Fluorescence in situ hybridization-based detection of Salmonella spp. and Listeria monocytogenes in complex food matrices

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Fluorescence in situ hybridization-based detection of *Salmonella* spp. and *Listeria monocytogenes* in complex food matrices

by

Bledar Bisha

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Current methods for detection of *Salmonella* spp. and *Listeria monocytogenes* in food are culture-based methods and require performing numerous steps, between preenrichment, enrichment, selective plating, identification, and confirmation. Conducting these procedures can take several days; they require extensive manual labor and large amounts of media and reagents which can increase the cost of the testing. Molecular-based rapid high throughput methods can present a valid alternative to these methods, allowing for timely and sensitive detection of these bacterial pathogens before the contaminated products can reach the consumer, helping to prevent the occurrence of foodborne listeriosis and salmonellosis. Fluorescence *in situ* hybridization (FISH) is a sensitive and robust molecular method that uses sequence-specific rRNA-targeted fluorescently-labeled oligonucleotide probes to specifically label whole, permeabilized bacterial cells. When coupled with fluorescence microscopy or flow cytometry for analysis, FISH can be a powerful tool for detection of bacterial pathogens in food.

My hypothesis was that we could develop rapid and sensitive FISH-based methods for detection of these two pathogens in complex food matrices. My research concentrated on four objectives: 1. Optimize use of existing FISH probes and hybridization conditions for detection of *Salmonella* spp. and *Listeria monocytogenes*; 2. Develop pre-analytical food sample preparation methods compatible with downstream approaches for whole-cell detection; 3. Utilize the results of objectives 1 and 2 to develop FISH-based assays for detection of *Salmonella* spp. and *L. monocytogenes* in foods; and 4. Establish the ultimate detection sensitivity of the developed methods.
Specifically, optimal combinations of existing *Salmonella*-specific probes were developed and applied for rapid (15 min) hybridizations of target cells. Use of these probe cocktails was integrated with pre-analytical sample preparation steps, including tangential flow filtration, adhesive tape sampling and immunomagnetic separation to enable sensitive detection of *Salmonella* spp. in complex food systems via flow cytometry or fluorescence microscopy. The food systems studied included alfalfa sprouts, fresh produce (tomatoes, jalapeño peppers, spinach, cilantro), and peanut butter. Pre-analytical sample preparation using pulsification also improved the signal-to-noise ratio for cytometric detection of *Listeria monocytogenes* in pork frankfurters via flow cytometry following FISH. In addition, use of the Pulsifier™ enabled detachment of surface-bound *L. monocytogenes* cells into minimal volumes of diluent, obviating the need for the subsequent cell concentration steps typically required prior to detection.

Results from this work suggest that, when paired with effective methods for upstream food sample preparation and with downstream analytical methods such as flow cytometry and fluorescence microscopy, FISH-based methods have great potential for rapid molecular detection of *Salmonella* spp. and *L. monocytogenes* in foods.
CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

Present methods for detection of *Salmonella* spp. and *Listeria monocytogenes* in food are culture-based and require performing numerous steps, between preenrichment, enrichment, selective plating, identification and confirmation. These lengthy procedures can take several days to carry out and are costly, requiring extensive manual labor and large amounts of media and reagents.

In light of the current fast-paced production and distribution practices of food products by the food industry, the development and application of alternative rapid methods for detection of *Salmonella* spp. and *L. monocytogenes* is needed to assure the effective and timely detection before contaminated food can reach the consumer. Early detection can help avoid the transmission of foodborne disease, lower the financial burden on the health care system, as well as reduce economic losses to the food industry, which stem from costly product recalls, loss of reputation, and litigation costs.

Molecular-based rapid methods can present a valid alternative to conventional detection methods, allowing for fast and sensitive detection of these two bacterial foodborne pathogens. Methods such as polymerase chain reaction (PCR) or immunassays are already finding wide acceptance across the food industry, since they provide faster analysis of foods for presence of these pathogens. However, these methods are still hindered by problems that prevent them from fully replacing traditional culture methods. PCR is prone to interference from inhibitors such as glycogen, calcium ions, phenolics, fat, and other organic compounds which are abundant in food. This requires careful and effective sample preparation, which
can remove interfering inhibitors from samples prior to analysis. Several steps are needed to perform these procedures, as nucleic acids need to be extracted before running PCR reactions, creating the opportunity for possible contamination. Also, amplification of sequences from non-viable cells is possible using conventional PCR, even though this pitfall has been overcome in an emerging PCR format, RT-PCR which uses RNA as the diagnostic target. Immunoassays on the other hand can be prone to cross-reactivity and low sensitivity. Both of these methods are still dependent on enrichment steps to propagate initial target cell numbers to levels detectable by the analytic method being employed. In addition, reagents can be expensive, increasing the overall cost of the microbiological analysis.

Fluorescence in situ hybridization (FISH) is a sensitive and robust molecular method that uses sequence-specific rRNA-targeted fluorescently-labeled oligonucleotide probes to specifically hybridize whole permeabilized bacterial cells. FISH protocols are easy to perform, extraction of nucleic acids is not required and probes and reagents needed to conduct this procedure are not costly. The natural abundance of ribosomes in an actively growing bacterial cell makes rRNA an attractive diagnostic target, assuring acceptable fluorescence signals, which facilitate detection even against naturally autofluorescent backgrounds. Recent developments in probe technology, such as peptide nucleic acid (PNA) probes or self-ligating (molecular beacons) probes have further improved the hybridization kinetics and decreased the number of preparatory steps needed to perform hybridizations, ultimately allowing for better signal-to-noise ratios and facilitates real-time analysis.

FISH can be coupled with fluorescence microscopy or flow cytometry to accomplish detection without need for cultivation, creating the basis for rapid and specific detection of bacteria. Fluorescence microscopy is a simple technology, which can be readily used by most
food microbiology labs; however, finding low numbers of target cells by microscopy can be
tedious and may require analysis of numerous microscopic fields. Typical flow cytometers
can analyze and characterize thousands of bacterial cells each second allowing for speeds of
analysis that can not be achieved by fluorescence microscopy. In addition, this technology is
capable of collecting multiparametric data as scatter characteristics and probe-conferred
fluorescence are collected and recorded from single bacterial cells illuminated by lasers. The
scatter characteristics can ultimately be correlated to intrinsic bacterial cell characteristics
such as cell size, volume and surface roughness, while specific fluorescent labeling of unique
diagnostic targets in bacterial pathogens allows differentiation from non-target microflora.
Pioneering work from Donnelly and Baigent in 1986 showed that combining flow cytometry
with *Listeria*-specific fluorescent antibody labeling permitted rapid detection of *L.
monocytogenes* in milk. More studies on cytometric detection of bacterial pathogens
followed, however the high cost of instrumentation has kept cytometry out of reach of food
microbiology labs. Recent developments in cytometric instrumentation have led to a decrease
in prices, and more user-friendly, semi-automated instruments some even capable of multi-
color analysis, are now commercially available. There is need for development of FISH-
based protocols, which combined with flow cytometric end-point detection, will provide an
alternative rapid detection technique to the food industry for detection of *L. monocytogenes*
and *Salmonella* spp.

My hypothesis was that we could develop rapid and sensitive FISH-based methods
for detection of these two pathogens in complex food matrices. My research concentrated on
four objectives: 1. Optimize use of existing FISH probes and hybridization conditions for
detection of *Salmonella* spp. and *Listeria monocytogenes*; 2. Develop pre-anayltical food
sample preparation methods compatible with downstream approaches for whole-cell
detection; 3. Utilize the results of objectives 1 and 2 to develop FISH-based assays for
detection of *Salmonella* spp and *L. monocytogenes* in foods; and 4. Establish the ultimate
detection sensitivity of the developed methods.

**DISSERTATION ORGANIZATION**

This dissertation is a compilation of work that includes a literature review (chapter 2)
pertaining to the research described here as four journal articles (chapters 3-6) followed by
general conclusions (chapter 7). It is the author’s intent to submit chapter 6 to Applied and
Environmental Microbiology. Chapter 3 has been accepted for publication by Biotechnology
Journal. Chapter 4 was published in Applied and Environmental Microbiology. It is the
author’s intent to submit chapter 5 to Food Microbiology. These papers have been presented
in three Annual General Meetings of the American Society for Microbiology and the United
FreshTech Meeting in support of our involvement in USDA Multi-State Research Project S-294 (Postharvest Quality and Safety in Fresh-Cut Vegetables and Fruits). Appendix A
contains a journal paper submitted to Journal of Food Protection, in which the author of this
dissertation was responsible for determination and characterization of the antimicrobial
activity of grape seed extract via staining with fluorescent viability stains followed by
fluorescence microscopy and flow cytometry. References can be found at the end of each
chapter with exception of this one and chapter 7, and follow the format of the aforementioned
journals.
CHAPTER 2. LITERATURE REVIEW

GENUS LISTERIA: LISTERIA MONOCYTOGENES

Historical Background

Listeria monocytogenes was first described by Murray et al. in 1926 (Murray et al., 1926). Following investigation of a disease that was causing death in rabbits, he isolated from their blood a bacterium that he believed had not been previously described. Murray observed that this bacterium caused large mononuclear leucocytosis, and assigned the temporary name of “Bacterium monocytogenes”. In 1927, Pirie isolated an organism from gerbils in South Africa that he decided to call “Listerella hepatolytica” (Pirie, 1927). Later it was determined that these were in fact, the same organism, leading Murray and Pirie to name the bacteria “Listerella monocytogenes”, which was later changed to Listeria monocytogenes.

Until 1948, Listeria monocytogenes was the only species comprising the genus Listeria. Subsequently that year the species L. denitrificans was included in the genus (Sohier et al., 1948), followed by L. grayi in 1966 (Larsen and Seeliger, 1966), L. murrayi in 1971 (Welshimer and Meredith, 1971), L. innocua in 1981 (Seeliger, 1981), L. ivanovii in 1985 (Seeliger et al., 1984), L. welshimeri in 1983, and L. seeligeri in 1983 (Rocourt and Grimond, 1983). After Stuart and Welshimer used DNA hybridization methods to show a high genomic homology between L. grayi and L. murrayi (Stuart and Welshimer, 1973), they proposed that these two species should be merged into only one. Listeria denitrificans was also excluded from the genus Listeria subsequent to 16s rRNAstudies that revealed differences with the other species of the genus Listeria and similarities with the coryneform bacteria (Rocourt et al., 1987).
Taxonomy of the genus *Listeria*

Currently six species comprise the genus *Listeria*: *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, and L. grayi*. This classification was based on DNA homology, 16s rRNA sequencing homology, chemotaxonomic characteristics, and multilocus enzyme analysis (Rocourt, 1999).

**Morphology and metabolism of genus *Listeria***

Listeriae are small gram-positive rods (0.5µm in diameter and 1-2µm in length), but they can also sometimes appear as cocci with a diameter of 0.5µm. They are nonspore-forming bacteria and they do not form capsules (Seeliger and Bokenmühl, 1968; Rocourt, 1999). Members of this species are motile by means of peritrichous flagella when they are cultured at 20-25°C but they show very weak movement or none whatsoever when grown at 37°C (Galsworthy et al., 1990; Rocourt 1999). They are catalase-positive (some strains were shown to not posses this characteristic), oxidase negative, aerobic, microaerophilic, and facultatively anaerobic. They use the Embden-Meyerhoff pathway to catabolize glucose aerobically or anerobically (Seeliger and Jones, 1986; Rocourt, 1999)

**Growth characteristics**

**pH.** Optimum pH for growth of *L. monocytogenes* ranges between pH 6 and 8. The minimum and maximum pH in which this pathogen can survive varies by strain, but some strains can survive in pH as low as 4.1 and as high as 9.6 (Jay, 2000).
**Temperature.** According to *Bergey’s Manual of Systematic Bacteriology* (Seeliger and Jones, 1986) the minimum temperature in which *L. monocytogenes* can grow is at 1°C. Growth temperatures for two strains of *L. monocytogenes* serotype 1/2 were shown to be as low as 0.5°C, but the minimum growth temperature of 1.1°C ± 0.3 was calculated as the mean of the minimum growth temperatures for 78 strains of *L. monocytogenes* (Juntila et al., 1988). The maximum temperature in which *Listeria* spp. can grow is about 45°C (Jay, 2000).

**Water activity (aw).** Minimum aw for growth of *L. monocytogenes* in foods is as low as 0.90 in certain conditions (Lou and Yousef, 1999).

**Selected virulence factors of *L. monocytogenes***

The PrfA virulence island harbors the majority of the genes that control virulence factors involved in the pathogenesis process of *L. monocytogenes* (Kuhn and Goebel, 1999). Internalin (InlA) plays an important role in the adhesion of the pathogen in the epithelium of the gut (Gaillard et al., 1991). Listeriosin O (LLO) is believed to be an important virulence factor altogether with phospholipase C, making possible the escape of *L. monocytogenes* from the phagosome in the phagocytes (Kuhn and Goebel, 1999). ActA regulates the cell to-cell spread of the pathogen during invasion of the host cells, making it possible for *L. monocytogenes* to invade new cells (Mounier et al., 1990).

**Listeriosis in humans***

Various researchers have isolated *L. monocytogenes* strains from the fecal material of healthy, non-pregnant human carriers. The reported prevalence was especially high in persons who were in continuous contact with food or in laboratory workers (Bojsen-Møller,
Listeriosis is often manifested in non-pregnant subjects with underlying immunosuppressive conditions. Invasive disease can occur with complications in patients that are suffering from lymphoreticular malignancies, elderly people under therapy to suppress their immunity, persons affected by AIDS, and in other conditions that lower the natural immunity (Louria et al, 1967; Simpson et al., 1967; Buchner and Schneier, 1968; Bizet et al., 1989; Paul et al., 1994). Typical severe manifestations of the invasive form of the disease include sepsis, meningitis, encephalitis, and endocarditis (Bassan, 1986; Gellin et al, 1991; Slutsker and Schuchat, 1999).

Pregnancy is another high-risk factor favoring the onset of this disease, and listeriosis during the third trimester of the pregnancy is better documented, even though listeriosis may occur in all stages of the pregnancy (Bortolussi, 1990; Slutsker and Schuchat, 1999). In these women, signs that are similar to the common flu can be produced, and the condition is present in about two thirds of the infected subjects (Bortolussi, 1990; McLauchlin, 1990). Transplacental infection of the fetus may occur in pregnant women who are experiencing bacteremia, but also the vaginal route of infection is possible. The intrauterine infection of the fetus could result in stillbirths (Slutsker and Schuchat, 1999). Neonates can become infected with *L. monocytogenes in utero* and express the so-called early-onset form of listeriosis with more cases of sepsis then meningitis soon after birth. However the clinical form of the disease that is caused from transmission of the pathogen during passage in the birth canal or probably nosocomially is manifested weeks after birth with meningitis as a clinical sign (Gellin et al., 1991; Slutsker and Schuchat, 1999). There is also evidence that febrile gastroenteritis can occur in healthy hosts (Schlech, W.F., 1997). Serotypes 1/2a, 1/2b, and 4b are associated with more than 95% of human listeriosis cases (Graves et al., 1999).
Selected Outbreaks

From early August 1998 to January 6, 1999, a multistate outbreak involving rare isolates of serotype 4b, which shared an unusual pattern by ribotyping methods and pulsed-field gel electrophoresis (PFGE), caused 50 illnesses and 8 deaths, including 2 stillbirths. Four months after the initial start of the outbreak, the strain involved was isolated from an open package of hot dogs. The company issued a voluntary recall (CDC, 1998). From May 2000, four deaths, three miscarriages and twenty-nine sicknesses were linked to the consumption of contaminated deli meats that included turkey products (CDC, 2000). Eight perinatal and twenty-one nonperinatal cases were reported. On December 12, Cargill Turkey Products, Inc. (Waco, Texas) ceased marketing ready-to-eat foods and two days later issued a voluntary recall for processed turkey and chicken deli meat that could have been contaminated. Another outbreak occurred in the Northeastern United States causing 46 cases, 7 deaths and 3 miscarriages in eight states (CDC, 2002a). The outbreak was linked to sliced turkey deli meats marketed by the company Pilgrim’s Pride Foods, which recalled 27.4 million pounds of poultry products. *L. monocytogenes* was isolated from one intact food product, and from 25 environmental samples. The food isolate was different from the one isolated from the clinical cases, but two of the environmental samples shared indistinguishable PFGE pattern to the outbreak isolates.

Distribution of *Listeria monocytogenes*

*L. monocytogenes in the natural environment.* *L. monocytogenes* are ubiquitous bacteria that can be naturally found in soil, water, and plants, especially those that are
decaying (Rocourt and Seeliger, 1985). \textit{L. monocytogenes} has been isolated from soil, and it has been demonstrated that damp soils with higher moisture content are more likely to be a good survival environment for this organism (Welshimer, 1960; Fenlon et al., 1996).

According to Fenlon (1999), and based on the evidence of studies on the environmental distribution of \textit{L. monocytogenes}, it can be concluded that soil is not a natural reservoir for this pathogen, but more likely contamination can occur from one of the following sources: a) during harvesting of the grass from the sheath area, b) decayed plant materials, and c) contamination of the soil by animal manure. Contamination of the vegetation seems to occur during harvesting, because far higher numbers of \textit{L. monocytogenes} have been found in harvested plants compared to the plants that have not yet been harvested, linking the contamination again with the presence of the pathogen in the sheath area of the plants (Weiss and Seeliger, 1975; Farber et al., 1989; Fenlon et al., 1996).

Animal feeds have been implicated in harboring \textit{L. monocytogenes}. Silage has been shown to be a good environment for survival and multiplication of these bacteria, especially when the quality of this animal feed was low and pH>4.5 (Grønstøl, 1979). Infiltration of air in improperly baled silage can create a good growth environment for mold, which subsequently can cause a rise in pH of the silage and make it possible for \textit{L. monocytogenes} to grow to high numbers (Fenlon, 1986). \textit{L. monocytogenes} can survive and multiply for years in silage when pH rises to an unacceptable level (Dijkstra, 1971; Fenlon et al., 1996).

Fecal material is a reservoir for \textit{L. monocytogenes}. Gray and Killinger (1966) state that \textit{L. monocytogenes} can be found in the feces of 37 mammals. Humans can be symptomatic or asymptomatic carriers, with shedding rates of 1.8-9.0\% among healthy individuals (Ralovich, 1984). Consumption of contaminated feed has been linked to the
The presence of *L. monocytogenes* in animal feces, where lower incidences of excretion of the pathogen were observed in grazing sheep and cattle, compared to the ones that were fed silage (Fenlon et al. 1986; Low et al., 1995). Some increase in the excretion rates of *L. monocytogenes* in stressed animals has been showed by some authors (Ralovich, 1984; Fenlon et al., 1996). Several studies have addressed the spread of *L. monocytogenes* in water and sewage and found the pathogen to be widespread in those environments (Watkins and Sleath, 1981; Dijkstra, 1982; Frances et al., 1991). To date no documented evidence exists of waterborne transmission of listeriosis in humans, but listeriosis has been experimentally transmitted in sheep (Gray et al., 1956; Fenlon 1999). Transmission of *L. monocytogenes* from sewage and water to certain foods is a possibility. An example of that are the high rates of infection that have been shown in mussels and oysters in ocean areas where contaminated sewage was deposited (Soontharanont and Garland, 1995).

**GENUS SALMONELLA**

**Historical Background**

Early work in 19th century by clinical pathologists in France following the observation of intestinal ulcerations in humans, which was subsequently linked to an infectious agent, was the first step towards further work leading to the isolation and characterization of the typhoid bacillus, the causative agent of typhoid fever (LeMinor, 1981; D’Aoust 1989). An independent group in the United States, Salmon and Smith (1885) isolated the formerly known *Bacillus cholera-suis* (now *Salmonella enterica* ser. *Cholerasuis*) from swine with hog cholera (LeMinor, 1981) subsequent development of
suitable serological detection techniques in the beginning of the 20\textsuperscript{th} century. Using the Kaufman-White antigenic scheme, more than 2,451 serovars are included in this genus (Popoff et al., 2004; Andrews and Bäumler, 2005; D’Aoust and Maurer, 2007).

**Taxonomy of the genus *Salmonella***

The taxonomy of genus *Salmonella* has undergone many changes over the years before coming to the current classification of two recognized species within the genus, *Salmonella enterica* and *Salmonella bongori* (former subgroup V). Most serovars are included in the species *S. enterica* with only a few serovars included in the other species. *S. enterica* is grouped into six subspecies based on genomic relatedness and biochemical tests. These subspecies are delineated by names or roman numerals as subs. *enterica* (I), subs. *salamae* (II), subs. *arizonae* (IIIa), subs. *diarizonae* (IIIb), subs. *houtenae* (IV), and subs. *indica* (VI). Under this classification the original name of *Salmonella typhimurium* as described by the Kauffmann scheme (Kauffmann, 1960), becomes *Salmonella enterica* subspecies *enterica* serovar Typhimurium, or in a shortened form *S. Typhimurium*. Serological identification is performed using antisera for somatic (O) lipopolysaccharides (LPS) on the surface of the outer membrane, flagellar (H) antigens, and in the case of serovars Typhi, Paratyphi C, and Dublin the capsular (Vi) antigen (Brenner et al., 2000; Andrews and Bäumler, 2005; D’Aoust and Maurer, 2007). For epidemiological analysis, phage typing can be used to further differentiate between isolates within serovars allowing determination of epidemic clones (Anderson et al., 1978).
Morphology and metabolism of genus *Salmonella*

*Salmonella* spp. are rod-shaped, gram negative and facultatively anaerobic bacteria. Except for *Salmonella* ser. Pullorum and ser. Gallinarum, all the other members of the genus are motile by means of peritrichous flagella. Salmonellae are chemorganotrophic, grow best at 37°C and produce gas and acid when they ferment glucose and other carbohydrates. Furthermore they are oxidase negative, catalase positive, grow on citrate as a sole carbon source, can generate hydrogen sulfide, do not hydrolyze urea and decarboxylate lysine and ornithine (D’Aoust and Maurer, 2007).

**Growth characteristics**

**pH.** Optimum for growth of *Salmonella* is 6.5-7.5, however it has been shown that it can grow over a wide range of pH, from 4.5 to 9.5 (D’Aoust and Maurer, 2007).

**Temperature.** Optimal growth temperature is established as 35-37°C at neutral pH (Andrews and Bäumler, 2005). *S. Typhimurium* and *S. Enteritidis* have been shown to posess the ability to grow at low temperatures in foods stored at 2-4°C, as they have been shown to adapt to such low temperatures if previously exposed (D’Aoust et al., 1975; Airoldi and Zotola, 1988). While information on the upper temperature limit of growth remains to be elucidated, it has been shown that *S. Typhimurium* can develop mutations that can allow it to grow at temperatures as high as 54°C (Droffner and Yamamoto, 1992; D’Aoust and Maurer, 2007)

**Water activity (aₜ).** Generally it is recognized that *Salmonella* does not grow in aₜ < 0.93. Anaerobic conditions and increases in temperature have been shown to induce tolerance to high salt concentrations (D’Aoust, 1989; Anonymous, 1986; D’Aoust and Maurer, 2007).
Selected virulence factors of *Salmonella* spp.

Attachment and invasion of the enterocytes and M cells which is an important factor in the pathogenesis of *Salmonella* infection is regulated by the *inv* (invasion) pathogenicity island consisting of 30 genes. This locus regulates the process of signaling the host cells inducing changes that prepare the invasion by the pathogens, such as release of calcium ions and changes in conformation of the eukaryotic cell cytoskeleton. The internalization in the eukaryotic cell is mediated by the *Salmonella* tyrosine phosphatase SptP (Fu and Galan, 1998; Garcia-del-Portillo and Finlay, 1994; Ginocchio et al, 1994; D’Aoust and Maurer, 2007). Survival of *Salmonella* inside the phagocytes and epithelial cells is regulated by the SPI2 pathogenicity island and escape from the phagosomes by the SPI3 island. Plasmids encoding virulence, the fimbrial antigens, the LPS layer and porins also play an important role in the pathogenesis of *Salmonella* (Cirillo et al., 1998; Blanc-Potard and Groisman, 1997; D’Aoust and Maurer, 2007).

Salmonellosis in humans

More than 99% of human salmonellosis cases are caused by *S. enterica* subs. *enterica* (Aleksic et al., 1996). The disease is manifested clinically with typhoid fever, or in the case of non-typhoid salmonellosis with enterocolitis or bacteremia (Andrews and Baumler, 2005; D’Aoust and Maurer, 2007). Enteric fever is caused by serovars which use humans as reservoirs (*S. enterica* ser. Typhi, Paratyphi B, and Paratyphi C), thus disease is not considered as transmitted via food and it has been eradicated from the U.S. (Andrews and Baumler, 2005). Salmonellosis caused by *S. Dublin* and *S. Cholerasuis* manifests itself with
bacteremia. Spiking fever is a possible sign. Other serovars cause mainly enterocolitis and
disease manifests itself with signs that include nausea, vomiting and diarrhea one to three
days following infection. Necrosis may develop as a result of inflammation of the ileum and
colon and other complications such as septicemia may appear although rare (Saphra and

Selected Outbreaks

From February 1, 2001 to May, 2001 a multistate outbreak of salmonellosis
associated with consumption of alfalfa sprouts occurred in the U.S., which caused 32
illnesses, with the greatest number of cases recorded in California. An indistinguishable PFGE
pattern identified a S. Kottbus cluster which was traced back to the same production plant
that had imported the seeds from Australia in 2000. The review of the documentation showed
that seeds had been heat treated and decontaminated with sodium hypochlorite at 2,000 ppm
(CDC, 2002b).

Peanut butter was the source of an U.S. outbreak of salmonellosis caused by
S. Tennessee in 2006-2007, which infected a total of 628 people from 47 states. Identical
PFGE patterns were obtained from patients and unopened and opened jars of brand name or
generic peanut butter produced from the same Georgia plant. Product from this plant was
also exported to 70 different countries. The plant was eventually shuttered and the products
recalled. The inclusion of peanuts, peanut paste or peanut butter as an ingredient in a number
of products produced by other companies led to voluntary recalls to be issued for literally
thousands of products. The source of the contamination of the product remained unknown
(CDC, 2007).
As of August 25, 2008, a total of 1442 persons had been infected with a S. Saintpaul strain which was reported to have caused two deaths. Difficulties arose in establishing the vehicle of the outbreak and initially it was thought that Roma tomatoes had been the source of the outbreak causing unnecessary product recalls and losses to the industry. Ultimately it was established that the contaminated products causing the outbreak were in fact jalapeño peppers imported from Mexico (CDC, 2008).

Peanut butter emerged again recently as a vehicle for foodborne salmonellosis in the U.S., when peanut butter and peanut butter containing products were associated with an outbreak of salmonellosis caused by S. Typhimurium which infected 529 persons from 43 states. Two clusters of identical PFGE patterns were assessed and finally traced back to the Peanut Corporation of America’s plant in Blakely, Georgia which was shut down due to its substandard practices. Peanut butter jars were used in school programs and peanut butter paste employed by other processors to produce peanut butter crackers and other products. All those products were also recalled (CDC, 2009).

**Reservoirs of Salmonella spp.**

Salmonella serovars are found in a great number of vertebrates, from reptiles to mammals and birds, with S. enterica subs. I mainly isolated from birds and mammals. It has been suggested that the reason that subspecies I is found more often in the food supply is also the reason why it is isolated more often from foods and clinical isolations and is associated with foodborne disease. *Salmonella* serovars can cause disease in food animals, however these animals can often be asymptomatic carriers, shedding salmonellae via the fecal route, which allows for transmission of the disease from animal to animal, or via contamination of
feed, water, and equipment (Aleksic et al., 1996; Olsen et al., 2000; Hurd et al., 2001; Crump et al., 2002; Andrews and Bäumler, 2005).

METHODS FOR DETECTION OF SALMONELLA SPP., LISTERIA SPP. AND LISTERIA MONOCYTOGENES IN FOOD

CULTURAL METHODS

Listeria spp. and Listeria monocytogenes. The two most used methods used to detect L. monocytogenes are the US Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) method (USDA, 2002) and the Federal Drug Administration (FDA) method (Hitchins, 2000). The USDA-FSIS method involves a two-stage enrichment in modified University of Vermont Enrichment Broth (UVM), supplemented with acriflavin and nalidixic acid. Fraser broth is used for secondary enrichment followed by plating onto Modified Oxford (MOX) agar supplemented with colistin sulphate and moxalactam. Subsequent transfer in Horse Blood Overlay (HL) agar for further identification and confirmation is performed following growth in Brain Heart Infusion (BHI) broth via a series of biochemical and cultural tests, among which testing for motility in a Motility Test Medium, for production of esculinase on Bile Esculin Agar, gram stain, oxidase test, catalase test. Tests such as hemolysis, acid production from D-xylose, L-rhamnose, α-methyl-D-mannoside, and mannitol are used to differentiate species of the genus Listeria (Rocourt et al., 1983, Rocourt 1999). L. monocytogenes and L. seeligeri produce narrow zones of β-hemolysis, L. ivanovii produces wide zones of β-hemolysis, while L. welshimeri, L. innocua and L. grayi are not
hemolytic. Differences in acid production from D-xylose, L-rhamnose, and α-methyl-D-mannoside differentiate *L. monocytogenes* from *L. ivanovii* (Rocourt, 1999). The Christie, Atkins, Munch, Petersen (CAMP) test is considered as the definitive test for *L. monocytogenes*. The isolate is considered presumptive *L. monocytogenes* if it is CAMP positive with *Staphylococcus aureus* or *Rodococcus equi*, but it does not also determine whether the strain is virulent (McKellar, 1994, Jay, 2000). Typing is performed by serotyping, where thirteen serovars are distinguished within the species *L. monocytogenes*, and the serovars that are more often isolated are 1 and 4, with serovar 4b accounting for 65-80% of all strains. Serovar 4b is more commonly related to outbreaks whereas serovar 1/2 is more commonly found in foods (Jay 2000). Serological typing (serotyping) is used to differentiate strains of *L. monocytogenes* on the basis of the antigenic determinants that are expressed on the bacterial cell surface, including lipotechoic acids, membrane proteins and external organelles like fimbriae and flagella (Graves et al, 1999). The determinants are classified into O and H antigens (Seeliger and Hohne, 1979). Serotyping can be valuable in screening groups of isolates during outbreaks, but otherwise has a poor discriminating power compared to other subtyping methods (Graves et al., 1999).

Bacteriophage typing uses phages to type *L. monocytogenes* strains based on the susceptibility of some bacterial strains to particular phages and not to others (Rocourt et al, 1985). This method has a high discriminating power, and allows for rapid and mass screening when outbreaks occur, but more standardization work needs to be done to limit variability in results between laboratories (Graves et al., 1999).

In addition, Curtiss and Mitchell tested a bacteriocin called monocin that can be used to differentiate *L. monocytogenes* from *L. ivanovii*. The method showed poor discrimination
power (Graves et al., 1999). The discrimination power of this method was improved to a discrimination index of .99 when was used in combination with the phage typing method (Bannerman et al., 1996).

Molecular typing is performed via multilocus enzyme electrophoresis (MEE), chromosomal DNA restriction endonuclease analysis, ribotyping, DNA macrorestriction analysis by PFGE, random amplification of polymorphic DNA, repetitive element-based subtyping, and DNA-based subtyping (Graves et al., 1999; Swaminathan et al., 2007).

The FDA method for isolation of *Listeria* spp. differs slightly from the USDA FSIS method in that it uses *Listeria* Enrichment Broth (LEB) supplemented with acriflavin, nalidixic acid, and cycloheximide for primary enrichment followed by plating onto PALCAM, lithium chloride-phenylethanol-moxalactam (LPM) or Oxford agars. Identification is performed as described for the USDA-FSIS method.

**Salmonella** spp. *Salmonella* spp. is commonly detected using the USDA-FSIS method (Rose, 2001) and the FDA method (Andrews and Hammack, 1998). The FDA method involves preenrichment in lactose broth followed by selective enrichment in Tetrathionate (TT) broth and Rappaport-Vassiliadis (RV) medium. Selective plating is performed onto Bismuth Sulfite (BS) agar, Xylose Lysine Desoxycholate (XLD) agar, and Hektoen Enteric (HE) agar. Biochemical screening is performed by stabs in Triple Sugar Iron (TSI) agar and Lysine Iron Agar (LIA), followed by an array of tests for identification and confirmation. Some of these tests include motility testing, Methyl-Red, Vogues-Proskauer (MR-VP Test), Lysine Decarboxylase test, and ability to ferment sugars. Serological testing is performed using antisera for the O and H antigens. While the FSIS method uses the same
scheme for biochemical screening, identification and confirmation, and serological typing, it differs in the choice of media for preenrichment and selective plating. It uses Buffered Peptone Water (BPW) for preenrichment and Xylose Lactose Tergitol™ 4 (XLT4) agar or Double Modified Lysine Iron agar (DMLIA) and Brilliant Green Sulfa (BGS) agar for selective plating.

While cultural-based methods are widely accepted and used for isolation, detection and confirmation of *Salmonella* spp. and *L. monocytogenes*, it takes from 5-7 days to obtain results which limits control over distribution of contaminated food products.

### RAPID METHODS

There is a continued need in food microbiological analysis for methods that can provide comparable results to traditional methods, while permitting the possibility of speedier analysis with fewer preparation steps. This has led to extensive research in development of rapid tests (Feng, 2007). The desired properties of these alternative rapid methods have been discussed before and serve as guidelines for further development of such methods (Swaminathan and Feng, 1994; Fung, 1995; Notermans et al., 1997; deBoer and Beumer, 1999; Brehm-Stecher and Johnson, 2007; Feng, 2007). Such methods should impart rapidity, high accuracy with high specificity and sensitivity, be reproducible, easy to perform, be validated against standard methods preferably in “real life” scenarios found with naturally-contaminated samples, capable of being automated, minimally affected by interference from the sample matrix and of course, inexpensive. It is clear that such ‘ideal’
detection method does not yet exist, however many of the alternative rapid methods which are described below fulfill several of these desired characteristics.

Diagnostic targets chosen for the development of rapid tests for detection include rRNA, mRNA, and chromosomal DNA for nucleic acid-based methods, somatic, capsular and fimbrial antigens for immunoassay immunologic-based methods or virulence factors such as as expressed proteins important to bacterial pathogenesis such as phospholipase C in *L. monocytogenes* (Brehm-Stecher and Johnson, 2007)

**Immunological methods**

Immunological methods are built upon the premise of the specific binding of an antigen in a bacterial cell to monoclonal or polyclonal antibodies specific for those antigens. Several types of immunoassays are used and Feng (2007) groups them into: latex agglutination assays, gel immunodiffusion, immunomagnetic separation, Enzyme-Linked Immunosorbent Assay (ELISA) and immunoprecipitation. The direct fluorescent antibody assay also is another format of immunoassay which can be combined with fluorescence microscopy for detection of bacterial pathogens.

The most used method is the “sandwich” ELISA, which is performed by immobilizing antibodies onto a solid phase, capturing antigens from target cells, adding of a second antibody conjugated with an enzyme, followed by colorimetric determination of the reaction when the enzyme substrate is added (Fratamico and Bayles, 2005). Gel immunodiffusion assays find use in the *Salmonella* 1-2 test, which are small simple L-shaped devices that detect motile salmonellae, which migrate from their enrichment broth to contact
the flagellar antibody in a different compartment and form a strip which indicates the positive result (Feng, 2007).

Immunomagnetic separation uses selective antibodies specific for antigenic targets in the bacterial cell. These antibodies are conjugated to magnetic beads, and can be used to extract target cells when a magnetic field is formed, which allows for removal of the background inhibitors resulting in “sample clean-up”.

Immunoprecipitation (or lateral flow) assays work by correlating the positive reaction to precipitation of precipitable material such as colloidal gold conjugated to specific antibodies when they react with the present antigen in the target cell. A color reaction indicates a positive result (Fratamico and Bayles, 2005).

For latex agglutination assays, antibody coated latex beads are used and a positive reaction is perceived when agglutination occurs in the presence of the antigen (Feng, 2007).

Most immunologic-based assays target *Listeria* spp. and not *L. monocytogenes* (Churchill et al., 2006). The sandwich ELISA has been used to detect *L. monocytogenes* targeted with two flagellar monoclonal antibodies with a sensitivity of $10^5$ CFU ml$^{-1}$, however the assay remained genus specific (Kim et al., 2005). VIDAS, a commercial enzyme-linked fluorescent assay detection system, detected heat killed *L. monocytogenes* following a three day procedure with an accuracy of 97% and detection sensitivity of $10^4$-$10^5$ cells ml$^{-1}$ (Sewell et al., 2003). Mansfield and Forsythe (2000) combined immunomagnetic separation and ELISA to detect *Salmonella* spp. in skimmed milk powder within 24 hr using monoclonal antibodies for the *Salmonella* LPS but could not achieve a detection sensitivity higher than $10^5$-$10^6$ CFU ml$^{-1}$. 
There are several potential advantages and disadvantages associated with use of immunological detection methods. The fact that antibody-based methods are indeed used in confirmation in conjunction with culture methods is an advantage, because it facilitates acceptance of immunological rapid detection methods. Immunological methods are prone to semi-automation and full automation as is the case of the commercially available VIDAS system, which reduces hands-on time, increases sample throughput and decreases time needed to release the product. Another clear advantage is the relatively short assay time, which for ELISA can be two to three hours (Baylis, 2003; McCarthy, 2003). Among disadvantages, the low sensitivity (~10^5 cells ml^-1 for ELISA) is one of the most important. This increases the total assay time, because rapid immunological techniques basically need to be coupled with enrichment steps that can take between 24-48 hr to perform. Specificity is limited by potential cross-reactivity of antibodies with non-target microflora, which when closely-related to the target cells might express the same antigens targeted by the specific monoclonal or polyclonal antibodies. Food components can interfere with the assay, as is the example of peroxidases present in food, which might cause a positive reaction even in absence of the target. Lastly, the cost of immunoasays is still higher than that of cultural methods. Results obtained by immunological methods are regarded as presumptive (McCarthy, 2003).

**Nucleic acid-based methods**

Nucleic acid methods employ techniques to specifically recognize and amplify nucleic acid material (DNA, rRNA, mRNA) in the target cells which is unique to those cells. Important nucleic acid-based methods are polymerase chain reaction (PCR) and its
variations, nucleic acid sequence-based amplification (NASBA), DNA hybridization and fluorescence in situ hybridization (FISH). FISH will be covered in more detail in a later section.

PCR’s basic mechanism involves the use of a DNA polymerase enzyme and oligonucleotide primers to amplify a specific region of DNA to multiple copies. This is done through a series of repeated cycles (denaturation at 95°C, annealing at 55°C and extension at 72°C) in the presence of cofactors and Mg\(^{2+}\). Primers can be designed to target rRNA encoding genes and genes that encode proteins, including virulence factors, allowing the amplification of target sequences by 10\(^6\)-fold in a matter of hours (Fratamico and Bayles, 2005; Brehm-Stecher, 2007; Feng, 2007). For PCR of \textit{L. monocytogenes} the most commonly used diagnostic target has been the \textit{hlyA} gene, which encodes LLO (Ryser et al., 1996; Blais et al., 1997, Norton et al., 2001). Other targets have included the \textit{iap} gene, which encodes a protein associated with invasion (Cocolin et al., 2002; Schmid et al., 2003), 16S rRNA (Call et al., 2003), and \textit{inlB}, which encodes internalin B (Ingianni et al., 2001; Pangallo et al., 2001). For \textit{Salmonella} spp., the most common diagnostic target employed has been the \textit{invA} gene, which is highly conserved within the genus (Rahn et al, 1992). For specific serovars, diagnostic targets have been sequences encoding antibiotic resistance (Carlson et al., 1999) or phage types (Hermans et al, 2005), as well as genes or sequences encoding surface antigens (Luk et al., 1993; Herrera-Leon, 2004).

Variations to conventional PCR include real-time PCR, conventional or real-time reverse transcriptase PCR (RT-PCR) and conventional or real time multiplex PCR. Real time PCR assays combine the amplification and detection in one step allowing for on-line monitoring and thus speedier detection. RT-PCR uses mRNA as a target and can also be
performed in real time, allowing for determination of cell viability, since mRNA is a very unstable molecule upon cell death. Multiplex PCR allows the amplification of two or more target sequences via use of two or more primer sets, which permits detection of several bacterial cells in one reaction and can eventually supply more information on the sample analyzed (Fratamico and Bayles, 2005).

Jung et al (2003) were able to detect spiked *L. monocytogenes* in frankfurters with a PCR protocol targeting the inlAB gene, following an enrichment of 16 hours and reaching a detection sensitivity of 10 CFU 25 g⁻¹ (with a limit of detection of 10⁵ CFU ml⁻¹ in pure culture). Simultaneous detection of *L. monocytogenes*, *S. Typhimurium*, and *E. coli* O157:H7 was achieved in fresh produce following 30 hours enrichments with a detection sensitivity of 1 cell ml⁻¹ for *Salmonella* and *E. coli* O157:H7 and 10²-10³ cells ml⁻¹ for *L. monocytogenes* (Bhagwat, 2003). Klein and Juneja (1997) used RT-PCR targeting the *iap* gene transcript to detect as few as 3 cells of *L. monocytogenes* per gram ground beef following a total procedure of 54 hours. Morin et al. (2004) employed multiplex RT-PCR to detect *S. Typhi*, along with *E. coli* O157:H7 with a sensitivity of 30 cells.

DNA hybridization will be referred here as the method applied to bacterial colonies following nucleic acid extraction (whole cell detection will be covered in the section on FISH). DNA hybridization (or dot-blot) is performed by transferring colonies to a solid support, followed by cell lysis, denaturation of the DNA and hybridization. An alternative format uses hybridization in solution and changes in fluorescence signal a positive reaction (Fratamico and Bayles, 2005). An example of the DNA hybridization technique is given by the GeneTrack commercial kit, which uses a dipstick with a capture probe specifically
targeting rRNA encoding genes and is available in *Salmonella* and *Listeria* formats as well as for *Campylobacter* and *E. coli* O157:H7 (Fung, 2002).

NASBA, like PCR, is also applied to extracted nucleic acid, but differently from PCR NASBA is an isothermal reaction which does not require going through amplification cycles and can be performed in tubes not requiring equipment such as a PCR heat block. In addition it offers the advantage of being applicable to mRNA targets allowing for a realistic assessment of viability (Churchill et al., 2006; Fratamico and Bayles, 2005; Feng, 2007). NASBA has been employed to detect *L. monocytogenes* in meats and seafood using 16S rRNA sequence as well as *hlyA* mRNA, allowing for a sensitivity of 10 CFU 60g\(^{-1}\) of meat (Uyttendaele et al., 1995; Blais et al., 1997). This method was also used with the NucliSens kit to detect *S. enterica* in meat, poultry and other foods by targeting mRNA transcribed from the *dnaK* gene (Simpkins et al., 2000; D'Souza and Jaykus, 2003).

There are several potential advantages and disadvantages associated with use of nucleic acid-based methods. Among advantages, speed of analysis can be listed as the major benefit, allowing for a significant decrease in the sample analysis time. Theoretically methods such as PCR are able to amplify even one target sequence logarithmically to a detectable number of copies. Also nucleic-acid based methods show good correlation with culture-based methods (Sanderson and Nichols, 2003). Disadvantages include the need for enrichment which can not be completely overcome, and the interference to the assays such as PCR from inhibitors present in food such as phenolics, polysaccharydes, glycogen, calcium ions, fat an others (Rossen et al., 1992; Powell et al., 1994; Bickley et al., 1996; Liu, 2007). Inability to distinguish between live and dead cells has been listed as a potential disadvantage of nucleic acid-based methods such as PCR and NASBA, where target nucleic acids have been shown
to persist for 30 hours following cell death by heat inactivation (Birch et al., 2001; Sanderson and Nichols, 2003). Other possible disadvantages are: susceptibility to contamination characteristic of amplification-based techniques, degradation of RNA during extraction in RNA-based methods such as NASBA leading to false-negative results, and the large number of manipulation steps needed to perform methods such as NASBA (Sanderson and Nichols, 2003).

**Molecular sub-typing**

Extensive coverage of molecular typing methods of bacterial pathogens for epidemiological surveillance is beyond the scope of this review. PFGE, ribotyping, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and multilocus sequence typing (MSLT) are available tools which can be used in epidemiological surveillance of foodborne pathogens (Churchill et al., 2006). PFGE has been employed as the gold standard for subtyping *Salmonella* (Bender et al., 2001; Barrett and Gerner-Smidt, 2007) while MLST was found to compare favorably to PFGE for subtyping of this pathogen (53). PFGE has also been the common subtyping method for *L. monocytogenes*, while riboprinting, AFLP and MLST (including a modification that allows simultaneous subtyping of three virulence and three virulence associated gene foci-MVLST) have also been used (Zhang et al., 2004; Barrett and Gerner-Smidt, 2007).
**Other approaches and future detection technologies**

Other approaches to rapid detection involve the use of chromogenic agars which is a method based on the culture method; however media contains chromogens which are cleaved from specific enzymes in target cells and produce color changes that indicate a positive reaction and can produce faster identification. Miniturization of biochemical tests is also a rapid alternative to conventional biochemical testing which decreases time and labor needed to perform identification and confirmation tests. Flow cytometry will be covered more in detail in a later section.

Technologies that can be considered as technologies in development include microarrays, which are nucleic acid-based methods and biosensors that are mainly immunologic-based methods. Extensive research has focused on these two approaches for rapid bacterial detection; however they are still prone to low sensitivity and interference from food matrices (Fratamico and Bayles, 2005; Feng, 2007).

**SAMPLE PREPARATION FOR RAPID MOLECULAR DETECTION**

Sample preparation for rapid molecular detection is an important step, which can assure the successful detection if performed properly or complicate the performance of the test. An example of such need for pre-processing of sample would be the employment of PCR to detect pathogenic bacteria in food, which is limited by the small sample size, the presence of inhibitors and need for concentration of the target organism (Bej and Mahbubani, 1994). Concentration, separation and purification of bacterial cells from the complex food matrix in which they are found can effectively aid final detection via rapid detection methods.
and technologies by removing assay inhibitors or concentrating the target cells to detectable numbers (Swaminathan and Feng, 1994; Wilson, 1997; deBoer and Beumer, 1999; Jaykus, 2001; Stevens and Jaykus, 2004). Sharpe (2003) lists four different approaches for detection of bacterial pathogens in foods:

1. Extraction of whole cells with subsequent concentration and identification
2. Detection using cell phenotypes such as serological and enzymological characteristics
3. Chemical extraction as is the case for extracted nucleic acids
4. Direct detection of pathogens in food following transfer of a property or a label to the target cells, which will allow for them to be detected against the background noise supplied by the food sample.

These approaches will have to be considered and validated for each particular detection method and food matrix taking into account the method and sample variability in order to enhance detection capabilities and sensitivity of the detection methods. Stevens and Jaykus (2004) have pointed out three important issues that should be considered in order to overcome problems associated with practical employment of rapid molecular methods for detection of bacteria in food as follows:

1. Pathogen separation from the particulate matter in samples
2. Effective removal of assay inhibitors from the sample
3. Reduction in sample size without compromising cell viability

Several strategies have been developed to address the above-mentioned issues which can be grouped into physical, chemical, physico-chemical, and biological sample preparation methods (Stevens and Jaykus, 2004). An example of a physical method is given by the study of Neiderhauser et al. (1992). This group used differential centrifugation in order to enhance
the PCR detection of *L. monocytogenes* in meat by 1,000-fold, by basically centrifuging food samples at 100 x g to remove the larger particulate matter, followed by centrifugation at 3000 x g to harvest cells. The use of pulsification which uses sample agitation as compared to sample crushing by paddle-like action of homogenization is a modification of a physical sample preparation method that can enhance molecular detection since less debris is generated by this process (Sharpe, 2001). In our work, pulsification significantly enhanced the signal to noise ratio for flow cytometric detection of *L. monocytogenes* on pork frankfurters following pulsification and PNA-FISH when compared to the traditional sample preparation method-homogenization (Bisha and Brehm-Stecher, unpublished data). Other physical sample preparation methods that have been developed include more centrifugation methods (high speed and density gradient), filtration, ultrasound, spraying, gas bubbles, and more (Sharpe, 2003; Stevens and Jaykus, 2004). We have used physical separation and concentration methods such as tangential flow filtration and differential centrifugation to enhance the sensitivity of FISH for detection of *Salmonella* spp. in seed sprouts in combination with flow cytometry or used a simple adhesive tape method to effectively remove and detect *Salmonella* spp. from fresh produce surfaces, subsequent FISH and fluorescence microscopy or flow cytometry (Bisha and Brehm-Stecher, unpublished data; Bisha and Brehm-Stecher, 2009). Chemical methods of separation and concentration have also been used and range from adsorption and desorption methods, dielectrophoresis, and biphasic partitioning (Sharpe, 2003; Stevens and Jaykus, 2004). Pedersen et al. (1998) used a bi-phasic partitioning method employing 5% dextran and polyethylene glycol to effectively detect *L. monocytogenes* and *S. enterica* ser. Berta from smoked sausage via PCR without interference from inhibitors and particulate matter. Physico-chemical methods are a
combination of chemical and physical methods for cell extraction and concentration and an example is given by the employment of metal hydroxides (Stevens and Jaykus, 2004). *L. monocytogenes* and *S. enterica* ser. Enteritidis have been effectively concentrated in reconstituted non-fat dry milk increasing the detection sensitivity in a study involving endpoint detection via RT-PCR (Lucore et al., 2000). Finally, biological methods which are based on immunoaffinity also supply a valid alternative for sample preparation (e.g. immunomagnetic separation). Due to its high specificity, IMS has been incorporated in the official method for detection and identification of *Salmonella* spp. and *E. coli* O157:H7 in foods (AOAC, 1995; Stevens and Jaykus, 2004). Circulating immunomagnetic separation has been a recent development to the more traditional IMS methods, allowing the analysis of large sample sizes. We have been able to combine circulating immunomagnetic separation with FISH and fluorescence microscopy for direct detection of *Salmonella* spp. in peanut butter- a high fat content food, which allowed for decreased interference from fat and non-specific binding of the probe, which can result in high background fluorescence (Bisha and Brehm-Stecher, unpublished data).

**FLUORESCENCE IN SITU HYBRIDIZATION FOR BACTERIAL IDENTIFICATION AND CHARACTERIZATION**

*In situ* hybridization (ISH) was developed as a method that involved hybridization of radiolabeled DNA or 28S RNA to *Xenopus* oocytes followed by detection via microradiography (Pardue and Gall, 1969; John et al., 1969). This method allowed for whole-cell detection via hybridization with nucleic acids within the target cell without
altering the morphological integrity of the cells (Moter and Göbel, 2000). ISH was introduced to bacteriology by Giovannoni et al. (1988), who used radioactively labeled DNA probes targeting rRNA of bacteria with subsequent microscopic examination.

DeLong et al. (1989) introduced the use of fluorescent labeled probes which replaced the radioactive labeled probes for in situ detection of bacteria, hence the name Fluorescence In Situ Hybridization (FISH). Fluorescent probes supplied a number of advantages compared to radioactive labeled probes, among which enhanced safety, improved resolution, reduction in detection steps and due to the possibility of using dyes of different excitation and emission spectra, possibility of detection of several target sequences in one sample.

Oligonucleotide probes are designed in silico by aligning them with known target sequences in databases by using the probe matching tools in resources such as for example the ARB (from latin word ‘arbor’ meaning tree) database (Ludwig et al., 2004) or the Ribosomal Database Project II (RDP-II) (Maidak et al., 2001). Commonly, oligonucleotide probes which are generated in an automatic synthesizer are between 15 and 30 base pairs (bp) long (Moter and Göbel, 2000). For direct fluorescent labeling, probes are commonly labeled with fluorochromes chemically at the 5’ end via an amino linker or less often labeled at the 3’ end enzymatically using a terminal transferase. Typical fluorochromes used for probe labeling are fluorescein-derivatives (e.g. fluorescein isothiocyanate or FITC), rhodamine dyes (e.g. Texas Red) or cyanine dyes like Cy3, Cy5 and Cy7, which typically produce brighter staining (Moter and Göbel, 2000).

The fundamental principle in FISH is the specific hybridization of complementary nucleic acid sequences within whole permeabilized cells via fluorescent labeled probes for detection (Moter and Göbel, 2000). FISH assays are overwhelmingly performed by using
rRNA as a target. This diagnostic target is desired because it is genetically stable, contains both highly conserved and variable regions, and it is present in a high number of copies in each target cell (Woese, 1987; Moter and Göbel, 2000). The fact that rRNA contains both variable and conserved regions allows for development of probes that can differentiate target cells at different taxonomic levels. Probes can discriminate between large taxonomic groups (Archea, Bacteria, and Eukarya) or down to the specific genus or species (Moter and Göbel, 2000; Amann and Fuchs, 2008). While either 5S, 16S and 23S of bacteria can be used as a diagnostic target for oligonucleotide probes, 16S rRNA has more commonly been used for such purpose. The reason for such an occurrence is the availability of large databases for 16S rRNA of bacteria which have been deposited following PCR amplification and sequencing during the recent two decades covering basically about 8,000 species of Bacteria and Archea and amounting to about 500,000 SSU rRNA entries (Amann and Fuchs, 2008). The 5S rRNA contains only ~120 nucleotides and does not provide enough variability for discrimination via hybridization with oligonucleotide probes, however 16S rRNA which contains ~1,600 nucleotides serves as a more suitable diagnostic target. When 16S rRNA does not provide enough variability to accomplish inter-species and intra-species differentiation, 23S rRNA which contains ~3,000 nucleotides can provide enough variability to allow for those discriminations. While the lack of extensive deposited 23S rRNA sequences has accounted for only limited use of 23S rRNA as a diagnostic target in environmental studies, 23S RNA of main foodborne bacterial pathogens has been validly described, thus it can be successfully used as a target for FISH. Accessibility of target regions of the 16S rNA and 23S rRNA of *E. coli* and 18S rRNA of *Sacharomyces cerevisiae* to oligonucleotides probes has been studied in three large systematic studies (Fuchs et al., 1998; Fuchs et al., 2001; Behrens et al., 2003).
It was determined that the tertiary structure of the ribosomes did not affect the hybridization efficiency, probably due to the denaturation and destabilization caused by fixation and treatments at high temperatures during hybridization, but instead secondary structures were in fact the higher order structures that affected accessibility to the oligonucleotide probes. Regions that provided the hybridization efficiencies ranging from low to high were mapped in detail and can be taken into account when probes are designed. However, in a latter study Yilmaz et al. (2006) designed a thermodynamic study relating the hybridization efficiency of 16S rRNA of *E. coli* to overall Gibbs free-energy change ($\Delta G^\circ_{\text{overall}}$), and determined that there are no truly inaccessible regions of 16S rRNA, but these regions can be made accessible by designing probes with $\Delta G^\circ_{\text{overall}}$ of less than 13 kcal/mol.

FISH-based assays are performed by following these common steps: 1. fixation, 2. preparation, 3. hybridization, 4. washing, and 5. visualization and documentation. Fixation is performed in order to permeabilize the cells allowing for the entry of the probes as well as to prevent nucleic acids from degradation. This procedure is carried on using cross-linking fixatives like aldehydes (e.g. formaldehyde or paraformaldehyde) or precipitating fixatives such as methanol or ethanol. Precipitating agents are the fixative of choice when fixation of cells possessing a thick cell wall such as gram positive bacteria is desired, while aldehyde-based fixatives perform very well with gram negative microorganisms. Fixation causes loss of cell viability, and even though FISH of live cells has been shown by Silverman and Kool (2005), the possibility of uptake of oligonucleotide probes by live cells was contradicted by latter work (Amann and Fuchs, 2008). The second step, sample preparation, may involve treatment of gram positive bacteria with compounds which improve the permeability of cells to probes (e.g. lysozyme or lysostaphin) (Schönhuber et al., 1997; Wagner et al., 1998; Moter
and Göbel, 2000) or for example simply coating of slides with gelatin (Amann et al., 1990) or other coating agents, ethanol dehydration of samples air-dried onto slides or de-waxing of the paraffin-embedded preparations (Boye et al., 1998). The preparation step might not be necessary if no special treatments are needed to conduct a complete FISH procedure. The third step is hybridization, which basically involves annealing of the probe to its target sequence in the cell rRNA. Hybridizations are conducted under stringent (high degree of homology between probe and target sequence) conditions. Basically hybridization is conducted using preheated hybridization buffer containing the probe. The stringency of hybridization can be varied by varying the formamide concentration in the hybridization buffer, the temperature of hybridization or the salt concentration. In order to assure a successful hybridization, the temperature of the hybridization must be maintained below the melting temperature of the probe in order for annealing of the probes to target sequences to occur. An empirical formula can be used for calculating the $T_m$ of an oligonucleotide in relation to its GC content: $T_m$ (in °C) = 2(A+T) + 4(G+C) (Suggs et al., 1981). Formamide in the hybridization buffer weakens the hydrogen bonds of the target sequence, practically lowering the $T_m$, while decreasing its concentration will increase $T_m$. Formamide can play another role in hybridizations, by destabilizing the secondary structures of rRNA in the ribosomes and improving accessibility of the probes to the target sequences. However formamide is a harmful compound by inhalation, thus it should be used with caution or other measures to control stringency can be taken when possible. Simply varying the salt concentration in the hybridization buffer can help adjust stringency. Other compounds such as sodium dodecyl sulfate (SDS) or ethylenediaminetetraacetic acid (EDTA) when used in the hybridization buffer improve penetrability of the target cells. Washing is performed in
order to mediate removal of the unbound probe. It is performed in wash buffer containing elements which were previously described for hybridization buffers while keeping stringency under control. **Visualization** can be performed by either fluorescence microscopy or following mounting of the samples in a mounting medium with or without added agents which prevent fading of the fluorochromes under intense illumination or by cytometry. These detection and documentation methods will be described more in detail in subsequent sections.

Several variations to FISH have been described, which have been used to improve the sensitivity of this method or increase the hybridization efficiency and some of them will be covered later in this writing. One approach uses hybridization with several monolabeled oligonucleotides targeted to different sequences (Glöckner et al., 1996) to increase the signal intensity. Using oligonucleotides labeled with horseradish peroxidase followed by catalyzed reported deposition of fluorescent-labeled tyramide (CARD-FISH) has been shown to improve signal intensity (Schönhuber et al., 1997; Pernthaler et al., 2002); however, special and harsh pretreatment of whole cells in order to improve uptake of the large enzyme complexes is needed. DNA oligonucleotides can be modified by designing two complementary sequences on both sides of the probe sequence to permit formation of a stem-loop in solution. These structures are called molecular beacons (MBs) and they are labeled with a fluorochrome in one end and a quencher at the other end, thus they only fluoresce when they are annealed to the target sequence, but not when they are unbound, increasing the signal-to-noise ratio (Tyagi et al., 1996; Xi et al., 2003; Lenaerts et al., 2007). Peptide nucleic acids (PNA) bring upon another exciting development in probe technology. PNAs are molecules that mimic DNA, but instead are in possession of an uncharged, achiral backbone
consisting of repetitive units of $N$-(2-aminoethyl) glycine. These unique characteristics imparted by PNA molecules allow them to better penetrate thick cell walls of gram-positive cells; they also improve hybridization kinetics, make their employment independent of salt concentration, make them resistant to nucleases, which can be present in food matrices, as well as increase the accessibility of regions of the ribosome which are inaccessible to DNA probes (Egholm et al., 1993; Demidov, 1994; Stender et al., 2002; Brehm-Stecher et al., 2005).

Potential problems with FISH might arise from the fact that 16S rRNA can be too conserved, not allowing intra-species or interspecies differentiation (Fox et al., 1992), however as previously mentioned, 23S rRNA can serve in these cases as a more suitable diagnostic target that provides more region variability (Amann and Ludwig, 2000). Generally, the possibility of hybridization of the specific probes with rRNA of unknown microorganisms, which contain similar sequences with the target microorganisms exists (Amann and Ludwig, 2000), however this is not likely when FISH of well characterized bacterial pathogens such as *L. monocytogenes* and *Salmonella* spp. in their environmental niche is attempted. Upon designing probes *in silico*, they should be carefully checked against closely-related microorganisms and microorganisms found in the same environmental niche to confirm that the hybridizations are indeed specific at the set hybridization conditions. Problems that occur with low signal intensity, due to the low number of ribosomes and subsequently target rRNA subsequent stress and injury (Amann et al., 1995) might be overcome by including brief resuscitation and non-selective enrichment steps. Problems with target accessibility have been discussed above, and should be taken into consideration when designing oligonucleotides for probing of bacteria.
In conclusion, FISH allows for specific and sensitive labeling of target whole cells in a relatively short time which makes its use desirable in rapid microbiology. This method can be combined with end-point analysis methods such as epifluorescence microscopy or flow cytometry to process and document a large number of target cells rapidly.

**FLUORESCENCE MICROSCOPY AND FLOW CYTOMETRY**

**Fluorescence Microscopy.** Microscopic evaluation of bacteria has been in use for a long time and basically it signaled the beginning of microbiology as a separate field, when for the first time Antonie van Leeuwenhoek was able to visualize via microscope microorganisms that could not be seen prior to the invention of the microscope. Several microscopy methods such as bright field microscopy, confocal laser scanning microscopy, electron microscopy, and fluorescence microscopy have widespread use in microbiology, including food microbiology. Principles underlying fluorescence microscopy will be briefly described later in this section. Microscopes are defined by their capacity to magnify the observed objects (magnification) and their resolving power (lens property). A fluorescence microscope is designed based on two types of light, incident or transmitted, with epifluorescence microscopy (incident light-based instruments) being more commonly employed in food microbiology (Rayborne and Tortorello, 2003). Fluorescence in principle entails the excitation of a compound (e.g) fluorochrome by a short wavelength followed by emission in a longer wavelength. The difference between excitation and emission wavelengths, which is an intrinsic property of the fluorochromes when they are excited by an illumination source, is called a Stokes shift and is an important parameter in choosing
fluorochromes in fluorescence microscopy (Guilbault, 1990). A number of fluorochromes can be employed in fluorescence microscopy and some of them were mentioned in the section describing FISH. These include nucleic acid stains and fluorochromes that can be conjugated to cell-specific antibodies or oligonucleotides. For extensive lists of stains that can be employed in fluorescence microscopy or flow cytometry see Davey and Kell, 1996 and Shapiro, 2003. The excitation and emission wavelengths are controlled by filters and dichromatic mirrors to produce the desired output, and illumination is provided by a light source which can be a mercury, halogen or xenon lamp. Rayborne and Tortorello (2003) list two advantages of epifluorescence microscopy over fluorescence microscopy based on transmitted light; first enhanced performance at high magnification which are used for microbial cells, and second, due to the illumination coming from above, better penetrability of opaque, thick specimens allowing its coupling with filter-based fluorescent techniques such as direct epifluorescent filter technique (DEFT).

Flow cytometry. Flow cytometry as a method was developed many years ago by Moldovan (1934) and was basically a photoelectric method of counting cells flowing in a capillary tube on a microscope stage (Davey and Kell, 1996). Over the years there were several developments in the technology of flow cytometry with Gucker et al. (1947) developing an instrument to count dust particles which is often considered to be the real cytometer, and the development of the Coulter Counter in mid 1950s, which used conductivity to differentiate cells by volume and could be used for counting (Davey and Kell, 1996). Kamentsky et al. (1965) used spectrophotometry for the first time to study DNA of mammalian cells in a stream of fluid and the first use of flow cytometry to analyze bacteria dates back to 1978 (Hutter and Eipel, 1978). While initially flow cytometry was not
considered a technique suitable for analysis of microbial cells, Steen and Lindmo (1979) demonstrated the value of this technique for this purpose by designing an instrument illuminated by a sensitive argon laser, which contained an open sheath fluid chamber with laminar flow (Davey and Kell, 1996).

Flow cytometers are typically composed of one or more lasers for excitation (commonly argon lasers), which illuminate cells passing singly in the laminar flow fluid (sheath fluid) at a stable distance from the illumination source in a chamber (flow cell) that can be an optical quartz or cover slip surface flow cell. This controlled flow (hydrodynamic focusing) is achieved via control of the pressure by maintaining a precise pressure differential (Raybourne and Tortorello, 2003).

A summary of the process of detection of cells via flow cytometry is described below. As cells pass through the flow cell and are illuminated, light is scattered at a low angle in regard to the illumination beam (forward light scatter) or at a 90° angle (side scatter). A correlation between forward scatter and cell size or side scatter and granularity/surface roughness has been found, thus these simple characteristic can be used to characterize cells without need for labeling, or as a discrimating characteristic in multi-parameter analysis. Scattered light at 90° (side scatter or SSC), as well as fluorescence emitted from excited fluorochromes (e.g. in oligonucleotides, antibodies, nucleic acid stains) used to label cells, are detected via photomultiplier tubes (PMTs) subsequent passage through a series of filters. Forward scatter (FSC) is commonly detected via a photodiode. The signals are subsequently converted to electronic signals. These signals are then amplified by linear or more commonly logarithmic amplifiers. Logarithmic amplifiers are more suitable for analysis of microbial cells as they allow the analysis of more broadly distributed events.
Data plots are generated subsequent conversion of electronic impulses from analog to digital and multiparametric data on single cells can be collected (Davey and Kell, 1996; Alvarez-Barrientos et al., 2000; Shapiro, 2000; Shapiro, 2003). The collection of so much information on single cells which includes size, granularity and response to the probes allows for further analysis of samples via gating, permitting detection of populations of cells with similar characteristics. These characteristics can be used to selectively enrich target cells by fluorescence-activated flow sorting (FACS) in specialized research flow cytometers capable of imparting a charge to cells with desired characteristics and further separating them in separate vessels in order to enrich rare events; however such instruments can be expensive and are not needed to accomplish the relatively simple task of microbial detection.

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CHAPTER 3. FLOW-THROUGH IMAGING CYTOMETRY FOR CHARACTERIZATION OF SALMONELLA SUBPOPULATIONS IN ALFALFA SPROUTS, A COMPLEX FOOD SYSTEM

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Abstract

We recently developed an approach combining fluorescence in situ hybridization (FISH) and flow cytometry for detecting low levels of Salmonella spp. (~10³ cells/ml sprout wash) against high levels of naturally occurring sprout flora (~10⁷ –10⁸ CFU/g sprouts). Although this “FISH and flow” approach provided rapid presence/absence testing for Salmonella in this complex food system, it was not capable of more nuanced tasks, such as
probing the phenotypic complexity of the microbes present in sprouts or determining the physical interactions of *Salmonella* with these microbes, or with sprout debris. In the present study, we have combined rapid FISH-based labeling of *Salmonella* spp. in sprout washes with flow-through imaging cytometry (FT-IC), using the ImageStream® 100, a commercial FT-IC instrument. This approach enables image-based characterization of various subpopulations within these samples. Here, we demonstrate the ability of FT-IC to unambiguously identify cells, cell aggregates and other events within these subpopulations based on both cell morphology and hybridization status after reaction with a *Salmonella*-targeted probe cocktail. Our ability to directly explore the nature of these events expands the layers of information possible from cytometric analyses of these complex samples and clearly demonstrates that “a picture is worth a thousand dots”.

1 Introduction

Seed sprouts, including alfalfa, broccoli, and radish sprouts are microbiologically and physically complex foods that present real challenges to methods for rapid detection of pathogens. The same wet, warm, aerobic conditions needed to sprout plant seeds also promote the growth of a robust and varied microflora that grows to levels not found in any other type of non-spoiled produce [1, 2]. Mesophilic plate counts as high as $10^9$ CFU/g have been reported for sprouts purchased from retail stores and cells may be present as free-living cells or in biofilms associated with sprout surfaces [1, 3]. Although bacteria are dominant, yeasts and molds can also be found in sprouts, sometimes at concentrations as high as $10^6$ CFU/g [4]. Protozoan parasites, including *Cryptosporidium* oocysts and *Giardia* cysts, have been detected in sprout samples as well [5]. Despite the high numbers for direct plating, it
has been estimated that only about 10% of the microbial flora present on sprouts may be cultivable [3]. Together, these data suggest an almost staggering microbial complexity for this otherwise “fresh” and ready-to-eat food. Culture-independent methods for characterizing bacterial communities occurring on retail alfalfa sprout samples identified 15 families of bacteria and up to 27 different genera, including some potential human pathogens [6]. Gram negative bacteria, primarily from the families Enterobacteriaceae and Oxalobacteraceae were most abundant [6]. The prevalence of Enterobacteriaceae potentially increases the difficulty for detection of Salmonella spp. in sprouts, as background flora in this food mainly consists of non-target cells that are both physiologically and phylogenetically similar to Salmonella.

Within the past decade, sprouts have been implicated in a number of multistate or international foodborne disease outbreaks caused by pathogens such as Salmonella, Escherichia coli O157:H7, Bacillus cereus, and Yersinia enterocolitica [7]. We recently developed a FISH-based method for flow cytometric detection of Salmonella spp. in seed sprouts (Bisha and Brehm-Stecher, unpublished data). This method combines steps for pre-analytical sample preparation, use of an optimized dual probe cocktail and an abbreviated hybridization step that produces bright staining of Salmonella cells within 15 min. Although our “FISH and flow” method proved to be a powerful means for detecting relatively low numbers of Salmonella against very high levels of natural sprout microbiota, we could not use this approach for direct observation or characterization of sprout microflora, their phenotypic complexity, or the physical interactions of Salmonella cells with these microflora, or with sprout debris. Conventional flow cytometers generate volumes of data and can be used to great advantage in characterizing complex microbial populations. However, these
instruments essentially translate cells into a shorthand of pulses and dots that ultimately cannot convey the same level of detail as would a simple microscopic image [8, 9, 10]. In response to this limitation, a new generation of “hybrid” cytometers has been developed, capable of collecting both light scatter and fluorescence information as well as image data on the same cells [9, 10]. At least three such hybrid systems are commercially available, including the ImageStream® instrument used here (Amnis Corporation, Seattle, WA). In this study, we combined our FISH-based assay for detection of *Salmonella* spp. in sprouts with flow-through imaging cytometry (FT-IC) using the ImageStream® platform for direct, image-based characterization of six different subpopulations occurring within these samples. As a whole cell method, a key advantage of FISH is its ability to preserve diagnostically important details on cell morphology and on physical or spatial associations between cells and/or non-cellular material. Traditional flow cytometers rely on light scatter and fluorescence to resolve events within a sample. Although light scatter is linked to cell morphology, this relationship is indirect. Non-cellular particulates from the sample matrix, or mineral crystals, dust, and bubbles in the buffers used for analysis may give rise to high background levels of scatter-based “noise” that complicate detection of small cells such as bacteria. The inaccessibility of valuable morphological data via traditional flow cytometry limits the full diagnostic application of FISH when these two approaches are combined. In contrast, our work highlights the capacity of FT-IC to provide clear visual documentation of discrete events that are not directly observable using conventional flow cytometry. As demonstrated here, FT-IC provides microbiologists with new and exciting opportunities for exploring the complexity of macroscopically mundane, but microscopically fascinating samples such as alfalfa sprouts.
2 Materials and methods

2.1 Bacterial cultures: *Salmonella enterica* subsp. *enterica* ser. Typhimurium ATCC 14028 was from the American Type Culture Collection (ATCC, Manassas, VA). Working plate cultures stored at 4°C were used to inoculate 10 ml of Trypticase Soy Broth (TSB) and cultures were grown at 30°C for 20-22 h. Cells were harvested via centrifugation and washed once in 0.1% peptone water (PW) prior to being used to spike sprout samples (below).

2.2 Preparation of alfalfa sprouts: Sprout samples were spiked with *S.* Typhimurium and processed for cytometry according to a procedure developed recently as part of an assay for *Salmonella* in seed sprouts (Bisha and Brehm-Stecher, unpublished data). Briefly, 25 g of fresh store-bought alfalfa sprouts were aseptically weighed out into filter Stomacher® 400 Circulator bags (Seward, Worthing, UK), and inoculated with ~10⁷ CFU/g *S.* Typhimurium. Inocula were left in contact with sprouts for ~2 h, then 225 ml of 0.1% PW were added and the mixture was homogenized for 1 min at 230 rpm in a Stomacher Circulator® 400 paddle blender (Seward Ltd., Norfolk, UK). The resulting homogenate was vacuum filtered in a single pass through four layers of sterile cheesecloth to remove large particulates (i.e. visible stems, leaves). Small (1.3 ml) portions of the filtered homogenate were centrifuged briefly at low speed (300 x g, 30 s) to remove any remaining large particulates. One milliliter portions of the supernatant were fixed for 30 min in 1 ml 10% buffered formalin, then resuspended in a 50:50 mixture of absolute ethanol and phosphate buffered saline (PBS) and stored until use at -20°C. Samples without added *Salmonella* were also prepared and analyzed as negative controls.
2.3 Scanning Electron Microscopy (SEM): The physical and microbiological complexity of sprout samples was investigated via SEM using the following sample preparation procedures. For “uncleaned” samples, 25 g of unadulterated sprouts (no Salmonella added) were homogenized in 225 ml 0.1% PW, and 1 ml portions of the homogenate were removed and pelleted via centrifugation (2,000 x g, 5 min). The supernatant was discarded and samples were fixed for 15 min in EM-grade glutaraldehyde (2.5% final concentration, Sigma-Aldrich), then resuspended in PBS and shipped to the University of Iowa’s Central Microscopy Research Facility (CMRF) for analysis. At CMRF, a drop of the fixed sample was applied to a poly-L-lysine-treated silicon chip, allowed to adhere for 5 min, then samples were fixed further in 1% osmium tetroxide, followed by dehydration in an ethanol series, sputter coating and viewing via SEM using an Hitachi S-3400N microscope. For “cleaned” samples, sprout homogenates were filtered through four layers of cheesecloth and subjected to a brief, low speed centrifugation (30 s at 300 x g) prior to fixation with glutaraldehyde and subsequent processing at CMRF.

2.4 Fluorescence In Situ Hybridization: Two 23S rRNA-targeted oligonucleotide probes previously developed for detection of Salmonella spp., Sal3 [11], and Salm-63 [12], were combined as described by Lantz et al., [13] and used as a dual probe cocktail at a total probe concentration of 5 ng/µl (2.5 ng/µl each probe). Probes were synthesized, end-labeled at the 5’-end with either Cy5 or 6-carboxyfluorescein (FAM) and purified via HPLC by Integrated DNA Technologies, Inc. (Coralville, IA). Cy5-labeled probes were used in experiments involving conventional flow cytometry and FAM-labeled probes were used for experiments involving microscopy or imaging cytometry. For each hybridization reaction, one hundred microliters of fixed sprout samples were pelleted (5 min, 2,000 x g) and
resuspended in 100 µl of hybridization buffer (0.7 M NaCl, 0.1 M Tris [pH 8.0], 0.1% SDS, 10 mM EDTA) containing the dual probe cocktail. Samples were hybridized for 30 min on a heat block set to 55°C, followed by a 30 min wash step at the same temperature in 500 µl hybridization buffer without probe. Hybridized samples were pelleted and resuspended in a 50:50 mixture of PBS and absolute ethanol, cooled to -20°C and shipped on ice via overnight courier to Amnis Corporation (Seattle, WA). Once received, samples were placed and held at -20°C until used, for up to a week. Samples remained liquid under these storage conditions due to their ethanol content.

2.5 Stability of hybridization (storage study): As noted above, sprout samples were prepared and hybridized in our lab, then shipped overnight to Amnis for analysis. To determine the lifetime of probe-conferred fluorescence, and thus the acceptable window between hybridization and analysis, we performed an initial storage study comparing two potential storage regimes: frozen and refrigerated. For the frozen treatment, hybridized samples were resuspended in a 50:50 mixture of PBS and absolute ethanol and held at -20°C as described above. Refrigerated samples were stored at 4°C, and received an additional post-hybridization fixation step (30 min in 10% buffered formalin) in an effort to “cement” hybridized probes in place via cross-linking, reducing their diffusive loss during storage at this higher temperature. Samples stored using both regimes were held up to a week and assayed periodically for fluorescence intensity via fluorescence microscopy and digital microscopy, with a photograph of the “time zero” sample (fresh hybridization) used as a reference for comparison at each sampling interval.

2.6 Flow cytometry: Hybridized sprout samples were examined using either a Becton-Dickinson FACSCanto flow cytometer with red (633 nm) excitation or with an
ImageStream® 100 FT-IC with blue (488 nm) excitation (Amnis Corporation, Seattle, WA). Data obtained via conventional flow cytometry were analyzed using FlowJo software (v. 8.7.1, Tree Star Inc., Ashland, OR). For FT-IC, data were examined using the Image Data Exploration and Analysis Software package (IDEAS™, v. 3.0, Amnis). As noted above, once samples were received at Amnis, they were placed at -20°C until used. Prior to analysis, tubes were vortexed to resuspend cells and break up loosely associated flocs or aggregates. One hundred microliter samples were pelleted via centrifugation (2,000 x g, 5 min), were washed in 100 µl PBS + 0.5% (w/v) bovine serum albumin (BSA), centrifuged again, then resuspended in 50 µl PBS + 0.5% BSA prior to running on the ImageStream® system. Prior to collecting data, a compensation matrix was created using single-color controls and was used to correct for spectral crosstalk. Samples were mixed with the SpeedBead™ reagent (an internal control for imaging quality) and files containing 100,000 events were collected. After data collection, the beads were gated out, smaller data files containing ~7,000-10,000 events were created and dot plots of side scatter (Y axis) vs. fluorescence intensity (X axis) were generated (Fig 2, panels B and C). Six unique subpopulations were chosen from the resulting dot plots for image-based exploration.

3 Results and discussion

Seed sprouts are surprisingly complex microbial niches, and represent unique challenges to methods for rapid detection and characterization of pathogens such as *Salmonella* spp. Not only are seed sprouts populated by large numbers (up to 10^9 CFU/g) of predominantly gram negative bacteria, but they are physically complex, as well, containing a size continuum of particulates. Figure 1 visually depicts these two levels of complexity.
Panel A (100 µm scale bar) clearly shows the diversity of large sprout particulates present in “uncleaned” sprout samples (see section 2.3 of “Materials and methods” for details). These plant structures provide a large surface area to which microbial cells can bind. Panel B shows a view of “cleaned” sprout samples at higher magnification (10 µm scale bar), highlighting the high load, morphology and adherent nature of the indigenous sprout flora. Although these cells can all be described as “rods”, they are varied in size and width, differences that could stem either from cell type or age (or both). Note that even in the “cleaned” sample, there is still ample surface area to which these cells can adhere. As described in previous microscopic studies of sprouts [1, 3] and confirmed in our work, native sprout microbiota is present as both loose aggregates of surface-associated cells, and as classically-defined biofilms, with several layers of cells embedded in an extracellular matrix (data not shown). No obvious yeast cells were seen using SEM or conventional microscopy, and although we have previously observed an unidentified, motile, grazing protozoan in fresh sprouts from one manufacturer (Bisha and Brehm-Stecher, unpublished observation), no protozoa were observed in the samples analyzed via SEM or FT-IC. In order to determine shipping and storage conditions that would retain FISH-based staining during transport and pre-analytical storage, we conducted a storage study, as described in Materials and Methods. Results from this study indicated that storage of already hybridized cells in a 50:50 mix of ethanol and PBS at -20°C was superior to post-hybridization fixation with refrigerated storage for maintaining intensity of probe-conferred fluorescence. Cell preparations held at -20°C remained very bright for up to one week after hybridization and these conditions were used throughout the rest of the study.
Next, we sought to compare our results from conventional cytometry with those obtained using FT-IC. The scatter plot in Figure 2, panel A typifies our hybridization results for alfalfa sprouts contaminated with S. Typhimurium when a standard flow cytometer (Becton Dickinson FACSCanto) was used. Several distinct populations can be seen. Population (a), which comprises most of the sample and is thought to consist of both non-target sprout microbiota and particulate matter, spans a large range of side scatter values. Population (b) increases in fluorescence intensity with increasing scatter values, and may represent large clumps of non-target cells or particulate matter that bind or entrap the Salmonella probe cocktail. Despite being markedly more fluorescent than the bulk non-target population, this population was easily differentiated from S. Typhimurium (population c) on the combined basis of side scatter and probe-conferred fluorescence.

The exact nature of population (d), which formed a “bridge” between the discrete low-scatter Salmonella population and higher-scatter populations, is unknown. Because the fluorescence intensity of this “bridge” coincided with that of the Salmonella population, we hypothesized that it could have resulted from Salmonella cells bound to a size continuum of sprout particulates, or that it could be an artifact caused by coincidence – one or more non-target cells passing in front of the detector at the same time as a Salmonella cell. This latter explanation is more probable - if the “bridge” was formed by binding of Salmonella cells to a size-distributed population of sprout particles, larger particles should bind more Salmonella cells, which would result in a rightward skew for the bridge at higher scatter values. While this could explain the population at the top of the bridge (upper right hand corner of the plot), the bridge itself is fairly straight. This, along with the high load of non-target microbiota
known to be present in sprouts (~$10^8$ CFU/g), suggests a coincidence-based explanation for this feature.

Still, for all the power of conventional flow cytometry, its ability to probe deeper and identify the events (cells, particulate matter, or cell-particulate complexes, etc.) responsible for these patterns is limited – at some point, a dot is simply a dot. Therefore, we sought to use FT-IC, a “hybrid” approach that combines aspects of conventional flow cytometry with imaging of each event detected, to further explore the nature of these populations [9, 10]. The optical configuration and principles of operation for the ImageStream® 100 are described in detail by Ortyn et al., [10]. Briefly, this instrument is capable of imaging cells simultaneously in six different modes: brightfield, darkfield and up to four fluorescence colors. Hydrodynamically focused cells are illuminated with a laser for darkfield and fluorescence imaging, and with a filtered white light for brightfield imaging. An objective lens is used to collect light from the cells, which is then passed through a spectral decomposition unit that separates the composite signal into discrete spectral bands. These are projected onto a charge-coupled detector, with each band trained onto a physically separate vertical “channel” on the chip’s surface. Over thirty-five morphometric and signal intensity features are then calculated for each image using a real time algorithm. Captured images are accessible through an interactive user interface - by highlighting an event (a dot), the operator is able to retrieve stored images of the corresponding cell or object. This approach combines the best aspects of flow cytometry and digital imaging technologies, enabling flow-through multimode imaging of individual cells in liquid suspension [8, 9, 10].

Figure 2, panel B highlights the power of the FT-IC approach for image-based confirmation of “dot” identity. With the FT-IC system we used (the ImageStream® 100
instrument from Amnis Corporation, Seattle, WA), highlighting of an event using a crosshair-shaped cursor calls up the images collected of this event and displays them according to operator-defined specifications. Images possible include bright field, dark field, up to four fluorescence colors and automatically generated overlays of individual image channels. The inset in Figure 2, panel B shows bright field (BF) and green channel fluorescence (FITC, or fluorescein) images collected of the multi-particle event highlighted with the arrow on the scatter plot. Although this event appeared as a single dot on the side scatter vs. fluorescence plot, the corresponding images provide further layers of informational content. Specifically, these images establish that this “single” event arose from the simultaneous detection of three particles: one non-target rod-shaped bacterium (left hand particle), one S. Typhimurium cell (center particle, stained green with the Salmonella-targeted FISH probe cocktail), and one small high-contrast sphere (right hand particle), possibly either a 1 µm calibration bead (SpeedBead™ reagent) or an end-on rod having a diameter of ~1 µm. Although the exact identity of this particle is not known, this example clearly shows that this image-based cytometry approach has substantial advantages over conventional cytometry for further exploring subpopulations of interest within physically and microbiologically complex samples such as alfalfa sprouts.

When we examined the side scatter vs. fluorescence plot of a Salmonella-contaminated alfalfa sprout sample, we identified at least six unique regions that we chose to explore further via FT-IC-based imagery (Figure 2, panel C). Clockwise from top, these regions are: Region 1 (high scatter, no fluorescence), Region 2 (high scatter, medium fluorescence), Region 3 (high scatter, intense fluorescence), Region 4 (low scatter, high fluorescence), Region 5 (low scatter, medium fluorescence) and Region 6 (low scatter, no
fluorescence). Using the IDEAS™ software, we explored images of events occurring within these regions, looking for images that typified each region.

Figure 3 is a montage of images of events occurring in Regions 1 – 6 (labeled R1 through R6 on Figures 2 and 3). Images of events occurring within each region are physically arranged to correspond roughly to the parent population’s position on the scatter plot in Figure 2, panel C. Brightfield images from Region 1 show that this region was typified by large (high-scatter) non-target bacteria, unidentified microbes or sprout debris. Because these cells or particles did not react with the Salmonella probe cocktail, these events remained dark in the green (fluorescein) channel. Region 2, was populated by large aggregates of non-target flora containing one or two S. Typhimurium cells or physically separate, but coincident events comprised of one S. Typhimurium cell and one or more non-target particles. The lack of green channel fluorescence for non-target cells in this panel highlights the specificity of the Salmonella probe cocktail. Although FT-IC provides additional data on particle morphology, the identities of some particles remain unclear. For example, the dark sphere immediately above the S. Typhimurium cell in the bottom frame of R2 may either be an end-on rod or a SpeedBead™ tracking particle, two objects having an expected diameter of ~1µm. Additional labeling, such as nucleic acid staining, could help resolve the identity of this particle. Region 3 contained large (high-scatter) aggregates of non-target bacteria or sprout debris and four or more S. Typhimurium cells. The large number of Salmonella cells contained within these aggregates led to their intense fluorescence signatures. It is not clear if this type of event is an artifact stemming from how we spiked Salmonella into the sprouts, or if natural biofilm-based growth of Salmonella spp. would result in similar subpopulations.
The events in Region 4 formed a discrete, low scatter/high fluorescence subpopulation, distinct from Region 5. Images revealed that Region 4 was comprised of large \textit{S. Typhimurium} cells or \textit{S. Typhimurium} cells in various stages of division. Large or dividing cells would be expected to contain higher levels of rRNA, in agreement with our observation of correspondingly brighter probe-conferred intensities for these cells. The bulk of the \textit{Salmonella} from these samples was contained in Region 5. These were present as single, non-dividing \textit{S. Typhimurium} cells. Lastly, the non-fluorescent, low scatter subpopulation in Region 6 was comprised of non-target cells of various shapes and sizes.

As shown in Figure 2 (panels A and B), the two side scatter vs. fluorescence outputs from the ImageStream® 100 and the BD FACSCanto are not superimposable, but contain similar elements, including a large non-target population, at least one clearly separated subpopulation of FISH-stained \textit{Salmonella}, and a density of high-scatter/high fluorescence events immediately above the main \textit{Salmonella} population. The FACSCanto output shown in panel A is comprised of \(\approx100,000\) events, the plot density level at which the “bridge” immediately above the \textit{Salmonella} subpopulation becomes visually apparent. It was the inaccessibility of this bridge feature to direct characterization via conventional flow cytometry that led us to seek FT-IC as an alternate means of exploring the subpopulations present in \textit{Salmonella}-spiked sprouts. Although ImageStream® files plotted at a density of 100,000 events more closely resembled the output from the FACSCanto, the type of manual image screening we performed in our lab necessitated the use of smaller “bite-sized” files of \(\approx7,000\) events (Fig. 2, panel B). The ability to directly explore the identities of individual events using FT-IC provided us with a unique window on the interactions of \textit{Salmonella} with non-target cells occurring within this complex, heterogeneous food sample. Our image-based
analysis of events in Region 2 of the ImageStream® output supports the theory that the bridge feature from the FACSCanto output can be explained by both clumping of target and non-target cells and by coincidence – passage of one or more non-target cells in front of the detector at the same time as a Salmonella cell.

4 Concluding remarks
A time-honored saying among flow cytometrists is that “dots don’t lie”. Although this may be true, our results suggest that they still can withhold information. We have shown here that FT-IC, combined with “phylogenetic staining” using Salmonella-targeted DNA-FISH probes, enables a more complete and direct visual exploration of this physiologically and phylogenetically complex food system than is possible using either traditional imaging approaches, such as microscopy, or conventional flow cytometry. The ImageStream® instrument used here was originally developed to bridge the gap between the relatively slow, but detailed imaging capabilities of confocal microscopy and the faster, but less information-rich analyses provided by traditional flow cytometry [10]. Because they enable high-throughput imaging of liquid sample suspensions, the use of such “hybrid” cytometry systems can provide unique insights into discrete phenomena occurring in complex samples such as alfalfa sprout washes, including information on cell-cell and cell-particle interactions and coincidence. As we have shown here, the use of Salmonella-specific FISH probes provides an additional layer of information, allowing differentiation of this organism within mixed populations. This can be especially useful in samples such as alfalfa sprouts, where physiological differences between target and non-target cells are either limited or nonexistent.
Recent studies on the prevalence of protozoa in foods and food processing environments and the potential protective effects of these protozoa on internalized pathogens suggest other areas in food microbiology where use of a combined FISH and FT-IC approach could provide valuable information. Protozoa such as *Tetrahymena pyriformis*, *Glaucosa* spp. and others have been isolated from spinach, lettuce and food contact surfaces within meat-cutting plants [14, 15]. These protozoa can engulf foodborne pathogens such as *Salmonella*, and serve as hosts, enabling the bacteria to multiply while simultaneously protecting them against inactivation by antimicrobials or physical treatments such as heat [14, 15]. The role that such protozoan hosts may play in the ecology of foodborne pathogens, or in protection of these pathogens against traditional methods of inactivation could be examined in further detail through the combination of FISH, additional physiological stains and analysis via FT-IC. The ability of instruments such as the ImageStream® to provide high-throughput, visually-rich information on complex samples is expected to be of great basic value for observing the activities of native, inoculated or contaminant microbial populations in foods, fermentations or other materials of interest to food biotechnologists.

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5 References


Figure 1. Scanning electron microscopy of sprout particulate matter and natural, adherent microflora. Panel A highlights the physical complexity of alfalfa sprout samples not treated to remove large particulates. Relatively large alfalfa plant structures can be seen, including columnar palisade parenchymal cells at center and collapsed root or stem structures (scale bar 100 µm). Panel B (scale bar 10 µm) shows the complex assemblage of rod-shaped bacteria typical of the natural flora of fresh store-bought alfalfa sprouts.
Figure 2. Comparison of sprout samples analyzed via both conventional and flow-through imaging cytometry. Panel A shows typical results for Salmonella-spiked alfalfa sprouts hybridized with a Cy5-labeled Salmonella probe cocktail and analyzed using a BD FACSCanto cytometer (100,000 events shown). Subpopulations a and b were ascribed to non-target sprout flora and particulate matter. Subpopulation c (not present in Salmonella-negative control samples) was comprised of hybridized S. Typhimurium cells. The identity of subpopulation d, a fluorescent “bridge” connecting the low-scatter Salmonella cells to higher
scatter events, could not be determined using conventional flow cytometry. Panel B demonstrates the ImageStream® 100 instrument’s capacity for direct visual probing of event identity within physically and microbiologically complex samples. The inset shows brightfield and green channel fluorescence images of the multiparticle event highlighted by the arrow. Panel C shows the division of the ImageStream® output into the six distinct regions used for the image-based exploration of this sample shown in Figure 3.
Figure 3. Representative images of events occurring in Regions 1-6. Image-based exploration of events occurring within each of the six regions shown in Figure 2 provided a unique window into cell or particle morphology and interactions of non-target events with FISH-labeled *Salmonella* cells. Images of events occurring within each region are physically arranged to correspond roughly to the parent population’s position on the scatter plot in Figure 2, panel C.
CHAPTER 4. SIMPLE ADHESIVE-TAPE-BASED SAMPLING OF TOMATO SURFACES COMBINED WITH RAPID FLUORESCENCE IN SITU HYBRIDIZATION FOR SALMONELLA DETECTION

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ABSTRACT

A simple adhesive tape-based method for sampling of tomato surfaces was combined with fluorescence in situ hybridization (FISH) for rapid culture-independent detection of Salmonella spp. Tapes could also be placed face-down on selective agar for on-tape enrichment of captured Salmonella cells. Overlay of cell-charged tapes with small volumes
of liquid enrichment media enabled subsequent detection of tape-captured Salmonella via flow cytometry.

In the past decade, Salmonella spp. have been implicated in multiple foodborne disease outbreaks tied to the consumption of fresh fruits and vegetables (19). In the U.S., tomatoes have been the most commonly implicated crop for produce-related salmonellosis, with twelve outbreaks occurring since 1998 (3, 19). Contamination of fresh produce can occur at any point in the farm-to-fork continuum, but environmental factors, such as use of contaminated irrigation water, runoff from adjacent animal production lots, activities of wild animals in fields and use of untreated manure as a fertilizer are key routes for the introduction of human pathogens (9, 19). Additional routes may include unsanitary practices by workers in the field or even intentional contamination of crops in the field. Although field environments provide greater opportunity for contamination to occur, contamination of tomatoes with Salmonella is also possible for crops grown in controlled (hydroponic) environments (21). The largest documented fresh produce-related outbreak of salmonellosis to date in the U.S. occurred during the summer of 2008. Although tomatoes were initially implicated, the source was difficult to pinpoint, and the outbreak strain was later recovered from jalapeño and serrano peppers grown in Mexico. Methods for detection of Salmonella on fresh produce can play an important role in mitigation of disease from outbreaks such as this by providing decision makers with timely data on the presence of this pathogen in contaminated foods.

Adhesive tape-based sampling methods have been used in clinical, environmental and
food microbiology, beginning in the early 1950’s (4, 10, 13, 17) and have recently been combined with an rRNA-targeted whole-cell method for fluorescent labeling of specific microbial cells (fluorescence in situ hybridization, or FISH) for culture-independent analysis of microbial communities present on the surfaces of stone monuments (15). We have extended this approach to the rapid sampling of fresh produce surfaces for detection of *Salmonella* spp., using tomato as a model system. In addition to tomatoes, we found the method could also be used to sample and detect *Salmonella* artificially inoculated onto jalapeño pepper, cilantro and spinach surfaces and that cell-charged tapes could be enriched further on *Salmonella*-selective agar, or in low-volume (0.5 ml) liquid culture followed by flow cytometric analysis.

Tomatoes (“red tomatoes on-the-vine”, not waxed or oiled, average weight 135g), jalapeño peppers, cilantro and spinach were obtained from a local grocery store and confirmed to be negative for *Salmonella* via culture. Square regions (1 cm² each) were drawn on produce surfaces with a fine-tip permanent marker using a sterile paper template. *Salmonella* spp. (overnight cultures of sers. Typhimurium ATCC 14028 or Newport, *Salmonella* Genetic Stock Centre SARB 36, washed and resuspended in 0.1% peptone water) were spot inoculated within each 1 cm² region. Final cell densities ranged from ~10⁰-10⁷ CFU cm⁻². For tomatoes, inocula were applied to skin at either the top (adjacent to the stem scar) or bottom (adjacent to the blossom scar) of the fruit. For spinach and cilantro leaves, the tops of the leaves (adaxial sides) were used. For some samples, mixtures of individual *Salmonella* strains and *R. glutinis* ATCC 32765 were also spot inoculated in the same fashion (Fig 1). Microbial inocula were allowed to attach by drying onto the tomato surfaces for ~3 h at 25°C prior to tape-based sampling. Although preliminary experiments suggested that
generic office-grade transparent tape may be suitable in this application, we focused on two commercially available adhesive tapes intended for microbiological use: Fungi-Tape™ (Scientific Device Laboratory, Des Plaines, IL) and CON-TACT-IT® sampling tape (Birko Corporation, Denver, CO). Microorganisms were sampled by placing Fungi-Tape™ or CON-TACT-IT® tape onto inoculated areas, applying gentle and even pressure to ensure full contact of the sampling tape with the produce surface, followed by removal of the tape-cell complex (Fig 2A). In some experiments, after making lifts of cells from tomato surfaces, tapes were placed onto Xylose Lysine Tergitol™ 4 (XLT-4) agar plates, which were then inverted and incubated for 8 h at 35°C for on-tape formation of microcolonies. Following incubation, adhesive tapes were first pressed gently against the agar surface to bind any loosely adherent cells and the tape-cell complex was removed. Prior to further processing (for fixation, hybridization and microscopy or on-tape liquid culture), cell-charged tapes were mounted (with generic transparent tape) onto microscope slides, sticky side facing upwards. All inoculation and tape-based sampling experiments were repeated three times, using two serovars (Typhimurium and Newport); experiments on recovery efficiency of tape-based tomato sampling using S. Newport were carried out in duplicate and were repeated three times; cytometry experiments were performed twice.

Liquid phase enrichment (liquid surface miniculture) was performed by placing a CoverWell™ perfusion chamber (model PC1R-2.0, non-sterile, Grace Bio-Labs, Bend, OR) on top of a slide-mounted tape and filling the chamber with 500 µl growth media (Trypticase Soy Broth, TSB or Buffered Peptone Water, BPW), preheated to 37°C (Fig 2B). The flexible silicone base of this type of chamber allowed formation of a water-tight seal, yielding closed, media-filled chambers whose bottom surfaces were comprised of Salmonella-charged tapes
mounted, sticky side up onto microscope slides. Perfusion chamber inlet ports were sealed using transparent adhesive tape and the chambers were incubated at 37°C for 5 h.

Prior to FISH, tape-bound cells were fixed for 30 min at 25°C by covering the sample contact area with 500 µl 10% neutral buffered formalin (Sigma). After fixation, the formalin was discarded and the tape was washed once in 1x phosphate buffered saline (PBS), then dehydrated in ethanol (a 50, 80 and 95% (v/v) series, exposure for 3 min to 300 µl ethanol at each concentration) prior to hybridization. For fixation of liquid cultures, the entire 500 µl volume was transferred into a 1.5 ml microcentrifuge tube, pelleted for 5 minutes at 2,000 x g, then resuspended in 0.5 ml 10% buffered formalin (Sigma Chemical Company) and fixed for 30 minutes at 25°C. Fixed samples were harvested via centrifugation (5 min, 2,000 x g), the supernatant was discarded and cell pellets were resuspended in 0.5 ml of cell storage solution (a 50:50 mix of PBS:absolute ethanol), then stored at -20°C until analyzed.

Two oligonucleotide probes previously developed for detection of *Salmonella* spp., Sal3 (20) and Salm-63 (14), were combined as described by Lantz et al., (18) and applied as a dual probe cocktail at a total concentration of 5 ng/µl probe (2.5 ng/µl each probe). In mixed flora experiments with *R. glutinis*, a universal Eucarya probe, EUK 516 (1) was also used at 5 ng/µl. Probes were synthesized and HPLC-purified by Integrated DNA Technologies (Coralville, IA) and were labeled at the 5’ end with fluorescein or Texas Red (for microscopy work) or with Cy5 (for flow cytometry experiments). For most experiments, samples on tapes were hybridized for 15 minutes at 55°C using a moisture-sealed slide incubation chamber (Slide Moat™ model 240000, Boekel Scientific, Feasterville, PA). Briefly, 500 µl volumes of hybridization buffer (0.7 M NaCl, 0.1 M Tris [pH 8.0], 0.1% sodium dodecyl sulfate, 10 mM EDTA, containing probe, preheated to 55°C) were applied to
the surface of the tape and the chamber’s lid was sealed, creating a moist, temperature-controlled environment within the chamber. After 15 min, the lid was removed and samples were briefly rinsed with probe-free hybridization buffer, preheated to 55°C. Tapes were then processed for microscopy, as described below. In initial tests, and for Figure 1, hybridization and washing (30 min each) were carried out in a hybridization oven (Bambino, Boekel Scientific), inside sealed 50 ml polypropylene centrifuge tubes. Due to the limited throughput of this approach, subsequent hybridizations were carried out using the Slide Moat™, which allowed analysis of multiple (> 20) slides and also provided direct contact heat transfer. For hybridization of cells grown using liquid surface miniculture, fixed cells (entire 500 µl samples, in cell storage solution at -20ºC) were pelleted (5 min, 2,000 x g) and resuspended in 100 µl of probe-containing hybridization buffer. Samples were hybridized at 55ºC on a heat block for 30 min, followed by a 30 min wash step at the same temperature using 500 µl hybridization buffer without probe, then analyzed via cytometry.

Hybridized cells on tapes were counterstained for 10 minutes in the dark with ~30 µl mounting medium containing 1.5 µg ml⁻¹ 4',6-diamidino-2-phenylindole (DAPI) (VECTASHIELD® HardSet™, Vector Laboratories, Burlingame, CA), then mounted with a coverslip and examined using a Leitz LaborLux S microscope equipped with a Canon PowerShot A640 consumer-grade digital camera controlled by Axiovision software (v. 4.6, Carl Zeiss Microimaging, Inc., Thornwood, N.Y.). Raw Tagged Image Format (TIFF) outputs from green (fluorescein) and red (Texas Red) channels were adjusted for brightness and contrast to appear as they did via microscopy and composite images were made using Adobe Photoshop®. Flow cytometry of liquid surface miniculture samples was performed on a Becton-Dickinson FACSCanto flow cytometer with red (633 nm) excitation, using bacterial
side scatter to trigger event detection. Samples were run for 3 min at “low” flow rate (10 µl min). Flow cytometry data were analyzed using FlowJo software (v. 8.7.1, Tree Star Inc., Ashland, OR).

Since its introduction in 1930, “Scotch”-type adhesive tape has been adopted for a number of interesting “off label” uses, including use in the household for removal of lint from garments, in forensic science for lifting fingerprints from surfaces and in the clinic for sampling and detection of intestinal parasites or their eggs via anal tape lifts or for sampling of pathogenic fungi from skin (4, 10). In environmental microbiology, adhesive tape has been used for sampling of microbes from leaf surfaces for subsequent microscopic or cultural analyses (17) and tape-based sampling is an accepted technique in food microbiology for monitoring of food or environmental surfaces (12, 13). For example, the use of CON-TACT-IT® tape is suggested in the Compendium of Methods for the Microbiological Examination of Foods as an alternative to Replicate Organism Detection and Counting (RODAC) plating for estimating the sanitary condition of food processing environmental surfaces (12), and this tape has also been combined with acridine orange staining for sampling and analysis of microbial populations on beverage dispenser tips via fluorescence microscopy (16). Extending the approach further, La Cono and Urzí (15) combined tape-based sampling with on-tape FISH for the detection and characterization of microflora present on the surfaces of historic stone monuments and suggested the approach for use on other surfaces, including food contact surfaces. However, in addition to inanimate objects (i.e. cutting boards, countertops, floor tiles, processing equipment, etc.), the surfaces of many foods themselves may become contaminated with human pathogens. In the U.S., tomatoes and other fresh produce have been implicated in a number of recent outbreaks of salmonellosis, therefore, we
sought to examine the utility of this “tape-FISH” approach for sampling and direct detection of *Salmonella* spp. on tomato and other fresh produce surfaces.

We found that two commercially available microbiological sampling tapes (FUNGITAPE™ and CON-TACT-IT®) could be used to remove *Salmonella* spp. and other microorganisms from the surfaces of tomatoes (with greater than 99% recovery efficiency determined for *S. Newport* at an inoculum level of 10⁷ CFU cm⁻² using FUNGI-TAPE™, data not shown) and that *Salmonella* cells could be detected via FISH performed directly on the tape. Use of this tape-FISH approach was also demonstrated for other types of produce considered at risk for contamination with *Salmonella* spp., including jalapeño peppers, spinach and cilantro (data not shown). The limit of direct detection via fluorescence microscopy was 10³ CFU cm⁻² - the practical limit of detection for manual microscopy (2), and all procedures (surface sampling, cell fixation, dehydration, hybridization, counterstaining and detection) could be carried out within ~1.5 h. We also found that *Salmonella* spp. could be enriched at a tape-agar interface by simply laying cell-charged tapes face down on selective agar plates. Substantial microcolony formation was observed after only 8 h at 37°C (data not shown). Alternatively, non-sterile perfusion chambers could be sealed over slide-mounted sampling tapes, allowing liquid surface miniculture-based enrichment of sampled cells in non-selective broths. Trypticase Soy Broth (TSB) was superior to Buffered Peptone Water (BPW), both in its ability to support the growth of *Salmonella* spp. and in promoting release from the tape into liquid miniculture (Fig. 3).

Although the ultimate level of detection was not determined for the combination of liquid surface microculture and flow cytometry, a relatively low number of cells (10³ cm⁻²) could be detected directly from TSB-washed tapes and substantial enrichment of *Salmonella* spp. was
observed after a brief enrichment in liquid surface miniculture (500 µl volumes, 5 h enrichment at 37°C), even in the absence of visible turbidity (Fig. 3). Our work highlights the potential for tape-FISH to provide rapid and specific detection of *Salmonella* spp. on fresh produce surfaces, even in the presence of non-target organisms.

As a simple approach for sampling, adhesive tape methods have a number of potential advantages: they are easy to learn, use and troubleshoot and the raw materials or equipment needed are inexpensive and widely available. They are portable enough to facilitate testing in the field or in a food production environment and are non-destructive (15). Because they have the potential to save both time and money, use of such simple methods may free up limited resources, enabling more frequent or extensive testing to be done. Additionally, tape-based sampling comprises elements of both sample preparation and sample presentation. That is, the same action (contact with the food surface) accomplishes both removal of attached organisms from the surface and two-dimensional presentation of the cells on an optically clear film, facilitating downstream processing, such as staining (colorimetric, fluorescent, FISH) and direct examination via microscopy. Of special benefit to FISH-based analyses is the fact that microbial cells are removed from the host tissues, which could be a significant source of interference with probe-conferred fluorescence, due to the intense autofluorescence often seen in plant tissues (5, 6, 8).

Tape-based detection approaches have long been used in environmental microbiology for examination of plant-associated microorganisms, such as fungi present on leaves (10, 17). A key benefit of this application is that the spatial relationships of the sampled organisms from the leaves are preserved as a “mirror image” *in situ* on the tape (15, 17). The FISH approach has been used to great advantage in environmental microbiology for cultivation-
independent analyses of complex microbial consortia and FISH has also been used as a valuable tool for studying the spatial arrangements and physical interactions of specific microbes occurring in foods, such as artisanal cheeses (7, 11). Because FISH is a culture-independent approach, tape-FISH can theoretically be used for *in situ* examination of target cells on fruit or leaf surfaces, without the need for culture. Because multiple probes can be used, the presence and physical location of more than one phylotype can be determined and followed simultaneously (Fig 1).

In their study on the colonization of cilantro leaves by *Salmonella* Thompson, Brandl and Mandrell (5) found that low inocula of this organism were able to reach high cell densities when the leaves were stored under humid conditions. *S.* Thompson formed distinct microcolonies or large mixed-species aggregates with other enteric species commonly found as epiphytes on cilantro, such as *Pantoea agglomerans*. In the study of Barak and Liang (3), co-colonization of tomato plants with the plant pathogen *Xanthomonas campestris* pathovar vesicatoria led to significantly higher populations of *S. enterica* compared to plants colonized by *S. enterica* alone, suggesting cooperative activities of these two organisms during growth on these plants. Metabiotic interactions between proteolytic molds and *Salmonella* spp. have also been documented for raw, ripe tomatoes, with the metabolic activities of spoilage molds and concomitant physical degradation of tomato surfaces enhancing the growth of *S. enterica* (23). In light of these studies, culture-independent techniques capable of preserving spatial information on relationships between target cells, competitive or cooperative microflora and host structures are expected to be of great value to basic research on pathogen-produce interactions. Our tape-FISH protocol may therefore be leveraged as a basic research tool and, when coupled with enrichment, as a rapid and simple approach for sampling and screening
for *Salmonella* on fruit, herb or leafy greens surfaces in support of routine control measures or as a tool for outbreak investigation.

Several factors can potentially impact the efficiency of cell capture or release by the tape, including serovar-dependent differences in cell surface properties, the mode of attachment (i.e. non-specific adhesion, or adhesion mediated by specific structures, such as pili or flagella), the presence of soil on or moisture content of the sample surface, and whether microbial cells are present in a monolayer or in a firmly-attached biofilm (6, 13, 17, 22). Because different brands of commercially available tapes are expected to be formulated with different adhesives, they may also vary in their adhesive properties or compatibility with living cells, which could also impact cell recovery, release or growth. As noted, we were able to recover *S. Newport* artificially inoculated onto untreated tomatoes (no waxes or oils) with greater than 99% efficiency at an inoculum level of \(10^7\) CFU ml\(^{-1}\) using Fungi-Tape\textsuperscript{TM}, and cells remained culturable as determined by agar and liquid surface miniculture enrichment.

As a sampling method, tape-based removal of microorganisms from vegetable surfaces faces some practical challenges. In principle, FISH is capable of single-cell sensitivity, but, as reviewed by Amann et al. (2), bringing a single FISH-labeled cell into view under the microscope is technically challenging, with an inverse relationship existing between the number of target cells present and the time needed to find them. Therefore, rapid and reliable detection of fewer than \(10^3\) cells per cm\(^2\) is not practical using manual microscopy (2), a result that we confirmed for tape-FISH in our work. This is expected to remain a limitation of simple, manual microscopy, but developments in automated microscopy or use of scanning laser cytometry could be effective means for reliable identification of lower levels of target cells occurring on hybridized tapes.
One potential limitation of our tape-FISH approach is that salmonellae may be randomly distributed over produce surfaces and might be missed, depending on which surface is tested. In the testing of beef carcasses, sampling is narrowed to well-defined regions (i.e. brisket, flank, rump) previously established to harbor the highest microbial loads. Therefore, in testing of certain types of produce (such as cilantro), it may therefore be possible to focus sampling on well-defined regions of plant surfaces that may preferentially harbor *Salmonella* spp., such as the vein structures on leaves, or the stem scar of tomatoes (5). The use of such rational sampling approaches may increase the likelihood of detecting *Salmonella* spp. or other pathogens on the surfaces of some types of produce via tape-FISH.

Tape-based sampling methods have long been used in the separate fields of environmental, food and clinical microbiology. Therefore it is fitting to recognize that Tape-FISH, as described here, may have potential applications at various points along the production-to-consumption-to-disease continuum, in other words, from “farm to fork to physician”. We have described the use of Tape-FISH for detection of *Salmonella* spp. on the surfaces of tomatoes, jalapeños, spinach and cilantro and have shown for tomatoes that this dual sampling and sample presentation approach can also be combined with brief enrichments using either *Salmonella*-selective agar (XLT-4) or non-selective broth culture (TSB). In the latter application, we found that because the tape-cell complex is essentially two dimensional, we could perform a liquid surface miniculture step by overlaying a minimal volume of broth on the tape after it was affixed to a microscope slide. In this application, tape-based sampling effectively represents a means for cell concentration prior to enrichment. Enrichment of even relatively few cells in a small volume, with subsequent analysis of the entire volume may be a promising means for facilitating earlier detection of
target cells, as no subsequent concentration step (filtration, centrifugation, etc.) is needed. In addition to its use for detection, the Tape-FISH technique may also be a valuable research tool for exploring events occurring during the colonization of tomatoes by *Salmonella*, or the interplay between spoilage microflora and *Salmonella* and the role of such metabiotic interactions on establishment and persistence of infection (3, 23). It is hoped that the established and familiar nature of adhesive tape-based techniques, combined with our simple and streamlined approach for FISH-based staining of target cells will enable more rapid adoption of the tape-FISH approach by food microbiologists who may not be familiar with or currently using whole cell molecular techniques.

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Figure 1. Tape-FISH for detection of *Salmonella* spp. in mixed culture from tomato surfaces. Tomatoes were spiked with a mixture of *S.* Typhimurium (10<sup>7</sup> CFU cm<sup>-2</sup>) and *R.* glutinis (10<sup>6</sup> CFU cm<sup>-2</sup>), then sampled with adhesive tape after drying. Tapes were hybridized for 30 min with a combination of probes targeting *Salmonella* spp. (Sal3/Salm-63 cocktail, green label) and eukaryotic cells (EUK-516, red label). These results demonstrate the utility of tape-FISH for simultaneous visualization of the distribution and interactions between multiple phylotypes occurring together on produce surfaces.
Figure 2. Tape-based sampling of tomato surfaces and liquid surface miniculture.

Microorganisms artificially spiked onto tomato surfaces were sampled using sterile adhesive tape, as shown in panel A. Tapes were applied with gentle and even pressure, ensuring full contact of the sampling tape with produce surfaces, followed by removal of the tape-cell complex for subsequent processing. Panel B illustrates filling of a perfusion chamber prior to enrichment via liquid surface miniculture. The bottom surface of the chamber was comprised of a *Salmonella*-charged tape, mounted sticky side up. After filling with 500 µl of non-selective broth (TSB or BPW, as described in the text), chambers were incubated for 5 h, followed by cell harvesting, fixation, hybridization and analysis via flow cytometry (see Fig. 3).
Figure 3. Tape-FISH combined with liquid surface miniculture for rapid detection of *Salmonella Typhimurium* on tomatoes via flow cytometry. Adhesive tape was used to remove *S.* Typhimurium from tomato surfaces inoculated with $10^3$ cells cm$^{-2}$. As described for Fig. 2B, cell-charged tapes were mounted face-up on microscope slides, perfusion chambers were placed on top of the tape, then filled with non-selective broths. Liquid surface minicultures were incubated for 5 h, mixed via up and down pipetting using gel loading tips, processed for FISH and then analyzed via flow cytometry. When TSB was used, it was possible to detect *Salmonella* directly from tapes (0 hr, TSB). Despite a lack of visible
turbidity, substantial enrichment was possible after only 5 h of non-selective preenrichment in TSB (5 hr, TSB). These data show the utility of FISH and flow cytometry in combination with adhesive tape-based sampling for the rapid detection of *Salmonella* on contaminated tomatoes.
CHAPTER 5. IMPROVED SAMPLE PREPARATION FOR DIRECT CYTOMETRIC DETECTION OF *LISTERIA MONOCYTOGENES* ON PORK FRANKFURTERS

A paper to be submitted to Food Microbiology

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ABSTRACT

A key problem in food microbiology is the detection of specific microorganisms within physically and microbiologically complex food matrices. Flow cytometry is a detection technology which allows the rapid analysis and discrimination of microbial populations according to single cell light scatter and fluorescence characteristics. Although an attribute of flow cytometry is to distinguish microbial cells from acellular particles, excess
particulate matter may still interfere with detection by adsorbing available probe, rebinding suspended bacteria or, depending on the size of the particles, clogging the cytometer. As a means to circumvent these complications, we investigated the use of an alternative means (The Pulsifier®) for preparation of pork frankfurter samples spiked with *Listeria monocytogenes* NADC 2045 prior to fluorescence in situ hybridization and cytometric analysis. No significant differences were observed between pulsification and homogenization (p = 0.44) in their ability to detach the *L. monocytogenes* from spiked samples, but pulsification was substantially less destructive, yielding clearer homogenates having markedly lower particulate loads. This resulted in significantly improved signal to noise ratios (p < 0.001), further enhanced by application of a blocking agent. Pulsification allowed for further processing of samples via membrane filtration and use of minimal amounts of buffer for cell detachment, which in return permitted a direct detection sensitivity of ~10^2 cells g^-1.

**INTRODUCTION**

Flow cytometry is a detection technology that allows for rapid analysis and discrimination of complex microbial populations according to single cell light scatter and fluorescence characteristics. It can therefore be used to distinguish between cellular and acellular particles in liquid samples, and when combined with an appropriate molecular probe, to specifically detect microbial cells. Flow cytometry can be effectively used in combination with fluorescence in situ hybridization (FISH), which is a rapid nucleic acid-based method that can be employed to specifically recognize permeabilized whole microbial cells. However, performing FISH on gram-positive bacteria can be difficult due to the
presence of negatively charged and thick cell walls typical of this group of microorganisms. Lengthy preparatory steps, which include application of proteinase K digestion and lysozyme, are needed in order to permeabilize gram-positive cells to DNA-based oligonucleotides commonly used in FISH. An alternative to DNA-based oligonucleotides, the peptide nucleic acids (PNA) are molecules that mimic DNA, but instead are in possession of an uncharged, achiral backbone. These unique characteristics imparted by PNA molecules increase their ability to penetrate cell walls of gram-positive cells and the hybridization kinetics, make them resistant to nucleases, which can be present in food matrices, as well as increase the accessibility of regions of the ribosome which are inaccessible to DNA probes (Brehm-Stecher et al., 2005, Stender et al., 2002). Although a strength of flow cytometry is to distinguish microbial cells from acellular particles, appropriate steps should be taken to minimize the generation of excess particulate matter, as it may interfere with detection by adsorbing available probe, rebinding suspended bacteria or, depending on the size of the particles, clogging the cytometer.

A typical approach used within the food industry for macerating food samples and detaching adherent bacteria is homogenization by “stomaching”. However, homogenization can generate a high background of particulate matter that may interfere with detection. An alternative approach involves the use of the Pulsifier® (Microgen Bioproducts, Ltd.). Using a rapid, vibratory agitation, the Pulsifier® detaches food-adherent bacteria as efficiently as homogenization, yet in less time (15 sec vs. 1 min) and with less sample destruction, minimizing the generation of particulate matter. We have found that that pulsification of pork wieners artificially contaminated with Listeria monocytogenes yields microbial counts comparable to homogenization, but results in markedly clearer supernatants. An additional
advantage of the clearer supernatant gained using pulsification is that the liquid is filterable. Although simple filtration is a useful means for removing any remaining particulate matter, filtration may also be used as a means for cell concentration, leading to further enhancement in detection sensitivity.

Because adequate sample preparation is key to the success of any assay, including our “FISH and Flow” approach, we are interested in optimizing such pre-analysis preparatory steps for the assay we are developing. In this work we investigated a simple and effective protocol for optimization of sample preparation via pulsification and PNA-FISH of *Listeria monocytogenes* NADC 2045 (ScottA) on pork frankfurters, followed by cytometric analysis using a research-type cytometer (FACSCanto) and further application in a simpler, task-dedicated instrument optimized for routine testing, Rapid Bacteria Detector 3000 (RBD 3000).

**MATERIALS AND METHODS**

**Chemicals.** Chemicals were obtained from Sigma-Aldrich (St. Louis, MO) or from Fisher Scientific (Itasca, IL), unless otherwise stated. Microbiological media were from Difco Laboratories (Detroit, MI) or Remel (Lenexa, KS).

**Cultures and inoculum preparation.** *Listeria monocytogenes* NADC 2045 (ScottA) and *Brochothrix thermosphacta* ATCC 11509 were activated from -20°C glycerol stocks and kept on trypticase soy agar (TSA) slants at 4°C prior to being used in experiments. A loopful from refrigerated slants was used to inoculate 10 ml fresh trypticase soy broth (TSB) in screwcap borosilicate tubes followed by static incubation for 20-22 h at 30 and 25°C for *L. monocytogenes* and *B. thermosphacta*, respectively. A second transfer was performed by
transferring 100 µl from the overnight growth into a fresh TSB tube followed by incubation. One milliliter portions were transferred into 1.5 ml DNAse & RNAse free polypropylene microcentrifuge tubes (Biologix, Lenexa, KS) and centrifuged at 2000 x g for 5 min. The supernatant was then discarded and cells were first washed twice in 0.1% (w/v) peptone water (PW) and either fixed or resuspended in an equal amount of PW for use in inoculating the food samples.

**Comparison of pulsification and homogenization via plating.** Vacuum packaged commercially available pork frankfurters (containing 8 individual links weighing ~52 g each) were obtained from a local grocery store. The original packages were opened with scissors, then the links were cut into 25 g portions before being removed and placed in filter Stomacher® 400 Circulator bags (Seward, Worthing, UK) for use with a Stomacher® 400 Circulator Stomacher or Whirl-Pak bags (Nasco, Fort Atkinson, WI) for use with PUL100 Pulsifier® (Microgen Bioproducts, Ltd, Camberley, UK). Aseptic techniques were followed through all the above described steps and scissors, knifes, and tongs used in the procedure were pre-sterilized via autoclaving. Aliquots (250 µl) of stationary-phase *L. monocytogenes* serially diluted in 0.1% PW to achieve final concentrations of ~10^6 CFU g^{-1} were spiked onto several spots on the surface of the samples and gently massaged from the outside of the bag to promote the spread of the inoculum on the surface. The bags were placed within a laminar flow hood and left there for ~2 h at 25°C to allow for attachment of the spiked cells. Detachment by homogenization was performed by adding 225 ml PW, followed by pummeling for one minute at 200 rpm in a Stomacher® 400 Circulator. Pulsification was conducted by adding 75 ml of the buffer and running the instrument for 15 sec. Homogenized samples were prepared for FISH by filtering through four layers of cheese cloth and
differential centrifugation at 300 x g for 30 seconds in order to precipitate the particulate matter generated by homogenization of the sample, followed by extraction of the supernatant for further processing. No filtration or differential centrifugation steps were performed on pulsified samples.

**Minimal diluent for bacterial detachment via pulsification.** All procedures were conducted as described in the section above, except whole links were used in all experiments instead of 25 g portions, and the level of contamination of ~10⁶ CFU link⁻¹ (~2 x 10⁵ CFU g⁻¹) was achieved by spiking an aliquot of 100 µl PW containing the inculum, then subsequently treated the same as described above. Then 2, 20 or 50 ml buffer were added to the samples prior to pulsification.

**Application of blocking agent and membrane filtration.** Denhardt’s solution [1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, and 1% (w/v) bovine serum albumin] provided at 50x initial concentration was added at a 5x concentration in 1 ml PW to samples in 1.5 ml polypropylene tubes followed by incubation at 25ºC for 30 min prior to fixation. Pulsified samples processed for evaluation of minimal diluent amounts needed to perform bacterial detachment, were also filtered through 20 µM CellTrics® monofil nylon filters (Partec, Munster, Germany). Treatment with Denhardt’s solution was furthermore tested following membrane filtration.

**Pure culture and spiked sample fixation.** Stationary phase cells from overnight growth in 1 ml portions in 1.5 ml microcentrifuge tubes were spun down at 2000 x g for 5 min and the supernatant was discarded. The pellet was resuspended in the remaining liquid followed by resuspension in equal volumes of 1:1 mixture of absolute ethanol and 0.2 µM filtered phosphate buffered saline solution (pH 7.2). Fixation was performed for 30 min at
25°C; the sample was then stored in the fixative, which served as storage buffer at -20°C until use. Fixation procedures were conducted in the same way for spiked food samples, however stomached samples were exposed to filtration and differential centrifugation prior to fixation as described above.

**Microbiological analysis.** Following serial dilutions in PW, inocula were track plated in duplicate onto Modified Oxford Agar (MOX) containing 0.1% Modified Oxford Antimicrobial Supplement. Samples were tested via plating prior to use in experiments to verify the presence or absence of *Listeria* spp. Incubation was conducted for 24 h at 35°C and typical colonies were counted and expressed as CFU g⁻¹ or CFU link⁻¹.

**FISH and probe conditions.** A *Listeria* spp. specific LisUn-11 PNA probe modified at the 5’ end with Cy5 and two “O” linkers (Cy5-OO-AAG GGA CAA GCA GT), developed in a previous collaboration with Boston Probes, Inc. (Bedford MA) was obtained from Panagene (Daejeon, Korea) in lyophilized form and brought into solution in 50%, N, N dimethyldimethyl formamide (DMF) in 0.1 µM filtered molecular grade water. The stock solution was further diluted in 50% DMF to create the working solution of 100 µM which was kept in the dark at -20°C until use in FISH.

FISH was performed in 100 µl portions of sample from storage buffer, which were previously centrifuged (2,000 x g, 5 min) and the supernatant discarded. Cell pellets were resuspended in 100 µl hybridization buffer (20 mM Tris [pH 9.0], 100 mM NaCl, 0.5% sodium dodecyl sulfate) containing approximately 100 pmol ml⁻¹ of the probe. Hybridization reactions were performed in 1.5 ml polypropylene microcentrifuge tubes on an Eppendorf® Thermomixer R heat block (Fisher Scientific) set to a constant temperature of 55°C.
Hybridizations were conducted for 30 min, after which 500 μl wash buffer (10 mM Tris [pH 9.0], 1 mM EDTA) preheated to the hybridization temperature were added to each reaction. Washing was carried on for 30 min at 55°C with intermittent vigorous vortexing, then spun down (2,000 x g, 5 min) and resuspended in 0.5-1 ml PBS for analysis in a FACSCanto cytometer or 1.3 ml phosphate buffer for cytometric analysis via RBD 3000. Samples were kept in the dark at 2-8°C until they were analyzed by cytometry.

**Flow cytometry.** Excitation in the FACSCanto cytometer (Becton Dickinson, Mountain View, CA) was achieved via a red (633 nm, 17 mW HeNe) laser. The fluorescence signal was detected using a 660/20 bandpass filter, side scatter via a 48/10 bandpass filter and forward scatter via a neutral density filter. Data was collected as list mode data (lmd) files then exported as FCS 2.0 files for further analysis. Data collection was triggered on side scatter (threshold 200). Cytometric detection was also performed in a RBD 3000 (Advanced Analytical Technologies, Inc., Ames, IA). The RBD 3000 is equipped with a single 488 nm excitation source and has fixed optics. Two parameters, side scatter and fluorescence were collected and data were collected and stored in lmd format. The Weasel (WEHI, Melbourne, Australia) software was used to convert the lmd files to FCS files for further analysis. The FACSCanto instrument was run based on time at low flow rate, which permits the analysis of approximately 10μl of sample per minute. The RBD 3000 cytometer was run for 2-5 min on time permitting the analysis of 100 - 250 μl of sample. Data were analyzed using FlowJo software (version 4.6.2, Tree Star, Inc., Ashland, OR).

**Statistical Analysis and Reproducibility.** All experiments were conducted in triplicate, including microbiological analyses, hybridizations and flow cytometric analyses. The geometric means (GM) for fluorescence as well as the robust coefficients of variation
(CV) were extrapolated from the cytometric output via analysis with FlowJo. Direct comparisons between samples were performed between samples using the Chi-Squared [Chi(T)] test to compare univariate histograms. Significance level was set at 0.99.

The General Linear Model (GLM) analysis was conducted to compare microbiological analysis data. Means were compared via the Analysis of Variance (ANOVA) for significant differences. Significance level was set at 0.95. When significant differences were found, pairwise comparisons between means were conducted using Student’s t-test.

RESULTS

Bacterial detachment and sample preparation for cytometry by pulsification. No significant differences (p = 0.44) between pulsification and homogenization for detachment of *L. monocytogenes* on pork frankfurters were observed as confirmed by plating. Figure 1 shows dot plots of pork frankfurters inoculated at levels of ~10^6 CFU g\(^{-1}\) which were subsequently prepared by pulsification or homogenization with or without addition of Denhardt’s solution as a blocking agent as well as the respective uninoculated negative controls. To allow for a direct comparison between the two sample preparation methods, the homogenized samples were pre-processed by filtering and differential centrifugation to remove background particles generated during homogenization. Initial dilution in PW for homogenization was 1:10, while for pulsification was 1:4, thus the cytometer run time was adjusted accordingly to allow the observation of comparable target population numbers. Side by side comparisons shown in panels B and E demonstrate differences in the cytometric output for stomached and pulsified samples. While the target populations in both samples were not expected to differ, considering that samples prepared by either method were spiked
at the same levels, approximately two times as many positive events were observed in the positive events gate for pulsified samples compared to stomached samples. On the other hand, the positive population in pulsified samples corresponds to 6.55% of the total events collected compared to merely 0.26% in stomached samples, clearly indicating the effect of increased particulate matter generated by homogenization on the cytometric output. The lower number of events in the target population in stomached samples might be explained by the exclusion from the positive population gate of events which appeared to be highly fluorescent but displayed higher scatter characteristics. This could be due to target cells associated to particulate matter generated by homogenization. We have characterized such a phenomenon via imaging cytometry in alfalfa sprouts contaminated with salmonellae (Bisha and Brehm-Stecher, 2009). Another factor that might account for lower numbers of target events in stomached samples is the non-specific binding of the probe to background debris, which depletes the probe levels available for specific hybridization of the target cells, possibly increasing the number of unstained target cells.

Cytometric outputs for stomached and pulsified samples were compared for statistical differences as shown in Figure 2, Panel A. Using unstained cells as reference, the samples were gated on side and forward scatter in order to produce univariate fluorescence histograms. Plot differences were calculated using the Overton subtraction logarithm and direct pair wise comparisons were conducted using the Chi-Squared test with a level of significance of 0.99. Pulsified samples were significantly different from stomached samples (p < 0.001), with the highest difference indicated at value 13.8 on the fluorescence scale, which corresponds to the target population. This indicates the possibility for increased
sensitivity of cytometric detection on pulsified samples as compared to stomached samples. The plot difference also shows significantly lower background counts in pulsified samples.

**Application of Denhardt’s solution.** The effect of the application of Denhardt’s solution as a blocking agent to pulsified and stomached samples is shown in Figure 1, Panels C and F, respectively. Addition of Denhardt’s solution resulted in an increase in positive events in both stomached and pulsified samples. The number of positive events in stomached samples increased from 203 to 242, while the number of positive events in pulsified samples increased from 419 to 620. The increase in the absolute numbers of positive events indicates that the addition of the blocking agent allows for an increase in available probe for specific hybridizations, probably by not allowing the non-specific binding of the probe to debris and non-target cells. While an increase in absolute positive events was noted, a disproportionally higher increase in percent of total events of the target populations in the samples prepared by either method was observed. In pulsified samples, the addition of Denhardt’s solution increased the ratio of the target to non-target events from 6.55 to 11.2% and in stomached samples from 0.26 to 0.43%. It is clear that main effect of the blocking agent is exerted by suppressing the background noise. Panel B in Figure 2 shows the statistical comparisons between pulsified samples with or without added Denhardt’s solution. Samples treated with Denhardt’s solution were significantly different (p < 0.001) from non-treated samples. The analysis revealed that the main difference was observed at value 8.66, thus confirming the role of Denhardt’s solution as mainly a suppressant of background clutter. As a result it became clear that sample treatment with Denhardt’s solution improved the cytometric output by both increasing the target signal and decreasing the contribution of background events.
Effect of pulsification on cytometric detection via RBD 3000. Figure 3 shows dot plots generated from the cytometric analysis of pulsed or stomached samples treated with Denhardt’s solution (Panels B and C, respectively) and the positive control, hybridized L. monocytogenes NADC 2045 hybridized with LisUn-11 (Panel A). The gate was constructed based on the positive control to encompass 80% of the events and exclude outliers. Cytometric outputs for stomached and pulsed samples differed considerably, with only ~36% of the positive events appearing in the gate for stomached samples as compared to ~71% in pulsed samples. However, the task of discriminating between true positives and false positives in stomached samples becomes difficult as clearly the number of events in the positive gate appears high in scatter and can not be clearly differentiated from non-target events based on the side scatter characteristics. This instrument is built for routine testing and it does not collect events above a set fluorescence threshold, thus it becomes important that the non-target background is suppressed. From our analysis we deducted that depressing generation of debris by pulsification allows for an improvement of the cytometric output via this instrument.

Pulsification in minimal buffer volumes. Considering the fact that pulsification decreases the amount of debris generated during cell detachment, we evaluated lower amounts of buffer for cell detachment via pulsification. When 2, 20 or 50 ml of PW were used to pulsify whole pork frankfurters spiked at ~ 10^6 CFU link⁻¹, we confirmed by plating that there were no significant differences in bacterial detachment when these amounts were used (p > 0.05), allowing us to further investigate by flow cytometry the effect of using minimal diluent quantities on the sensitivity of cytometric detection. Panels A, B and C in Figure 4 shows dot plots for samples pulsified in 50, 20, and 2 ml PW, respectively. The
numbers of positive events increased significantly as the buffer volume used in pulsification as decreased. The number of positive events for samples pulsified in 50, 20 and 50 ml PW was 152, 234 and 2166 events, respectively. Employment of membrane filtration decreased the background noise in samples pulsified in the lowest amount of diluent; however that was not true for samples pulsified in other volumes of buffer. Indeed the main background suppressing effect was achieved by using Denhardt’s solution. The increase in the target events allowed for an increase in sensitivity of our detection protocol, permitting the detection of ~ 500 cell g⁻¹ in samples pulsified in 2 ml diluent.

**DISCUSSION**

Use of pulsification for detachment of microorganisms from foods has been evaluated by other authors in foods ranging from produce to muscle foods and microorganisms ranging from coliforms to oocysts (Fung et al., 1998; Sharpe et al., 2000; Kang et al., 2001; Moriarty et al. 2004). Generally the authors found very good correlations between the ability of pulsification to detach microorganisms from food and the standard methods. This is in agreement with the results that we obtained via plating on pulsified pork frankfurters spiked with *L. monocytogenes*. Our finding that pulsification generates less debris and clearer suspension compared to homogenization also seems to be in agreement with other authors’ findings. Homogenization uses a paddle-like action to macerate food, which tears the sample matrix releasing the microorganisms in liquid for subsequent analysis. Stomachers became really popular (~40,000 instruments in use today) following studies that found that homogenization generated comparable counts with samples processed using lab blenders, but imparted advantages that included reduction of debris generated by the sample preparation.
process (Sharpe and Jackson, 1972; Tuttlebee, 1975; Jay and Margitic, 1979). Pulsification does not destroy the food sample, as it uses shock waves to release the microorganisms into the diluent instead of the paddle-like action exerted by homogenization. The absence of major food debris in pulsified food samples can allow for further processing and concentration of samples by membrane filtration without causing cell loss and clogging of filters (Sharpe et al., 2000). It has been suggested before that the reduced generation of debris by pulsification might make this sample preparation method attractive for use with methods such as ATP bioluminescence or polymerase chain reaction, which can be negatively affected by presence of inhibitors (Fung et al., 1998). To our knowledge there has been no prior research conducted on the effect of pulsification on molecular detection of foodborne bacteria. We sought to evaluate the use of pulsification to enhance the flow cytometric detection following PNA-FISH of *L. monocytogenes* on pork frankfurters, a model meat system. Pulsification significantly reduced the amount of debris in samples, allowing for increased signal and reduced background noise, facilitating detection via flow cytometry. Another consequence of the low amount of particulate matter generated during sample preparation by pulsification is that lower amounts of diluent could be used for bacterial detachment from the food sample, thus enhancing the sensitivity of the detection method by lowering the initial dilution of the target cell numbers in the sample. The sensitivity of a detection method can be hampered by the dilution of the initial numbers of cells, lowering the validity of the method even though it may still impart high specificity. In food microbiology, samples are commonly prepared using homogenization or even blending. The need for processing liquid samples in order to conduct detection of the target cells released in the liquid commands the use of at least 1:10 dilution of the sample in an appropriate buffer.
This is mainly due to the fact that these sample preparation methods are destructive in nature and generate significant amounts of debris, which would seriously interfere with the sample analysis if not diluted considerably in a buffer system. However, this initial dilution of the target microorganisms also lowers the sensitivity of the detection method. Pulsification generates only a fraction of the debris produced by other sample processing instruments, which allows for minimal amounts of buffer to be used in order to release the cells into liquid. We found that buffer amounts as low as 2 ml were sufficient to mediate detachment of *L. monocytogenes* from pork frankfurters, allowing for a sensitivity of flow cytometric detection of \(~10^2\) CFU g\(^{-1}\). Using Denhardt’s solution (Denhardt, 1966) significantly further enhanced the cytometric detection by mainly suppressing the background noise. Pulsified samples could readily be filtered through membrane filters, and while this was not found to significantly facilitate detection in our study, it might find use with other types of food samples in which greater amount of debris could possibly be generated.

In conclusion, we have developed a simple and effective protocol for detection of *L. monocytogenes* on a model meat system (pork frankfurters). This protocol involves sample preparation steps such as pulsification, use of blocking agents and membrane filtration which could be coupled with PNA-FISH and flow cytometry for specific detection of *L. monocytogenes* on pork frankfurters. We validated our protocol using two types of instruments for end-point detection, a FACSCanto cytometer which is designed for research purposes and a simple, fixed optics instrument designed for use in routine bacterial detection, the RBD 3000. We conclude that the FISH followed by cytometric detection can be significantly affected by sample preparation methods. We drastically improved the sensitivity
of cytometric detection by introducing simple modifications to common sample preparation methods for food microbiological analysis.

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REFERENCES


Figure 1. Flow cytometric detection of *L. monocytogenes* on pork frankfurters following sample preparation by homogenization or pulsification with or without application of Denhardt’s solution. Panel A shows uninoculated homogenized pork frankfurters hybridized with LisUn-11-Cy5. Panels B and C show homogenized pork frankfurters inoculated with ~10^6 CFU g⁻¹ *L. monocytogenes* subsequently hybridized with LisUn-11-Cy5 with or without added Denhardt’s solution, respectively. Panels D, E and F
show pulsified uninoculated, inoculated, and inoculated treated with Denhardt’s solution samples, respectively. Cytometric detection via FACSCanto.

![Image of histograms showing cytometric detection comparison](image)

**Figure 2. Direct Comparison of pulsification and homogenization and evaluation of Denhardt’s application for cytometric detection of *L. monocytogenes* NADC 2045 on pork frankfurters.** Panel A shows univariate histogram overlays of inoculated homogenized (black) and inoculated pulsified (blue) samples as well as the plot difference (green). Panel B shows univariate histogram overlays of inoculated pulsified (black) and inoculated pulsified with added Denhardt’s solution (blue) samples as well as the plot difference (green). Pulsification was superior to homogenization as significantly higher target events and lower non-target events were collected (p < 0.001). Denhardt’s solution addition significantly improved the output by mainly decreasing the background fluorescence (p < 0.001).
Figure 3. Flow cytometric detection of *L. monocytogenes* by RBD 3000 on pork frankfurters following sample preparation by homogenization or pulsification. Panel A shows the cytometric output for hybridized cells of *L. monocytogenes* NADC 2045, while
Panels B and C, present the output for hybridized inoculated pulsified and homogenized samples respectively. The percentage of positive events is shown on the side of the positive gate. Detection via this instrument is designed to be triggered by fluorescence, thus signals which manifest fluorescence intensity below the threshold of the instrument are not collected and are not available for analysis.

Figure 4. Increased sensitivity of cytometric detection following pulsification in minimal buffer volume. Panels A, B, and C represent spiked samples pulsified in 50, 20 and 2 ml buffer, respectively. Clearly using low volumes of buffer permitted a substantial increase in target events as shown in the figure, which increases the sensitivity in the case when direct detection is attempted on low-level naturally contaminated samples. Using low volumes of diluent allowed for a detection sensitivity for our FISH and Flow method of \( \sim 10^2 \) CFU g\(^{-1}\) on pulsified inoculated pork frankfurters.
CHAPTER 6. FLUORESCENCE IN SITU HYBRIDIZATION FOR SENSITIVE QUALITATIVE DETECTION AND IDENTIFICATION OF SALMONELLA SPP. IN PEANUT BUTTER

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ABSTRACT

Peanut butter has been traditionally considered a microbiologically shelf-stable food product, due to the low water activity and the application of heat treatment prior to packaging. However it has recently emerged as an important vehicle for transmission of salmonellosis with two major outbreaks occurring in between 2006 and 2009. Rapid
detection methods can present a valid alternative to the traditional detection methods and reduce the chance of the contaminated product reaching the consumer. Although a number of rapid and specific methods for microbial detection have been developed in recent years, their routine application can be frustrated by the complex nature of certain food matrices. With its high fat content and viscosity, peanut butter is a good example of such a challenging matrix.

**Fluorescence in situ** hybridization (FISH) is a specific and robust method, which can detect whole cells in complex matrices, and when coupled with fluorescence microscopy or flow cytometry it can allow for rapid and sensitive detection of pathogens in food. However, the sensitivity of FISH can also be negatively affected by the specifics of the matrix in which the target cells are found. We evaluated the effectiveness of FISH combined with fluorescence microscopy or flow cytometry for detection of *Salmonella* spp. in peanut butter. Sample preparation methods for FISH such as Circulating Immunomagnetic Separation (cIMS) and non-selective enrichment media were assessed followed by development of protocols which permitted the rapid detection of as few as 0.5 CFU g⁻¹ of *Salmonella* spp. in peanut butter.

**INTRODUCTION**

*Salmonella* spp. has been rarely associated with foodborne disease outbreaks caused by consumption of peanut butter. Indeed, until recently the only documented such outbreak has been the one caused from *Salmonella* ser. Mbandaka in Australia in 1996 (Scheil et al., 1998). Recently however, two well-publicized consecutive outbreaks of salmonellosis have occurred in the U.S. The first one was linked to the consumption of peanut butter contaminated with *Salmonella* Tennessee and infected a total of 628 persons across 47 states,
while the second involved peanut butter and peanut butter products contaminated with *Salmonella* Typhimurium and infected a total of 529 persons from 43 states and one in Canada (CDC, 2007; CDC, 2009). Although peanut butter is a low aw product which undergoes a heat treatment prior to packaging, the possibility of salmonellae surviving improper heat treatment or contaminating the ready-to-eat product prior to packaging still exists as proved by the recent outbreaks. Salmonellae have been shown to survive for long periods of time in peanut butter (Burnett et al., 2000; Park et al., 2008). Indeed it has been shown that high fat content might exert a protective effect against heat inactivation of *Salmonella* spp. in food, while the adaptation to low aw has also been indicated to increase their heat resistance (Juneja and Eblen, 2000; Mattick et al., 2000). Considering the fast-paced production and distribution of food products, the development and application of alternative rapid methods for detection of *Salmonella* spp. is needed to assure the effective and timely detection before it reaches the consumer, avoiding foodborne disease and product recalls (Maki, 2009). Fluorescence *in situ* hybridization (FISH) which uses rRNA in whole permeabilized cells as a diagnostic target has great potential as a method for in situ detection of bacteria (Amann et al., 1995; Brehm-Stecher, 2008). FISH can be combined with fluorescence microscopy or flow cytometry to accomplish detection without need for cultivation, thus creating the basis for rapid and specific detection of bacteria.

We hypothesized that combining FISH with fluorescence microscopy or flow cytometry would allow for rapid and sensitive detection of *Salmonella* spp. in peanut butter. A protocol was developed which entailed artificial spiking of 25 g portions of peanut butter with various levels of *Salmonella* spp., subsequent non selective enrichment for up to 18 h, followed by FISH on slides for fluorescence microscopy [with or without Circulating Immunomagnetic
Capture (clIMS capture) and liquid hybridizations for flow cytometry. Following short non-selective enrichments (8 h) as few as 0.5 CFU g$^{-1}$ *Salmonella* spp. were detected with a total sample preparation time of about one hour. We concluded that FISH can be successfully employed for detection of *Salmonella* spp. in peanut butter.

**MATERIALS AND METHODS**

**Microbiological media and reagents.** Microbiological media were obtained from Difco (Detroit, MI) or Remel (Lenexa, KS) and reagents from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Itasca, IL) unless otherwise specified. Paramagnetic beads coated with *Salmonella*-specific polyclonal antibodies were obtained from Matrix Biosciences (Golden, CO) and HPLC purified DNA oligonucleotides from Integrated DNA Technologies (IDT, Coralville, IA).

**Bacterial strains and culture conditions.** For spiking food samples, the following serovars were used: *Salmonella enterica* subs. *enterica* ser. Typhimurium ATCC 14028, ser. Mbandaka ATCC 51958; and the wild-type isolates, ser. Tennessee 2007026177 isolated from the 2007-2008 peanut butter, and ser. Typhimurium 200867028, which shares an indistinguishable pulsed-field gel electrophoresis (PFGE) pattern with the peanut butter outbreak of 2008-2009. In addition, the following strains which represented all seven DNA subgroups of *Salmonella* spp. were used in experiments to determine inclusivity of the developed FISH protocol and reactivity with the polyclonal antibody coated paramagnetic beads: *Salmonella enterica* subs. *enterica* ser. Typhimurium SA 3250 (I), subs. *salamae* SA 4406 (II), subs. *arizonae* SA4407 (IIIa), subs. *diarizonae* SA 4408 (IIIb), subs. *houtenae* SA 4409 (IV), subs. *indica* SA 4401 (VI), and *S. bongori* SA 4410 (former DNA subgroup V).
The closely related species, *Escherichia coli* ATCC 25922 was also used throughout all experiments as a negative control. Cultures were maintained as streaks on trypticase soy agar (TSA) plates following reactivation from -80°C glycerol stocks. Preceding experiments, they were grown statically in tryptic soy broth (TSB) at 30°C for 20-22 h, before being subjected to fixation or used to spike samples.

Artificial contamination of peanut butter samples and microbial analysis.

Creamy peanut butter was acquired from local vendors. 25 g portions were aseptically removed from the original containers and transferred to filter stomacher bags where they artificially contaminated with the inoculum which was washed once in 0.1% (w/v) peptone water (PW) prior to use. The contamination process was performed in several spots in the sample making sure to insert the tip inside the paste to make sure that the inoculum is delivered in different areas of the sample. Samples were then allowed to sit for ~3 hours following spiking with levels of salmonellae varying from ~ 0.5 to 10^7 CFU g^(-1). Microbial analysis to determine numbers of salmonellae was performed following homogenization in a Stomacher Circulator® 400 in 225 ml PW or enrichment broth for 1 min. Serial dilutions were made in PW, and than track plating was performed in XLT 4 (Xylose Lactose Tergitol™ 4) agar. Track plates were incubated at 35°C for 24 h. Typical colonies were counted and expressed as colony forming units (CFU) per gram or per ml (enrichments). Inocula were also plated prior to spiking to confirm the inoculum level.

Non-selective enrichment. 25 g samples spiked at low levels (~ 0.5 CFU g^(-1)) with washed cells were homogenized by homogenization in 225 ml Buffered Peptone Water (BPW), Universal Preenrichment Broth (UPB) or Lactose Broth (LB). In addition, samples prepared for analysis by flow cytometry were enriched in TSB and Terrific Broth (TB).
Enrichment was performed for up to 18 h and one-ml aliquots were sampled every two-hours from the aqueous phase of the enrichment or from the stomachate following 1 min homogenization and processed for fixation. The stomacher bag was then transferred to the Pathatrix unit for immunomagnetic capture and processed as described below.

**Immunomagnetic separation.** Flow-through immunomagnetic separation was carried out using a Pathatrix unit (Matrix Biosciences) in 250 ml volumes of samples which consisted of 25 g of peanut butter sample homogenized in 225 ml PW or enrichment broth. Immunomagnetic separation was also carried out on samples made by 1:5 dilution of enrichment containing salmonellae in fresh enrichment broth to potentially maximize recovery. Following manufacturers instructions, 50 µl antibody-coated paramagnetic beads were used for capture. Capture was performed for 30 min at 37°C, then samples were washed one in the unit with 100 milliliters of fresh PW or enrichment broth (same medium as the one in which the initial concentration was performed), followed by concentration for 5 min on a magnetic rack. The supernatant was removed, bead-cell complexes were transferred to 1.5 ml microcentrifuge tubes and washed once in 500 µl of phosphate-buffered saline (PBS) pH=7.2. Consequently they were concentrated again using a magnet and the supernatant discarded. Following concentrations using a magnet, gentle flicking was used to detach beads sticking to the walls of the tubes instead of vortexing to avoid perturbation of the cell-bead complexes. For inclusivity experiments and electron microscopy, cell suspensions in PW at a concentration of ~ 10^7 CFU/ml in one ml volumes were reacted on a rocking unit at 37°C for 30 min with 20 µl paramagnetic beads, then processed to be concentrated as stated above.
Fixation. Pure cells or food sample enrichments were pelleted at 2000 x g for 5 min, and then fixed in one ml volumes of 10% neutral buffered formalin. Fixation was performed for 30 minutes at 25°C; samples were pelleted again and resuspended in storage buffer (1:1 v/v mixture of PBS in absolute ethanol) and stored at -20°C until use in FISH. Cells captured by cIMS were fixed in 200 µl of the fixative, and then resuspended in equal volumes of storage buffer. For cIMS captured cells, pelleting was performed via magnet concentration and vortexing between steps was substituted by gentle flicking of the containing vessel.

Probes and FISH. A dual probe cocktail of DNA oligonucleotides specific for *Salmonella* spp. at a total concentration of 5 ng/µl probe (2.5 ng/µl each), Sal3 (5’-AATCACTTCACCTACGTG-3’) (Nordentoft et al., 1997) and Salm-63 (5’-TCG ACT GAC TTC AGC TCC-3’) (Kutter et al., 2006) was employed as described before (Lantz et al., 2008; Bisha and Brehm-Stecher, 2009). To evaluate the contribution of each probe on hybridization of the wild type isolates the probes were also used singly. Hybridizations and washing were conducted at 55°C. The composition of the hybridization buffer which also served as wash buffer was as follows: 0.7 M NaCl, 0.1 M Tris [pH 8.0], 0.1% SDS, 10 mM ethylenediaminetetraacetic acid). Hybridizations for end-point detection by microscopy were carried out using two concurrent protocols. The first protocol involved liquid hybridizations of 100 µl volumes of samples in a Thermomixer R heat block (Fisher Scientific), which following washing were either air-dried on microscope slides or resuspended in PBS and filtered via 1 ml syringes through 0.45 µm polycarbonate filters, then mounted on slides using Vectashield Mounting Medium H-1200 (containing 1.5 µg ml⁻¹ DAPI) and covered with a coverslip for counterstaining in the dark for at least 10 minutes. The protocol involved 15 min hybridizations and 15 min washes for cells coming directly from enrichments. To
avoid harsh treatment of cells attached to paramagnetic beads, the wash was reduced to a simple volume replacement for samples captured by immunomagnetic separation. The second protocol involved air drying 10 µl sample on slides, followed by dehydration in ethanol series [50, 80 and 95% (v/v)] absolute ethanol in distilled water, 100 µl volumes for 3 min each), then on-slide hybridization on a controlled humidity hybridization chamber (Slide Moat™ model 240000, Boekel Scientific, Feasterville, PA). Hybridizations were conducted for 15 min; samples were rinsed three times with pre-warmed washed buffer, and then washed for 5 min. 100 µl hybridization or wash buffer volumes were used at all times. Samples then were treated as described in the first protocol. This protocol was finally adopted for all experiments due to its simplicity and to minimize cell loss. For flow cytometric detection, 100 µl volumes of samples were subjected to liquid hybridization for 15 min with 5 min washes then resuspended in 500 µl volumes of PBS for further analysis.

Inclusivity and specificity of FISH using the dual probe cocktail were confirmed via hybridization of strains comprising all seven DNA subgroups each time that modifications to the FISH protocols were introduced. Hybridizations were run in parallel on samples digested with an RNase cocktail (RNase Cocktail™, Ambion, Inc., Austin, TX) for 30 min at 25°C. Hybridization with a nonsense probe (EUK-516) or with the dual probe cocktail against *E. coli* were also used to confirm specificity.

**Fluorescence microscopy.** Fluorescence microscopy was performed using a Leitz Laborlux S microscope equipped with a 100 W, 20 V mercury lamp for illumination. Slides with coverslips were examined using a 63x objective. Samples hybridized with fluorescein isothiocyanate labeled probes were observed using an I3 filter block (Leitz 513719, blue excitation range, BP420-490 nm excitation filter, RKP510 nm dichroic mirror and LP520 nm
suppressor filter), while samples hybridized with Texas Red labeled probes were observed using an N2 filter block (Leitz 513609, green excitation range, 530-560 nm excitation filter, RKP580 nm dichroic mirror and LP580 nm suppressor). Counterstaining with DAPI was observed using the D filter block (blue excitation, BP355-425 nm excitation filter, RKP455 nm dichroic mirror and LP460 nm suppressor). A Canon PowerShot A640 consumer-grade digital camera controlled with Axiovision software (v. 4.6, Carl Zeiss Microimaging, Inc., Thornwood, N.Y.) was used to take photographs, which were prepared in Adobe Photoshop for print in grayscale.

**Cytometry and data analysis.** Cytometry was performed using a FACSCanto flow cytometer (BD Biosciences). Excitation was performed by a 633 nm, 17 mW HeNe red laser. Fluorescence was detected via a 660/20 bandpass filter, side scatter via a 48/10 bandpass filter and forward scatter via a 2.0 neutral density filter. Collection was performed at low flow rate for 1 min, then to increase sensitivity of detection following gating on fluorescence for 3 min at high flow rate. Data was initially processed using FACSDiva software version 4.0 and then exported as FCS 2.0 files for further analysis. Files were analyzed using software package version 4.6.2 (Tree Star Inc., Ashland, OR).

**Scanning Electron Microscopy (SEM).** The procedure for SEM preparations has been described before (Bisha and Brehm-Stecher, 2009). Briefly, samples were fixed in 1 ml 2.5% glutaraldehyde, then resuspended in 1 ml PBS and kept on ice until analysis was performed at the University of Iowa’s Central Microscopy Research Facility (CMRF). Then sample portions of the fixed sample were allowed to attach to poly-L-lysine coated silicon chips, fixed in 1% osmium tetroxide, dehydrated in ethanol series, and following sputter coating observed using a Hitachi S-3400N electron microscope.
RESULTS

Refinement of FISH method and conditions. Figure 1 shows the results for typical hybridizations. Panel A shows the results for liquid hybridizations analyzed via flow cytometry. A Mean Fluorescence Intensity (MFI) ~14 times higher was achieved from hybridization of pure target cells (S. Typhimurium ATCC 14028) compared to the negative control (E. coli ATCC 25922). Panel B in Figure 1 shows the equivalent slide hybridization results for S. Typhimurium ATCC 14028 confirming bright specific hybridizations. Satisfactory hybridizations were achieved after only 15 min followed by a quick wash for 5 min. Table 1 shows the hybridization results for four main bacterial cultures used in the study (including E. coli as a negative control). Considering that the target sequence in the 23S rRNA of S. Tennessee contains a mismatch with the probe Sal3 (Nordentoft et al., 1997) we sought to evaluate the effectiveness of the dual labeling protocol by establishing the contribution of each of the probes on the hybridization efficiency. Bright hybridizations were achieved even for S. Tennessee when the dual probe approach was employed, although MFI for hybridizations with Sal3 in S. Tennessee was less than 50% of that of S. Typhimurium strains. Indeed, detection of S. Tennessee would have proved difficult if Sal 3 was used alone, since the MFI was merely twice as high as that of the negative control. The dual probe approach allowed for a satisfactory fluorescence signal in the S. Tennessee strain (~10 times higher than negative control), allowing us to use the FISH approach for discrimination of this strain which was involved in a salmonellosis outbreak major linked to the consumption of peanut butter.

Inclusivity and specificity of FISH. To establish the inclusivity of the method the developed FISH protocol was applied to cultures representing all DNA subgroups of
Salmonella spp. with positive results. FISH was also conducted on cells captured by antibody-paramagnetic bead complexes to establish possible interference from beads, confirming positive hybridization. We confirmed the specificity of the FISH protocol via hybridization of RNase treated cells or from cells hybridized with the nonsense probe (Euk516) and no non-specific staining observed for these hybridization conditions.

**Direct detection via fluorescence microscopy.** The excessive presence of fat in the analyzed samples interfered with the direct FISH assay. Indeed it was not possible to detect low levels of target cells directly from the samples unless immunomagnetic capture was performed. This was more significant when fluorescein isothiocyanate-labeled probes were used, which did not yield very bright hybridizations as when Texas Red-labeled probes were used. The polyclonal antibodies reacted with all *Salmonella* spp. cultures used in the study, and following fixation could be stored in storage buffer at -20°C without significant cell loss. Figure 1, Panels A and B show electron micrographs of fixed cells of *S*. Tennessee 2007026177 and *S*. Typhimurium 200867028, respectively, which have formed complex clusters with paramagnetic beads and have remained attached to them several days later following on-bead fixation. The morphology of the beads is more obvious in Panel B, and shows a variety of sizes. The beads did not interfere with fluorescence microscopy and no inherent autofluorescence or fluorochrome uptake could be visually identified via fluorescence microscopy. Panel C shows slide FISH for *S*. Typhimurium ATCC 14028 attached to paramagnetic beads. Capturing target cells from the 250 ml bag allowed for significant reduction in background material, particularly excluding fat from further sample processing and allowed for a detection sensitivity of ~10⁴ CFU g⁻¹ (or 10³ CFU ml⁻¹) via fluorescence microscopy. Beads could be concentrated on the slide via magnets (from ~ 1
cm² to ~ 2-3 mm²), which improved the chance of finding the target cells, however generally cells attached to paramagnetic beads formed three dimensional structures (as can be clearly seen from Panels A and B in Figure 2, which lowered the quality of the photos taken. Diluting enrichments 1:5 in fresh buffer did not maximize recovery, thus one whole homogenized samples were used for all experiments.

**Detection by fluorescence microscopy following enrichment.** Considering that direct FISH on homogenized peanut butter samples proved to be difficult, we considered sampling the aqueous phase which naturally formed under the top layer of lipids during enrichment at 37°C. Plating confirmed that numbers of target cells in the aqueous phase were not different from those in the homogenized samples. Results are shown in Figure 1 for samples enriched in BPW. Panels A, B, and C show uninoculated, spiked enriched for 10 h (confirmed by plating to be ~ 10⁶ CFU ml⁻¹) and spiked enriched for 12 h (confirmed by plating to be ~ 10⁷ CFU ml⁻¹) samples, respectively. Spiking levels were low, only ~0.5 CFU g⁻¹. Simply benefiting from the natural separation of phases in incubating peanut butter enrichments allowed us to detect this low inoculum level by fluorescence microscopy following only 8 h of enrichment (confirmed by plating to be ~ 10⁴ CFU ml⁻¹). cIMS did not improve the sensitivity of detection as compared to FISH on fixed aliquots from the aqueous phase, however for the earliest detection time-point it decreased the amount of fields that were necessary to be examined in order to accomplish detection. For samples enriched for 8 h or more simply hybridizing cells from aqueous phase proved satisfactory. Using high nutrient media such as TSB or TB did not significantly improve sensitivity over the course of enrichment.
Detection by flow cytometry following enrichment. Figure 4 shows the dot plots for 10 h enrichments of spiked peanut butter and the uninoculated controls in BPW. Panels A and B show hybridized stomachates of enrichments, while Panels C and D shows hybridized aqueous phase counterparts. Cytometric detection was possible in samples prepared in either form, however a ~ 2 fold improvement in signal-to-noise ration (S:N) was achieved in hybridized aqueous phase samples. This explains the difficulties that we encountered in direct detection of *Salmonella* spp. from stomachates via fluorescence microscopy, even though samples are diluted several fold for analysis for cytometry, which reduces the interference from background events. To allow for more sensitive detection, we applied gating on fluorescence and collected at high flow rate for 3 min, which allowed for analysis of ~70% of the sample. This permits the instrument to collect only interesting events above the set threshold of fluorescence and ensures the continuation of analysis for longer times allowing for more target events to be recorded if present. Figure 5 shows the effect of gating on the detection sensitivity of flow cytometric analysis. Panels A and B show ungated and gated samples which were enriched in TB for 10 h, while panels C and D show ungated and gated samples enriched for 8 h. Simply by gating the number of events collected in the ‘positive events’ gate increased from 5,209 events to 26,640 events and from 178 events to 1495 events, respectively. On the dot plots the target populations are expressed as percent of the total population and that number is significantly increased, allowing for the unambiguous detection of the *Salmonella* spp. spiked at low levels in peanut butter samples via flow cytometry following only 8 h of enrichment.
DISCUSSION

We evaluated the development of a FISH protocol that could be conveniently coupled with fluorescence microscopy or flow cytometry for end-point detection of *Salmonella* spp. in peanut butter. Enrichments in non-selective selective broths and cIMS were evaluated to prepare samples for FISH. To our knowledge this is the first time that immunomagnetic separation has been coupled with FISH for detection of bacterial pathogens in food. Other authors have investigated the combination of IMS with molecular methods for detection of foodborne pathogens. Seo et al. (1998) combined immunomagnetic separation with flow cytometry for detection of *E. coli* 0157:H7 in beef, apple juice and milk, while Mansfield and Forsythe combined IMS with ELISA to detect *Salmonella* spp. Coupling of IMS with polymerase chain reaction for detection of *E. coli* 0157:H7 and *Salmonella* spp. have also been described (Fu et al., 2005; Warren et al., 2007). All these studies found that the molecular detection could be enhanced by purification of target cells via IMS. This is in agreement with our findings. Direct detection of *Salmonella* spp. via FISH and fluorescence microscopy in peanut butter was aided by pre-processing via IMS which allowed for removal of interfering fat inherent in peanut butter. While generic IMS applications are limited by sample size (Sharpe, 2003), we employed cIMS which permits capture of target cells from whole 250 ml samples increasing the chances of detection. We found that during non-selective enrichment of *Salmonella* spp. in peanut butter a naturally occurring aqueous phase can be sampled to avoid interference to FISH and fluorescence microscopy from the bulk particulate matter (mainly fat) in peanut butter. While cell extraction methods that use aqueous two-phase partitioning and gradient separation to concentrate target cells or remove background interference have been described before (Stevens and Jaykus, 2004; Barra-
Caracciolo et al., 2005; Bertaux et al., 2007), this naturally-occurring separation is not induced by addition of reagents, thus is does not increase cost or require any preparatory steps.

We developed a robust FISH method for detection of *Salmonella* spp. in peanut butter which was coupled to fluorescence microscopy or flow cytometry for end-point detection. Sensitive and specific detection of low levels of target cells was accomplished following as little as 8 h non-selective enrichment with total sample preparation time of about an hour for fixation, hybridization and cytometry or microscopy. This method is significantly more rapid that conventional detection methods for *Salmonella* spp. in food and compares favorably with other detection methods in that is cheap and easy to perform while imparting a high sensitivity and specificity.

**REFERENCES**


Table 1. Hybridization results for the two peanut butter outbreak strains, the reference *Salmonella* strain ser. Typhimurium ATCC 14028 and E. coli ATCC 25922.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Result of Hybridization*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>ATCC 25922</td>
<td>15.9</td>
</tr>
<tr>
<td><em>Salmonella Typhimurium</em></td>
<td>ATCC 14028</td>
<td>81.3</td>
</tr>
<tr>
<td><em>Salmonella Tennessee</em></td>
<td>2007026137</td>
<td>80.4</td>
</tr>
<tr>
<td><em>Salmonella Typhimurium</em></td>
<td>200857028c</td>
<td>103.0</td>
</tr>
</tbody>
</table>

*Source: American Type Culture Collection, Manassas, VA*

*Source: University of Iowa Hygienic Laboratory, University of Iowa, Iowa City, IA. Commercial peanut butter outbreak strain, 2007.*

*Source: University of Iowa Hygienic Laboratory, University of Iowa, Iowa City, IA. PFGE identical with peanut butter outbreak strain, 2008.*

*Mean Fluorescence Intensity expressed in arbitrary units (a.u.)*

Figure 1. Typical DNA hybridization results. **Panel A** shows overlays of fluorescence histograms representing *Escherichia coli* ATCC 25922 and *Salmonella enterica* subs. *enterica* ser. Typhimurium ATCC 14028 liquid hybridizations with the dual probe cocktail Sal3/Salm63 labeled at the 5’ end with Cy5. The mean fluorescence intensity (MFI) was ~ 14 times higher in specifically hybridized cells as compared to the negative control indicating bright successful hybridizations. **Panel B** shows an image of *S. Typhimurium* ATCC 14028 slide hybridization with the dual probe cocktail Sal3/Salm63 labeled at the 5’ end with
Texas-Red (5 µM bar). For both liquid and slide hybridizations satisfactory results were achieved after only 15 minute hybridizations and 5 minute washes.

Figure 2. SEM micrographs of *Salmonella* spp. captured by antibody-coated paramagnetic beads and on-bead FISH. **Panel A** shows an electron micrograph of *S.* Tennessee 2007026177 attached to beads (5 µM bar). **Panel B** shows an electron micrograph S. Typhimurium 200867028 attached to beads (1 µM bar). **Panel C** shows an image of on-bead hybridized cells of *S.* Typhimurium ATCC 14028 (5 µM bar).
Figure 3. Fluorescence microscopy performed on cells coming from the aqueous phase of peanut butter enrichments in BPW at 37°C. Panel A shows slide hybridizations of uninoculated peanut butter enriched for 12 h. Panel B shows slide hybridizations of peanut butter spiked at 0.5 CFU g\(^{-1}\) with *S. Typhimurium* 200867028 and enriched for 10 h. Panel C shows slide hybridizations of peanut butter spiked *S. Typhimurium* 200867028 at 0.5 CFU g\(^{-1}\) and enriched for 12 h (10 µM bar).
Figure 4. Flow cytometric detection of S. Typhimurium 200867028 from homogenized BPW enriched samples or aqueous phase. Panel A shows hybridizations of homogenized uninoculated peanut butter enriched for 10 h. Panel B shows hybridizations of homogenized peanut butter peanut butter spiked at 0.5 CFU g⁻¹ and enriched for 10 h. Panel C shows hybridizations of aqueous phase of uninoculated peanut butter enriched for 10 h. Panel D shows hybridizations of aqueous phase of peanut butter spiked at 0.5 CFU g⁻¹ enriched for 10 h.
Figure 5. Effect on detection sensitivity of gating strategies. Panel A shows ungated peanut butter samples spiked at 0.5 CFU g\(^{-1}\) with \(S.\) Typhimurium 200867028, and enriched in Terrific Broth for 10 h Panel B gated peanut butter samples spiked at 0.5 CFU g\(^{-1}\) \(S.\) Typhimurium 200867028 and enriched in Terrific Broth for 10 h. Panel C shows ungated peanut butter samples spiked at 0.5 CFU g\(^{-1}\) \(S.\) Typhimurium 200867028 and enriched in
Terrific Broth for 8 h. Panel D peanut butter samples spiked at 0.5 CFU g⁻¹ S. Typhimurium 200867028 and enriched in Terrific Broth for 8 h.

CHAPTER 7. GENERAL CONCLUSIONS

CONCLUSIONS

It is well known the *Salmonella* spp. and *Listeria monocytogenes* are two of the most important bacterial pathogens causing foodborne disease. The continuous emergence of these two pathogens in foodborne outbreaks requires swift control of contamination of food products.

Considering the fast production and distribution of limited shelf-life food products, the requirement for rapid detection of these two pathogens in food is imperative. FISH has showed great potential for specific labeling of microbial cells. It has been employed in a variety of complex matrices, from soil, to sludge and oceanic waters to specifically identify target whole cells *in situ*. This method has been shown to be robust, easy to perform and can be coupled with flow cytometry, which is another promising technology for microbiological analysis, adding speed and discrimination power to the analysis. Potential application of FISH and flow cytometry for rapid detection of foodborne pathogens have attracted the interest of food microbiologists, however extensive studies which employ them for detection of microbial pathogens in food are missing.

We evaluated probes and hybridization conditions, optimized sample preparation procedures and developed FISH-based protocols for sensitive detection of *Salmonella* spp. and *L. monocytogenes* in a number of foods.
An approach was developed, which involved dual staining with DNA oligonucleotides, combining fluorescence in situ hybridization (FISH) and flow cytometry for detecting low levels of *Salmonella* spp. (~$10^3$ cells/ml sprout wash) against high levels of naturally occurring sprout flora (~$10^7$ – $10^8$ CFU/g sprouts) following short hybridizations (15 min). FISH was also combined with flow-through imaging cytometry (FT-IC) to obtain information on cell-cell and cell-particle interactions and coincidence. In another study we used the dual probe approach to detect as few as 0.5 CFU g$^{-1}$ of *Salmonella* spp. spiked in peanut butter via flow cytometry and fluorescence microscopy. We described for the first time an adhesive-based sampling method of fresh produce coupled with FISH for detection of *Salmonella* spp., which could be combined with agar or liquid miniculture enrichments for further analysis via fluorescence microscopy or flow cytometry. We successfully evaluated sample preparation methods such as immunomagnetic separation, tangential flow filtration, and pulsification for use with FISH. Tangential flow filtration and immunomagnetic separation improved the detection sensitivity of *Salmonella* spp. in seeded sprouts and peanut butter, respectively. Pulsification, significantly improved the flow cytometric output of *L. monocytogenes* on pork frankfurters following PNA-FISH.

We conclude that FISH has great potential for use as a basis for development of methods for rapid molecular detection of *Salmonella* spp. and *L. monocytogenes* in food.

**RECOMMENDATIONS FOR FUTURE RESEARCH**

More research will need to be conducted to validate the protocols that were developed here in naturally contaminated samples. We artificially spiked samples with inocula mimicking natural contamination by also using wild-type bacterial strains isolated from
foodborne outbreaks; however it will be important to evaluate the performance of the methods with ‘real life’ samples.

While some work has been conducted on the effect of stress and injury on detection of bacteria via FISH, comprehensive studies, which supply information on a great number of pathogens exposed to a variety of stresses are lacking. Future research should address this knowledge gap and provide insight on that effect, since foodborne bacterial pathogens are likely to have been affected by a number of stresses during growing, processing, storage and distribution.

Oligonucleotides are available which target more bacterial pathogens such as *Yersinia* spp, *Campylobacter* spp. and others or could be promptly designed for those that are lacking, so future research should focus on developing more protocols that focus on detection of other foodborne pathogens.

FISH and flow cytometric methods have the potential for multiplexing, thus more research should be conducted, which will optimize enrichment, fixation and hybridization protocols in order to accomplish detection of multiple pathogens from the same sample.
APPENDIX. LOW LEVELS OF GRAVINOL®-S GRAPE SEED EXTRACT DAMAGE THE LISTERIAL CYTOPLASMIC MEMBRANE AND CAUSE CELL LYSIS

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ABSTRACT

Grape seed extract (GSE) is a rich source of proanthocyanidins, powerful natural antioxidants also known to have wide-ranging bioactivities as anti-inflammatory, anticarcinogenic and antimicrobial agents. The known ability of GSE to rapidly inactivate L. monocytogenes, combined with its Generally Recognized as Safe (GRAS) regulatory status,
highlights its promise for control of *Listeria* in foods. The objective of this study was to characterize the antilisterial effects of GSE in more detail using Transmission Electron Microscopy (TEM), fluorescence microscopy and flow cytometry. The minimum inhibitory concentration (MIC) of a commercial GSE preparation (Gravinol®-S) against *L. monocytogenes* Scott A was found to be 1.25 mg/ml via broth microdilution. In plating studies, GSE at this level reduced cell viability by 6 logs within 10 min and TEM at 10x MIC over this same time interval indicated severe ultrastructural damage to cells. To gain clearer insight into how GSE affects *Listeria* spp., we evaluated the effects of sub-MIC levels of GSE against live cells of *L. innocua* via flow cytometry, using propidium iodide as a probe for membrane integrity. Surprisingly, even at ~1/100th MIC and only 2 min exposure, treatment with GSE caused rapid permeabilization and clumping of *L. innocua*, results that we confirmed for *L. monocytogenes* using fluorescence microscopy. The powerful antilisterial effects of this value-added plant extract, even at low concentrations, suggests its use as an effective and natural means for control of *L. monocytogenes* in foods and on food contact surfaces, either alone or in combination with additional antimicrobial hurdles.

**INTRODUCTION**

*Listeria monocytogenes* is an environmentally ubiquitous pathogen, found in soil, water and on decaying vegetable matter. Infection with *L. monocytogenes* causes listeriosis, a rare, but serious disease having a mortality rate of almost 30% (Painter and Slutsker, 2007). Populations at risk for contracting listeriosis include pregnant women, fetuses or neonates, and those with compromised immune systems, such as HIV-infected individuals or those undergoing cancer chemotherapy (Painter and Slutsker, 2007). Although
*L. monocytogenes* is widely distributed in the environment, nearly all cases of the disease can be traced to consumption of contaminated foods. Surveys indicate the presence of *L. monocytogenes* in foods ranging from raw and processed meats, to fruits and vegetables, fish and seafood and dairy products such as milk, soft cheeses and ice cream (Painter and Slutsker, 2007). The prevalence of *L. monocytogenes* in foods and food processing environments requires the development and effective use of antimicrobial hurdles capable of preventing its growth in foods.

At the same time, consumer demand for fresher foods containing fewer synthetic preservatives has driven the development of more “natural” antimicrobial treatments capable of improving food safety, extending shelf life and improving the quality of foods (Vigil et al., 2005). Compounds of interest for this purpose include chitosan, lysozyme, antimicrobial peptides and plant compounds such as essential oils and other phenolic-rich materials (Vigil et al., 2005). An *ideal* plant-derived food preservative would have several attributes: high antimicrobial activity against foodborne pathogens, antioxidant, stabilizing or other preservative effects on the food, no negative impact on the food’s organoleptic properties, Generally Recognized as Safe (GRAS) regulatory status and potential “nutraceutical” properties, where it may exert positive impacts on other aspects of human health, such as cardiovascular function or chemoprotection. The ideal compound should also be inexpensive and in the best-case scenario, it would be an environmentally “green” value-added product recovered from agricultural waste streams (Anastasiadi et al., 2009).

Recently, grape seed extract (GSE) has emerged as a rich source of food-grade plant phenolics with promising and wide-ranging bioactive properties (Nandakumar et al., 2008). Activities attributed to GSE include anti-cancer, anti-inflammatory, anti-nociceptive,
antioxidant and antimicrobial effects (Kaur et al., 2008; Nandakumar et al., 2008). In foods such as cooked meats, GSE is able to exert both antimicrobial and chemical preservative effects, reducing pathogen load while improving the color and shelf life of these products (Ahn et al., 2006; Rojas and Brewer et al., 2007). GSE, a value-added by product of the wine and grape juice industries, is commercially available from a number of manufacturers and has GRAS status.

The antimicrobial properties of GSE have been evaluated against *L. monocytogenes*, *Salmonella Typhimurium*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* O157:H7, *Aeromonas hydrophila* and other foodborne pathogens, both in vitro and in foods, and GSE has also been shown to be effective against *Helicobacter pylori* (Ahn, 2007; Anastasiadi, et al., 2009; Brown, 2009; Rhodes et al., 2006; Sivarooban et al., 2007; Sivarooban et al., 2008). GSE’s antilisterial activities appear to be particularly promising, with multi-log reductions in viable counts reported after only a few minutes exposure to GSE in vitro (Rhodes et al., 2006). Like other plant phenolic compounds or extracts rich in these compounds, the antimicrobial properties of GSE, including its primary targets and mode of action are poorly understood. Because GSE is a rich source proanthocyanidins (oligomers or polymers of flavan-3-ols such as (+)-catechin and (-)-epicatechin), its antimicrobial properties often attributed to “generic phenolic” activities such as enzyme inactivation, protein denaturation and alteration or destruction of the cell membrane (Vigil et al., 2005). As complex natural mixtures, extracts such as GSE may not have a single mode of action, but may act simultaneously on multiple cellular targets. The purpose of this study was to evaluate the antilisterial activity of GSE using multiple tools, including culture, TEM, fluorescence microscopy and flow cytometry in an effort to gain further understanding of the
antilisterial effects of this natural plant material. The information gained may be useful in the formulation of multi-hurdle antimicrobial compositions containing GSE and designed to inhibit the growth of *Listeria* spp. in foods or to rapidly inactivate them in food processing environments.

**MATERIALS AND METHODS**

*Cultures and growth conditions.* *Listeria monocytogenes* NADC-2045 (Scott A) was obtained from the culture collection of the Microbial Food Safety Laboratory at Iowa State University. *Listeria innocua* ATCC 33090 was from the American Type Culture Collection (Manassas, VA). Stock cultures were stored until use at -80ºC in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, Michigan), supplemented with 10% glycerol. At least two passages (18-22 h, 35ºC) in 10 ml volumes of BHI were made before use. For minimum inhibitory concentration (MIC), transmission electron microscopy (TEM) and time course plating experiments, *L. monocytogenes* NADC-2045 was grown in an Erlenmeyer flask containing 100 ml of BHI broth. After 18 h, the cells were harvested by centrifugation (10,000 x g, 10 min, 4ºC) using a refrigerated centrifuge (Sorvall® Super T21, Sorvall Product, L.P., Newtown, Connecticut), and washed once in sterile physiological saline (0.85% NaCl). Pelleted cells were suspended in fresh saline to give a final viable cell concentration of approximately 4.0 x 10⁹ CFU/ml, determined by plating onto BHI agar. For fluorescence microscopy (*L. monocytogenes*) and flow cytometry experiments (*L. innocua*), strains were cultured for 18-22 h at 30ºC and cells were harvested by centrifugation (2,000 x g, 5 min), then washed once in 0.85% saline prior to use.

*Grape seed extract.* The antimicrobial used in this study was a commercial
preparation of grape seed extract (GSE) (Gravinol®-S, Kikkoman Corporation, Tokyo, Japan). For MIC experiments, a stock solution of GSE (100 mg/ml) was prepared in BHI broth containing 10% (v/v) ethanol (to increase solubility of the extract). For plating and TEM experiments, a stock solution was made at the same concentration in autoclaved distilled water, also containing 10% ethanol. GSE is a complex natural plant product containing multiple molecular species. In an effort to separate potentially confounding pH effects from other biological activities we also measured the pH of a series of GSE solutions (1.5-1,250 µg/ml) made in 0.85% saline.

**Total phenolic content of GSE.** The concentration of phenolics in the antimicrobial was determined using the method of Price and Butler (Waterman and Mole, 1994). Briefly, this assay is based on reduction by phenolic compounds of iron from the ferric to ferrous state, with concomitant formation of the Prussian blue complex, [Fe₄Fe(CN)₆]₃, which is detected colorimetrically (Khiyami et al., 2005). A 1% solution of GSE in water/ethanol was prepared as above. Formation of the Prussian blue complex was measured with absorbance at 720 nm and total phenolic content was expressed as the number of catechin equivalents present in the 1% GSE sample. The total phenolic content of the sample is therefore the number of catechin equivalents x 100.

**Minimal Inhibitory Concentration of GSE.** The MIC of GSE against *L. monocytogenes* NADC-2045 was determined using the Bioscreen C Microbiology Reader (Growth Curves, USA, Piscataway, NJ), a combined incubator and automated turbidimeter. Cultures were incubated at 35°C for 18 h and optical density measurements (600nm) were taken every 15 min, with shaking prior to each reading. Final concentrations for GSE ranged from 0.005 to 5.0 mg/ml (0.0005 to 0.5%) and were obtained by serially diluting the stock
solution of Gravinol®-S (100 mg/ml) according to the Clinical and Laboratory Standards Institute (CLSI) methodology for preparing solutions of antimicrobial agents for use in broth microdilution susceptibility tests. Each well had a final volume of 200µl and contained a total concentration of $1 \times 10^5$ CFU of *L. monocytogenes*. Controls included BHI alone and BHI + 1.0% (v/v) ethanol and triplicate wells were used for all treatments. The MIC of the Gravinol®-S GSE for *L. monocytogenes* was defined as the lowest concentration that completely inhibited the growth of the pathogen after 18 h.

**Time course plating.** The antimicrobial activity of GSE at different concentrations was determined as a function of time with a time course plating assay. Briefly, washed cells of *L. monocytogenes* NADC-2045 GSE were suspended in 0.85% saline to obtain a concentration of approximately $10^8$ CFU/ml. Ten milliliters portions of this cell suspension were aliquoted into separate 50 ml polypropylene tubes and GSE was added to yield final concentrations of 1.25, 2.5, 5.0, or 10 mg/ml (0.125, 0.25, 0.5 or 1%). Tubes were incubated at 35°C in a gyratory shaker water bath (New Brunswick Scientific, Edison, NJ), with shaking at 5 rpm. At 10 min time intervals (0 – 120 min) an aliquot was taken and serially diluted (1:10) in 0.1% peptone water, then surface plated on BHI agar. Plates were incubated at 30°C for 24 h and bacterial colonies counted. Three independent replications of the experiment were conducted.

**Transmission electron microscopy.** As with the time course plating experiment, tubes containing 10 ml of saline were inoculated with the *L. monocytogenes* culture to a final concentration of approximately $10^9$ CFU/ml. GSE was added to a final concentration of 10 mg/ml (1%). Tubes were placed in the shaker water bath and incubated at 35°C for 5 or 10 min. Following incubation, cells were harvested by centrifugation (10,000 x g, 10 min, 4°C),
resuspended in fixative containing 2.0% glutaraldehyde (w/v) and 2.0% paraformaldehyde (w/v) in 0.1 M sodium cacodylate, then held for 48 h at 4°C. Samples were rinsed 2 times in sterile distilled water and pelleted after each step in a microcentrifuge (Beckman Coulter, Inc., Fullerton, California). This and all subsequent experiments except for resin block polymerization (below) were conducted at room temperature. The cells were post-fixed in 1.0% (w/v) osmium tetroxide in 0.1 M sodium cacodylate for 1 h, followed by a 5 min wash in distilled water and en bloc staining with 2.0% (w/v) uranyl acetate for 30 min. The samples were then dehydrated in a graded ethanol series, cleared with ultra-pure acetone, infiltrated, and embedded using a modified EPON epoxy resin (Embed 812, Electron Microscopy Sciences, Ft. Washington, Pennsylvania). Resin blocks were polymerized for 48 h at 70°C; thick and ultrathin sections were made using a Reichert Ultracut S ultramicrotome (Leeds Precision Instruments, Minneapolis, Minnesota). Ultrathin sections were collected onto copper grids and images were captured using a JEOL 1200EX scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, Massachusetts).

**Flow cytometry.** Flow cytometry experiments were performed in an effort to obtain information on the antilisterial activities of low levels of GSE over very short time scales. Because live cell cytometry of *L. monocytogenes* represented an aerosol hazard, we used *L. innocua* ATCC 33090, which is physiologically similar to *L. monocytogenes*, as a simulant for this pathogen. Cells of *L. innocua* were prepared and washed in saline as described earlier. One hundred microliter portions (~$10^8$ cells) of washed cells were pelleted via centrifugation, the supernatant was discarded and the pellet was resuspended in a small amount of the residual supernatant to ensure an even slurry of individual cells. Cells were
then resuspended in 1 ml portions of saline containing GSE at concentrations of 1.5, 15, 30, 50 or 100 µg/ml (0.00015 - 0.01%), with the highest level of ethanol in this series being 0.3%. Cells were exposed to GSE for up to 10 min at 25°C, and samples were taken at 2-, 4-, 8- and 10-minute intervals. Once sampled, cells were quickly (2 min) harvested by centrifugation and washed once in fresh saline without added GSE, then resuspended in a final volume of 250µl saline prior to fluorescent staining.

The membrane integrity probe propidium iodide (PI) (component B from the L13152 Live/Dead® BacLight™ Kit, Invitrogen Corporation, Carlsbad, CA), was prepared by dissolving the contents of 1 applicator in 5 ml 0.2µm filtered distilled water to form a 2x working solution. Two hundred and fifty microliters of this working stock were added to control or GSE-treated cell suspensions, mixed, then incubated in the dark for 15 min prior to cytometric analysis using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA). For each sample, data on cell scatter and PI fluorescence (488 nm excitation/670nm longpass emission) were collected for 20,000 events at a flow rate of 10µl/min. Controls included live cells with or without PI, cells treated with GSE for 10 min without PI staining, both stained and unstained isopropanol-killed cells, and a 50%-50% mixture of isopropanol-killed and live cells.

**Fluorescence microscopy.** For microscopy experiments, fluorescent staining of *Listeria monocytogenes* NADC-2045 was performed using the Live/Dead® BacLight™ Kit (Cat. No. L13152, Invitrogen Corporation, Carlsbad, CA). Ten microliter portions (~10^7 cells) of saline-washed cells were spread over a ~1 cm² area on poly-L-lysine coated microscope slides, placed in a BSL-2 biosafety hood and air-dried to facilitate cell attachment to slide surfaces. One hundred microliters of saline containing the GSE at concentrations of
1.5, 15, 30, 50, 100 and 1,250 µg/ml (0.00015 – 0.125%) were applied to the cells on the surface of the slide, completely covering the area containing the cells. Cells were exposed to GSE for 10 min, the antimicrobial overlay was discarded and slides were washed once with 100 µl fresh saline without GSE. Ten microliters of the Live/Dead® stain were applied to the sample, and the sample was sealed with a cover slip. Samples were viewed after 15 min of staining in the dark and again after 30 min. A live-dead control sample was prepared from a 50:50 mixture of live and isopropanol-killed cells, and stained as above.

**Statistical analysis.** For the MIC and time course plating experiments, data from three independent replications were subjected to statistical analysis. Data were analyzed using SAS 9.1 (SAS Institute, Cary, North Carolina). Differences between samples were determined using Tukey’s honestly significant difference pairwise test (p<0.05).

**RESULTS AND DISCUSSION**

Although the benefits of GSE as an antilisterial preservative in foods such as cooked ground beef and turkey frankfurters have been shown, descriptions of its activities have been limited to observed reductions in viable plate counts and gross ultrastructural damage seen via TEM for GSE applied at high concentration (1%) (Ahn et al., 2007; Sivarooban et al., 2007; Sivarooban et al, 2008). Here, we sought to probe the physiological effects of Gravinol®-S GSE at lower concentrations (as low as 0.00015%) and over time frames as short as 2 min. Modes of analysis included culture-based antimicrobial testing (broth microdilution and time course plating assays), TEM, fluorescence microscopy and flow cytometry, as described below. Our results highlight the rapid action and antilisterial efficacy of GSE, information that may be helpful in leveraging the benefits this extract in natural
antimicrobial hurdle systems.

**Physical properties of GSE: total phenolic content and pH.** The total phenolic concentration in GSE could not be measured directly, due to the extract’s natural pigmentation, which interfered with the spectrophotometric assay used. Therefore, a less pigmented 1% solution of GSE was used for this assay. The total phenolic content of the 1% solution Gravinol®-S, expressed as catechin equivalents, was 0.95. This coincides with the manufacturer’s claim of “up to 95% total polyphenols”. To test whether the observed antimicrobial activities were not simply attributable to plant acids potentially present in GSE, we measured the pH of a series of GSE solutions made in 0.85% saline. These were (micrograms per ml GSE in solution/observed pH): 1.5 µg/ml/pH 6.77, 15 µg/ml/pH 6.36, 30 µg/ml/pH 5.98, 50 µg/ml/pH 5.54, 100 µg/ml/pH 5.39 and 1,250 µg/ml/pH 4.73. These results indicate that GSE does contain acidic species and that these may lower the pH of the test system in a dose-related fashion. However, the disparity between the relatively small changes in pH seen for increasing levels of GSE and the observed antimicrobial effects of these higher levels suggests that simple pH effects are not responsible for the observed antimicrobial activities of GSE. The use of biological buffers or microbial growth media (which also have some buffering capacity) are expected to be effective means for addressing potential pH effects in future experiments.

**Antimicrobial activities of GSE in liquid media.** The MIC of Gravinol®-S was 1.25 mg/ml (0.125%), determined via broth microdilution assay in BHI broth. This was the concentration of GSE that effectively inhibited growth of *L. monocytogenes* at an initial inoculum of 5.0 log CFU per microtiter plate well. This compares favorably with MIC determinations made by others for *L. monocytogenes*, although a different strain and a
different GSE preparation (ActiVin™ powder) were used. A similar milligram-range MIC for a non-commercial GSE preparation was also recently reported for Helicobacter pylori (Brown et al., 2009). In our work, exposure of L. monocytogenes in 0.85% saline to Gravinol®-S at the MIC value of 1.25mg/ml resulted in a 6-log reduction in viable cell counts after only 10 min. This rapid and dramatic inactivation confirms the findings of Rhodes et al., (2006), who reported a similar effect against L. monocytogenes with GSE isolated from Vitis vinifera var. Ribier and standardized to 250-280 mg/ml gallic acid equivalents. We hypothesized that the precipitous drop in viability that occurs via plating might stem from immediate, gross and irrecoverable damage to listerial cell structures. We performed a TEM study to examine this hypothesis.

**Effect of grape seed extract on cell integrity via TEM.** Results of our TEM study confirmed our hypothesis that even very short exposure of L. monocytogenes Scott A to GSE has a substantial impact on cell integrity. Our study contrasts with that of Sivarooban et al. (2008), who used TEM to investigate changes in cell morphology for L. monocytogenes exposed for a longer period (3 h) to 1% of a commercial GSE (Mega Natural, Inc., Madera, CA) in growth media. Although we also used a 1% concentration for our TEM work, we exposed L. monocytogenes to GSE in 0.85% saline for minutes, vs. hours. Our endpoint for TEM (10 min) was chosen to coincide with the full loss of viability endpoint seen via time course plating, allowing us to relate plating results to the corresponding physiological phenotype.

TEM images of L. monocytogenes cells without addition of GSE (control treatment) show intact and homogeneously stained cells (Figure 1, panel A). In contrast, cells treated with 10 mg/ml Gravinol®-S for 5 min were characterized by heavy damage, loss of integrity
and accumulation of amorphous, electron-dark material on cell surfaces (Figure 1, panel B). This material could be interpreted as either intracellular material (DNA, protein) ejected from these or other cells in the sample, or as components of GSE bound to cell surfaces. Alternatively, this material might also represent a complex between externally bound GSE components and intracellular ejecta. Regardless of its identity, cells exposed to GSE for 10 min show further and more pronounced degradation, surface accumulation (Figure 1, panel C) and and lysis (data not shown).

**Flow cytometry.** Flow cytometry enables analyses of whole populations of cells on the basis of single cell characteristics, such as light scatter or reaction to externally applied stains (Brehm-Stecher and Johnson, 2004). Stains useful for cytometric assessment of antimicrobial activity include probes for membrane integrity such as propidium iodide (PI) or fluorescent respiratory substrates such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Brehm-Stecher and Johnson, 2004). Valuable information on the physiological effects of antimicrobials against live cell preparations, such as lysis or clumping, can also be obtained from analysis of cell light scatter during or after exposure to antimicrobials. Flow cytometry can be especially useful for detecting discrete phenomena, such as the presence of antimicrobial-sensitive or resistant subpopulations or for probing antimicrobial action over short time scales. Cultural analyses of GSE activity against *L. monocytogenes* demonstrated its efficacy against this pathogen, but did not yield information on its possible mode of action. Therefore, we sought to use flow cytometry to answer questions about the physiological effects of GSE as a function of both time and GSE concentration. Due to safety concerns about potential aerosol generation during these live cell cytometry studies, we used *L. innocua*, which is physiologically similar to *L. monocytogenes*. 
Our plating studies demonstrated drastic reductions in cell viability within 10 min for milligram levels of GSE and our TEM work showed that GSE directly affected cell structure and membrane integrity within this time period. We reasoned that use of lower levels of GSE might enable us to follow GSE-induced physiological changes in *L. innocua* as a function of time, and provide further insight into its antilisterial action. Therefore, we assayed lower (microgram) levels of GSE over a short time frame (0-10 min), and used PI as a probe for membrane integrity. Surprisingly, even with the relatively low levels of GSE and brief time intervals used here, GSE exerted dramatic and immediate physiological effects on *L. innocua*. The extent and immediacy of GSE’s effects limited our ability to determine discrete, time-sequenced steps that might occur during the inactivation of *Listeria* by this antimicrobial. However, an analysis of the effects of four different concentrations of GSE at the earliest time point investigated (2 min) provides valuable insight into the antilisterial action of GSE. At the lowest level tested (1.5µg/ml, ~1/1,000th of the MIC), a small subpopulation of membrane-compromised (PI-positive) cells emerged (Figure 2, panel A), highlighting the membrane permeabilizing effects of GSE even at this level. The light scatter properties of *L. innocua* cells were not affected at this level and appeared similar to controls (data not shown). However, a 10-fold increase in GSE concentration to 15.0µg/ml (~1/100th of the MIC) had a large effect on the scatter properties of the cells, with the increased side scatter seen in this sample indicative of cell clumping (Figure 2, panel B). Over the course of GSE concentrations examined here, the use of higher levels of GSE was marked by both increased clumping and increased PI staining. Specifically, PI-positive events ranged from ~2.5% of the total population with 1.5µg/ml to ~70% of the total population when 100µg/ml (~1/10th of the MIC) was used (Figure 2, panels A-D).
These observations for live *L. innocua* cultures were consistent with results obtained for *L. monocytogenes* using other methods, including TEM, fluorescence microscopy and time course plating. Together, these results indicate that GSE causes rapid and dramatic compromise of cell integrity, marked at lower levels of use by permeabilization of the cell membrane and at higher levels by cell clumping and lysis.

**Fluorescence microscopy.** To place our results for cytometry with *L. innocua* into context, we used a Live/Dead® staining protocol on GSE-treated *L. monocytogenes* and viewed these results using fluorescence microscopy. Mixtures (~50:50) of live and isopropanol-killed *L. monocytogenes* stained as expected, characterized by a mixed population of bright green (Syto 9-positive/PI-negative or live) and bright red (PI-positive or dead) cells (Figure 3, panel A). At the lowest level of GSE examined here (1.5 µg/ml), the majority of cells were bright red (dead or morbid) after 10 min exposure to GSE, with only a few green cells visible (Figure 3, panel B). Although we used poly-L-lysine coated microscope slides in an effort prevent or minimize GSE-mediated cell clumping, our microscopy results for *L. monocytogenes* were consistent with our cytometric analysis of *L. innocua*, with increasing levels of GSE leading to clumping (50 µg/ml, Figure 3, panel C) and ultimately, cell lysis. At the highest level (1,250 µg/ml), we were able to find cellular “ghosts” via light microscopy, but these were only dimly stained with PI, suggesting diffusive loss of nucleic acids from compromised and lysed cells (data not shown).

Interestingly, our microscopy samples were not static – when we revisited them after an additional 30 min of benchtop incubation, it was clear that GSE continued to exert an ongoing antimicrobial activity against treated cells, despite the fact that excess GSE had been removed via washing. At lower levels of GSE (i.e. 15 µg/ml) cells that had previously been
well spaced were now clumped and more brightly stained by PI. An example of clear cell clumping is shown in Figure 3 (panel C) and some degree of clumping was also apparent at the 1.5 µg/ml level (Figure 3, panel B). At higher levels of GSE (30 µg/ml), clumping and lysis were more apparent after the additional incubation period. A dose-related cell clumping effect for GSE was also recently noted by Brown et al. for *H. pylori* (Brown et al., 2009). This common observation may ultimately aid in explaining the mode of action for this natural extract.

The rapidity of GSE-mediated killing of *Listeria* spp., along with our observation of its continued activity, despite a removal step, suggests that the active components in GSE rapidly complex with target cells, where they remain bound, able to exert additional activities as a function of time. Rhodes et al. (2006) suggested that cationic species present in GS might interact with the negatively-charged surfaces of *Listeria* spp., akin to the action of antimicrobial peptides. Interestingly, Kondo et al. (2006) found that grape seed proanthocyanidins polymerize to form helical structures. One possible explanation for GSE activities may therefore include binding of and subsequent pore formation by cationic helical proanthocyanidin polymers, although such a theory would need to be tested experimentally.

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Figure 1. Transmission electron microscopy (TEM) of *L. monocytogenes* Scott A exposed to 10 mg/ml GSE in 0.85% saline. Treatments shown are control (no GSE, panel A), cells exposed to GSE for 5 min (panel B) and cells exposed to GSE for 10 min (panel C). Untreated control cells were intact and exhibited morphology typical of *L. monocytogenes* (panel A). Cells treated for 5 or 10 min showed signs of membrane damage and cell lysis.

Figure 2. Flow cytometric analysis of the effects of varying GSE concentrations against live cells of *L. innocua* 33090.
Figure 3. Live/Dead® staining of *L. monocytogenes* NADC-2045 (Scott A) treated with GSE.
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