTE foods. Although in February 2008 in was announced the FDA would revise the tolerance level for RTE foods that did not support the growth of the pathogen. Generally the foods that do not support growth: have a pH of 4.4 or less, are held or consumed frozen, have a water activity of 0.92 or less, or are processed to have listerial control measures *(2)*. The zero-tolerance policy is

still in place for many foods. End product testing and environment testing are used in the control off *L. monocytogenes (24).* The FDA recommended method for recovery of *L. monocytogenes* for foods includes two enrichment steps in University of Vermont broth (UVM) and then Fraser or 4-Morpholinepropanesulfonic acid buffered *Listeria* enrichment broth (MOPS-BLEB) followed by plating on modified Oxford (MOX), a selective and differential media. The environmental testing involves swabbing the processing area and following a similar enrichment and identification steps *(39)*

***Listeria monocytogenes* on RTE meats**

Many of the recent outbreaks on listeriosis and product recall have been linked to RTE meats, the reason is twofold: the pathogen ability to survive on RTE meats and product contamination. One study found the protein expression of *L. monocytogenes* differs when grown on deli-style turkey slice compared to brain-heart infustion (BHI) media. The protein expressed on the turkey meat enable the cells to grow more efficiently, by increasing energy metabolism, cell division rate, and protein synthesis. Other proteins were indentified to increase cold and osmotic stress adaptation *(29)*. This information in addition of other stress adaptations mentioned earlier allows the pathogen to survive and replicate on RTE meats.

RTE products are contaminated with *L. monocytogenes* by a variety of sources, including the air. According to Zhu et al, RTE meats are commonly contaminated during post-processing at the processing facility. The product is most at risk during the step(s) between the cooking or pasteurization and packaging *(50)*. The contamination of products has been linked to the equipment, workers, and the air. An outbreak of listeriosis in 1998 to 1999 was a result of post-processing contamination of RTE meats by dust from construction.

There were 100 cases of disease and 21 fatalities. The producer recalled over 35 million pounds of product, and lost 2 million dollars in sales *(14)*.

Listeria monocytogenes bioaerosols

Many studies have shown *L. monocytogenes* to survive in an airborne-like state or remain suspended as an aerosol. Foong et al. found that the pathogen was able to survive for up to 2 months on a dried, nutritionally depleted medium at 4 ˚C (conditions similar to an airborne state) *(16)*. In another study with *L. monocytogenes* attached to sterile sand, the researchers found a lower temperature and a higher relative humidity allows for better survival *(14)*. One study analyzed the fallout time of *L. monocytogenes* from aerosols, and found the strain Scott A remained suspended for 50 minutes to over three hours *(44)*. A *L. monocytogenes* surrogate persisted as an aerosol in pilot plant for 2.5 hours. It is feasible *L. monocytogenes* would remain suspended for a similar amount of time *(49)*.

L. monocytogenes is transmitted by the air. Goff focused on transmission of an aerosol and used a *L. monocytogenes* surrogate organism in a cold-air wind tunnel. The researchers found that at -16 to -18˚C a surrogate organism was able to survive the aerosol state and contaminate ice cream *(17)*. The contamination of RTE foods with the construction dust in the 1998-1999 listeriosis outbreak is further evidence of the ability of *L. monocytogenes* to be transmitted as a bioaerosols*(14)*.

Several studies have shown RTE foods contamination or potential contamination with *L. monocytogenes* bioaerosols. *Listeria monocytogenes* aerosols are capable of contaminating RTE meats *(49)*. The survival of the *L. monocytogenes* surrogate in the wind tunnel showed the potential for aerosol contamination of ice cream *(17)*. De Roin et al. used vector sand to

inoculate RTE meats. The population numbers decreased more slowly on the RTE meats when compared to the sand. It is thought *L. monocytogenes* cells on the RTE meats repaired from injury, survived or even proliferated *(14)*.

These studies show the importance of influencing factors on *L. monocytogenes* bioaerosols. One study showed a supportive media increases the time before aerosol fall out, and higher initial populations increased the number recoverable *(44)*. Another found that lower temperatures and higher relative humidity increases the survival in an airborne-like state *(16)*. These are factors are potential control methods of *L. monocytogenes* bioaerosols.

Conclusions

Despite the impact of these studies, little research has been done on *L. monocytogenes* bioaerosols. Standard methods for the recovery or enumeration are lacking. Few studies look for *L. monocytogenes* bioaerosols in RTE food-processing facility. In general more research is needed to close the gap in the knowledge regarding *L. monocytogenes* bioaerosols in food-production.

L. monocytogenes bioaerosols are a serious threat to food safety that requires a multidisciplinary approach to alleviate the problem. The ubiquitous nature of the organism, its ability to survive and its ability to be transmitted as bioaerosols necessitates more research into recovery and control methods on the part of microbiologist and food scientists. Consulting engineers will improve facility design and ventilation systems to prevent bioaerosols generation and dispersal. There is no quick and easy solution, awareness of the problem is the first step.

Chapter 3: Quantitative and qualitative analysis of airborne *Listeria monocytogenes* on ready-to-eat meats†

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† Mention of trade names of commercial products does not imply endorsement or recommendation by Iowa State University

Abstract

Listeria monocytogenes is a pathogen able to grow at refrigeration temperatures and is ubiquitous, making it a particular hazard for ready-to-eat (RTE) foods. Cases of listeriosis have been associated with airborne contamination of RTE meats. The objectives of this study were to quantitatively and qualitatively assess the potential for airborne contamination of RTE meats with *L. monocytogenes,* and to determine if cold storage for 28 days changes the population levels. Three strains of *L. monocytogenes* were attached to sterile sand and dusted into a vessel containing bologna slices and hot dogs. Three quantities of sand were used (1.0, 5.0 and 10.0 g). Half the samples were evaluated at day zero and the others 28 days later. RTE meat product were evaluated by spiral plating on chromogenic *L. monocytogenes* agar to determine colony forming units (CFU) per sample and then enriched in Modified University of Vermont broth (UVM) and 4-Morpholinepropanesulfonic acid buffered *Listeria* enrichment broth (MOPS-BLEB). The MOPS-BLEB enrichments were plated on Modified Oxford agar (MOX) and also analyzed using a commercially available PCR system. There was a significant difference in the potential contamination between the two product types using both qualitative methods. The quantitative data from the bologna showed no significant differences between the day 0 and 28 and the 5.0 g and 10.0 g samples. The qualitative analysis found no significant differences between the inoculum quantities and

population level after cold storage. This study illustrates the potential of airborne contamination of RTE meats. Airborne *L. monocytogenes* is a problem in RTE food production, and more research is needed to fully comprehend the issue, and how it can be prevented or controlled.

Introduction

Listeria monocytogenes is a food-borne pathogen. It is the causative agent of listeriosis, a disease with mortality rate of 20-40% and causes: stillbirth, miscarriages, meningitis and sepsis *(24)*. Those most at risk of listeriosis are pregnant women, neonates, newborns, the elderly and the immunocompromised. These individuals' immune systems unable to counteract the infection *(39)*. This disease has been linked to consumption of: soft cheese, raw dairy products, vegetables and ready-to-eat (RTE) meats *(48)*. In foods, microbes are controlled by pH, temperature and osmotic stress. *Listeria monocytogenes* has characteristics that enable it to survive these control measures, it tolerates a pH range of 4.2 to 9.5 *(48)*, and has acid stress adaptation mechanisms that enable it to survive in the stomach and other similar environments *(24)*. It grows at 4˚C, a temperature where most bacteria are metabolically inactive, and is able to survive salt water solutions of 13-16% *(18)*. RTE meats offer *L. monocytogenes* are rich growth media, this is why there is zero-tolerance policy on RTE foods that support the growth of the pathogen *(2)*

RTE meats can become contaminated by *L. monocytogenes* by a variety of means, including the air. Studies have shown *L. monocytogenes* ability to survive in an airborne state *(14, 16)*, remain suspended as aerosol for several hours *(44, 49)* and contaminate RTE foods *(17)*. One outbreak of listeriosis was linked to the contamination of the RTE meats with

construction dust that contained *L. monocytogenes (14)*. Evidence supports potential contamination of RTE meats and foods by *L. monocytogenes* from the air by bioaerosols

Bioaerosols are airborne microbes, liquids, or particles released from a living organism *(23)*, and are composed: bacteria, fungi, viruses, endotoxins, allergens, and pollutants *(33)*. The activities in food-production facility generate bioaerosols, including: cutting, grinding, washing, spraying, and the cleaning of equipment *(34)*. In abattoirs birds flapping their wings and workers removing cow hides are documented causes of bioaerosols *(25, 35)* . Employers are another source as they respire and scoff off skin cells *(34)*. Bioaerosols are controlled in food production facilities by proper facility design *(34)* and the use of air cleaning systems *(12)*. The bioaerosols of some food-processing environments are well studied, such as dairy *(21)*; however, RTE meat production facility are less studied and information in limited on the air environments *(5)*. The potential contamination to RTE foods from bioaerosols in not well known.

The objectives of this study were to determine the potential contamination to RTE meats from airborne *L. monocytogenes*, to establish if the contamination is inoculum quantity dependent, and what happens to airborne *L. monocytogenes* on RTE in cold storage for 28 days, and if potential contamination is the same for two different types of RTE meats.

Materials and Methods

Experimental design overview

Three strains of *L. monocytogenes* were attached to sterile sand and dusted onto 8 pieces of irradiated RTE meats. The sand was dusted at three inoculum quantities (1.0, 5.0

and 10.0 g), with each repeated in triplicate. Two of each type of RTE meats were evaluated at day zero and the other two were stored at 4˚C for 28 ± 1 day and then evaluated for *L. monocytogenes.*

***Listeria monocytogenes* strains and sand preparation**

The three strains of *Listeria monocytogenes* used in these experiments included ½b, Scott A, and 4c from the Food Safety Research Laboratory Culture Collection at Iowa State University. Each strain was purified on Modified Oxford agar (MOX; BD, Franklin Lakes, NJ) and verified with the Reveal Listeria Rapid Test (Neogen, Lancing MI).

The sand carrier was prepared as previously described by De Roin *et al (6),* with modifications. The three strains of *L. monocytogenes* were transferred into 9 ml of tryptic soy broth with 0.6% yeast extract (TSB+YE; BD) and incubated at 35˚C for 24 hours. One ml of each strain was transferred to 500 ml of TSB+YE, and incubated for 24 hours at 35˚C with shaking at 150 rpm. Following the incubation, the cells were harvested by centrifugation at 9,500 rpm for 30 minutes at 4˚C. The supernatant was poured off, and cells were resuspended in 500 ml of sterile phosphate buffered saline (PBS) (Sigma; St. Louis, MO), using a stir plate for five minutes. Cells were reharvested as previously described and resuspened in 500 ml of PBS. The cell suspension was combined with 600 g sterile dyed red sand (Tree House Studio, Oklahoma City, OK) in a sterile beaker. The sand was previously sterilized by autoclaving it twice. The sand mixture was covered with sterile foil and incubated at 35˚C with shaking at 175 rpm for 20 minutes. After 20 the shaking was discontinued, the sand mixture was incubated at 35˚C for 24 hours. After incubation the liquid was poured off and sand was filtered as described as De Roin et al *(14).* Following the filtration, the sand was divided into eight approximately equal proportions by mass (~75 g), and placed into sanitized

4 oz storage containers (Ziplock, Racine WI). Individual proportions of sand were further dried by spreading in an even layer into a sterile 12”X 5” nalogen tub (Fisher Scientific, Itasca IL) and covering with perforated sterile foil. The tub was placed into bio-safety cabinet and the fan in the biosafety cabinet was allowed to run for 1.5 hours. The dried sand was returned to 4 oz container, and stored at 25˚C until use. The cfu/g of sand was determined by taking a 1.0 g sample of the sand were placed into 9 ml of 0.1% peptone (BD), vortexing for 30 sec, and ten –fold serial diluted and duplicate plated on MOX, in triplicate.

Preparation of RTE Meats

Two types of commercially available RTE processed meats were used: thick sliced bologna and hot dogs. These meats were selected because their shape is representative of other RTE meat. The meats were repackaged in groups of four in 7” by 12”cryovac bags (Cryvac, Duncan, SC) and vacuum packaged using a Multivac vacuum packaging machine (model G-2; Kansas City, MO). The packaged meats were stored at -20˚C until three days before irradiation, and then meats were thawed at 4˚C. The meats were then irradiated at Iowa State University’s Linear Accelerator Facility, to an average absorbed dose of 4.95 kilogray (kGY) for the hot dogs and 4.19 kGY for the bologna to eliminate most of the naturally-occurring micro-flora. The irradiated meats were stored at -20˚C and thawed at 4˚C one day before use.

Inoculation vessel construction and RTE meat contamination

The inoculation vessel was a 12”x12”x12” high density tank with a lid (Fisher). An inlet port was created by drilling a hole in center of lid and 4 cm piece of silicone peroxide cured tubing with an inner diameter of 0.25” (Cole-Palmer, Vernon Hills, IL) was glued in (figure 1). The meats were placed according to the template (figure 2). The weighing of the

carrier sand and the inoculation of the RTE meat was performed in a biosafety cabinet. The sand carrier was weigh and placed into a centrobulb duster (U-Spray, Inc, Liburn, GA), and dusted onto the product. The centrobulb was weighed before and after dusting. Two hot dogs and 2 pieces of bologna were randomly selected for sampling at day zero, random sampling of RTE meats was determined by labeled pennies in opaque container. Day zero samples were aseptically transferred to 7.5" by 12" non-filtered whirl-pak bags (Nasco, Atkinson, WI) and on kept on ice until further processing. The inoculation of the meats with carrier sand was performed by one individual to lessen differences in the flow rate of the sand and the distribution onto the product. The remaining products were vacuum-packaged as before and stored at 4˚C for 28 ± 1 day. All the RTE meats were evaluated using the methods outlined next.

Quantitative Methods

The meats were aseptically transferred to whirl-pak bags for day zero samples (and to cryovac bags for day 28 samples) and 10.0 ml of 1.0% peptone was added. The products were massaged by hand for 1 minute to remove the sand, and 200.0 µl was spiral plated using the DW Scientific Whitley Automatic Spiral Plater (West Yorkshire, England) onto chromogenic *L. monocytogenes* media (R&F Labs, Downer's Grove, IL, in duplicate. The 200.0 µl samples were representative of approximately 20.0 cm^2 of hot dogs and 10.0 cm^2 of bologna. The plates were incubated for 72 hours at 35˚C. The cfu/ml was determined using the equation provided with the DW Spiral Plater. The total cfu for a product was determined using the following equation.

$$cfu = \frac{cfu}{ml} X \frac{Product\ Surface\ Area}{Sampled\ Surface\ Area}$$

Qualitative Methods

After the samples for quantification were taken, University of Vermont (UVM) broth (BD) was added to the whirl pak bags containing the products. The amount added was based on a 1:1 ratio of surface area of product to 1 ml of UVM (103 ml to hot dogs and 207 ml to bologna). This mixture was incubated at 30˚C for 22 hours. One tenth of ml was then transferred to 4-Morpholinepropanesulfonic acid (Sigma) buffered *Listeria* enrichment broth (MOPS-BLEB) (BD) and incubated for 24 hours at 35˚C, Following incubated, 0.1 ml was streaked on MOX, and incubated for 48 hours at 35˚C. The MOPS-BLEB enriched samples served as the template for a commercial PCR system. The commercial PCR (BAX, Dupont Qualicon) was carried out according to manufacturer's directions. Uninoculated media (UVM and MOPS-BLEB) was the negative control for the PCR and on the MOX plate. The positive control for the PCR was pure colonies selected from a MOX plate and suspended into the PCR lysis buffer.

Statistical Analysis

The spilt-plot analysis in (SAS Institute Inc; Cary, NC) was used for quantitative data obtained from the bologna samples at inoculum quantities 5.0 g and 10.0 g at both sampling dates. This analysis determines if cfu of the meat is a function of inoculum amount. The Mantel-Haenszel test in Win Episcope 2.0 was used for the analysis of qualitative data to: compare the percent positives between day zero and day 28, and compare the percent positive between the two product types. The Freidman test *(13)* was used to compare

inoculum quantity and percent positive of the qualitative analysis. The samples were blocked during the analysis to control for unequal distribution of carrier sand among the RTE meats.

Results and Discussion

The average cfu/g of sand carrier was $1.8x10^3$. The log mean and log standard deviation masses of the sand for each inoculum quantity into the inoculation vessel were 0.737 ± 0.021, 4.58 ±0.060 and 9.46 ±0.089 g, because of the residual sand that remained in the centrobulb inoculation device.

Quantitative analysis

Of day zero and day 28 all the hot dogs samples were below the detection limit of 100 to 10^5 cfu/ml for the quantitative analysis. With the exception of one sample, the bologna samples dusted with 1.0 g of sand were also all below the detection limit. These were excluded from further statistical analysis. The bologna data for both days at 5.0 and 10.0g (table 1) shows a large amount of variability in the recovery of *L. monocytogenes* for bologna. To obtain the log mean for one sample, the two plate counts were averaged and the log10 was determined.

The data were analyzed using the split-plot analysis method to determine if the cfu on the RTE meats correlated with the mass of sand. There was no significant difference between the 5.0 and 10.0 g, but a comparison of the day 0 to day 28 was suggested (p=0.0516) that 4˚C storage for 28 days may have an effect of the recovered populations. Comparisons of the inoculation quantities to recovered populations and the interaction of inoculum quantities and day were not significant. De Roin et al *(6)* found *L. monocytogenes* attached to sterile sand

was able to recover on RTE meats and multiply *(14)*. In this study it appeared that L. monocytogenes on some of 5.0 g bologna samples were not able to recover and the mean log cfu decreased from 6.96 to 3.00, while the 10.0 g bologna samples did not significantly change, from a mean log cfu of 5.51 to 5.86. This difference may be attributed to the populations on day 28 samples, were spreader colonies were encountered instead of the normal isolated colonies *(45)* because of poor media solidification. Another possible explanation for the difference from previously found by De Roin et al. is the carrier sand. In the this study, the sand was dried for an additional 1.5 hours compared to the study by De Roin et al. and stored in 25 ˚C, both treatments would result in additionally stressed cells. De Roin et al. showed storage of the carrier sand at 22 ˚C with 40% relative humidity had the lowest daily survival rate of the tested conditions, because the cells were still metabolically active at this temperature and more susceptible to injury *(14)*.

The chromogenic *L. monocytogenes* media used in this experiment was able to indentify *L. monocytogenes* from other bacterial species. The chromogenic reaction based on the breakdown of 5-Bromo-4-choloro-3-indoxyl-myo-inositol-1-phosphate by phosphatidylinositol-specific phosphpolipase, a virulence factor found in only *L. monocytogenes* and *L. welshmeri.* This media is both selective and differential, and has been found to more superior than both MOX and PALCAM media *(4)*. The selective properties of this media may have prevented injured or osmotically stressed cells from growing. This could explain why no *L. monocytogenes* was recoverable from the hot dogs. The typical method for recovery injured or stressed cells is to use nonselective media which will not further stress the cells and allow injured or stressed cells to grow. The discussion to use the

chromogenic media in this study was based on concerns about contamination of the sand carrier.

Qualitative analysis

The data for the qualitative analysis methods between the two methods correlated well, only two samples were PCR negative and culture positive. This difference likely due to in detections limits. Table 2 summarizes the number of positive samples from the qualitative methods. The trends seen the quantitative methods are similar to those seen with the quantitative analysis; the hot dogs have lower percent positive than the bologna, and the 1.0 g inoculum amount had the fewest positive samples for both RTE meats. There was no significant difference between the inoculum quantities using the qualitative methods. This suggests that the odds of the RTE meat being positive does not change when the inoculum amount changes. It was thought that the number of positive samples would increase with an increase in the pathogen population and quantities. This was not the case; the problem may be in the limited numbers of samples of each type RTE meat used. For example, if both the product 5.0 g and 10.0 g had all positives with only two RTE meat samples, more samples would be needed to find the difference in the potential contamination of the RTE product.

The statistical analysis showed no significant difference ($p=0.503$ for culture, $p=0.780$ for PCR) between the day 0 and day 28 samples based on the number of positive samples. This indicates the *L. monocytogenes* neither multiplied nor died and percent of positive samples did not change. These results seem to contradict the quantitative analysis results, where the p-value was suggestive of a difference between the days. The difference between the statistical tests is likely in the samples analyzed. The split-plot analysis for quantitative data used only the bologna dusted with 5.0 g and 10.0 g, while the second

analysis used all the samples. Given a difference group of samples analyzed it is not surprising the p-value differ. The results do contradict the results found by De Roin et al (6), but the difference in the storage of sand before RTE meat inoculation may have resulted in more stressed cells in this experiment.

There was a significant difference between bologna and hot dogs using all inoculum quantities and both days. The p-value for the culture method was 0.009 and for the PCR method is was 0.008. This means the percent of positive samples of the RTE meat products was higher from the bologna, indicating the bologna had a higher potential for contamination when compared to the hot dogs. One reason for the difference could be the larger surface for the bologna, 208 cm^2 to 103 cm^2. Additionally, the large flat surface of the bologna could more suitable for the attachment of *L. monocytogenes* than the rounded shape of the hot dog where the sand may have simply rolled off. Bologna and hot dogs also differ in the moisture contents, hydrophobicity and the external surface. Foong et al. showed the surface hydrophobicity of hot dogs was higher than bologna, and reported the difference was due to the external surface of hot dogs. The coagulated protein surface and a higher fat content make hot dogs more hydrophobic *(15)*. The intrinsic characteristics the RTE meats would impact the carriers sand ability to attach to the surface and allow microbial growth.

This is study shows the potential of using culture methods and commercial PCR for the recovery of airborne *L. monocytogenes* from RTE meats. This technique can be used as a springboard for the development of more sensitive methods. More work is needed to establish more concrete connections between inoculum quantities and amount of contamination of the product. A better understanding of how concentration of airborne *L.*

monocytogenes can affect RTE products can influence how RTE foods are produced and what controls measured are needed to ensure safe products.

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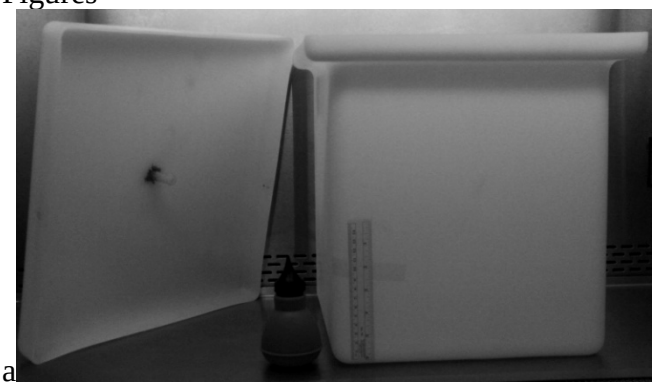
Figures

a  b

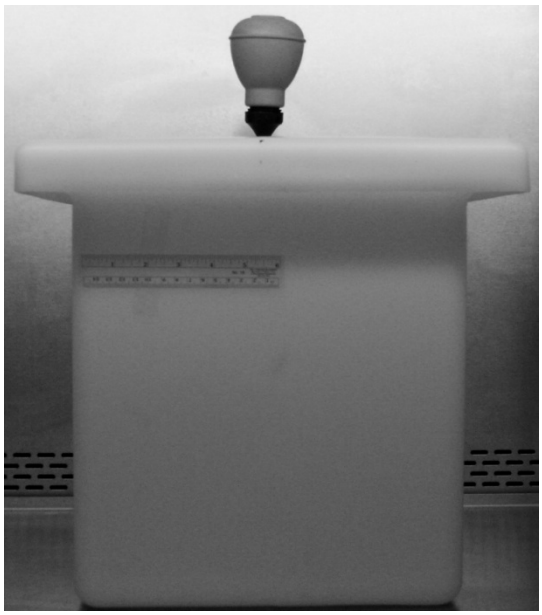


Figure 1

a. This is picture of 12” by 12” by 12”inoculation vessel and its lid. The lid with the 4 cm length of tubing glued into for the centrobulb to fit into. The 4.0 oz centrobulb is the center of the picture.
b. The inoculation vessel with lid on and the centrobulb into the tubing segment.

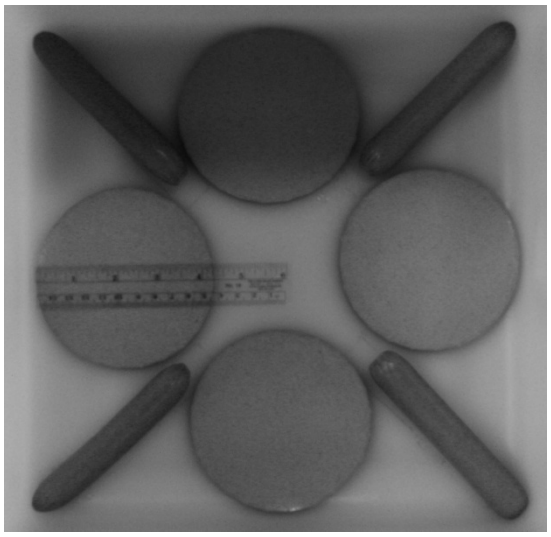


Figure 2
This is the arrangement of the RTE meat products inside the inoculation vessel, none of the products were touching.

Table 1 Mean log CFU for Bologna samples

Day	Dose	Mean Log CFU (standard deviation)
0	5	6.96 (2.14)
	10	3.99 (2.08)
28	5	5.51 (2.70)
	10	5.86 (0.60)

Table 2 Distribution of percent of positive over products, inoculum quantity and days

Product	Day	Inoculum Quantity (g)	% Positive Culture
Bologna	0	1.0	50.0
		5.0	100.0
		10.0	100.0
	28	1.0	83.3
		5.0	66.6
		10.0	100.0
Hot dog	0	1.0	0.0 (16.7)*
		5.0	66.6 (83.3)*
		10.0	33.3
	28	1.0	0.0
		5.0	0.0
		10.0	33.3

* When the methods differed the PCR method is listed in parenthesis.

Chapter 3: Concluding Remarks

The information in the literature review shows clearly the potential from the contamination of RTE foods by bioaerosols, and why *L, monocytogenes* is of particular interest. It also illustrates the shortcomings and knowledge gaps present in bioaerosols research. Too few studies have analyzed the air of RTE food-production facilities to determine the type and concentration of microbes present in the environment. Without this vital information the risk of food contamination is not well understood and is overlooked as a source of contamination. Technology has progressed to allow for the more rapid and accurate studies of bioaerosols without the dependence on culture techniques through the use of PCR. This type of technique has been applied to other environments for air analysis and needs to used in food-production environments as well.

The research manuscript has provided one technique for the evaluation of RTE meats contaminated with *L. monocytogenes* bioaerosols. These methods are slight modifications of standards. They have shown to be an effective means to assess to risk to product, and can continued to be used in future work. The results of the research are counterintuitive and more work is needed to determine the risk to RTE meats. In general, more work is needed in all aspects of bioaerosols of food-production, especially RTE products. The best approach for this future work is multidisciplinary, including microbiologists, food scientists, engineers and food processors