

Food Safety: ARS National Program 108

Retrospective Review Accomplishment Report For 2006-2010 Action Plan



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Major Accomplishments and their Impacts

1.1 Pathogens, Toxins and Chemical Contaminants Pre-Harvest

1.1.1 Methodology

Rapid, accurate, and sensitive methodology is needed for identification, detection, and quantification of zoonotic pathogens and chemicals that may be transmitted to humans. Identification and detection of other bacteria, such as commensals, is also important. It is essential to understand how these bacteria may influence foodborne pathogens within the animal and environment, or how these bacteria may become pathogenic. Because there are a wide range of species, subspecies, and serovars, with diverse phenotypic and genotypic properties, methods must advance to give scientists additional information about each organism. As the technology evolves, research studies can become more rigorous and enhances our understanding of the epidemiology and ecology of zoonotic organisms in food animals and their production environments. In parallel, methods for toxins and chemical contaminants have also improved. For example, acceptable precision and accuracy for all dioxin congeners can now be achieved. This achievement was impossible 10 years ago.

Innovative and advance methodologies are needed for research in order to identify and quantify the pathogens (and non-pathogens), toxins, and chemicals in both animals and their environments, and ultimately in foods and humans. These methods, in parallel, with new study methodologies for design and analysis, will help in the understanding of the ecology and epidemiology of the pathogens, to delineate the host-pathogen relationship of these zoonotic pathogens in food producing animals, and to develop effective interventions that can be implemented and evaluated by industry and researchers.

Selected accomplishments

- Understanding the mechanisms of colonization by E.coli O157:H7 in the gut is critical for finding new prevention and control strategies. Most studies investigating the mechanisms of colonization by O157:H7 in cattle are designed to monitor E.coli by enumerating these bacteria in cattle feces. Such enumerations can be compromised by the lack of discriminatory power of the media used in these studies. Scientists demonstrated that a genetically modified small plasmid of O157:H7 was able to successfully propagate and express green-fluorescence under conditions simulating the environment of cattle intestine. **Impact:** These plasmids could potentially be used for inoculating cattle during colonization studies for easy detection and enumeration of green-fluorescence expressing colonies. This method also has applicability to other types of studies, such as defining pathogen load or the measurement of E.coli on cattle hides.
- Genetically, Shiga-toxin producing E.coli O157 (STEC) strains are not all identical. There is genetic variation in human and cattle STEC O157, and this variation is important in understanding which serotypes are more virulent in humans. Scientists identified polymorphisms (SNPS) that vary between different STEC O157 strains. One of the polymorphisms is associated with the ability of an STEC O157 isolate to cause disease in humans; thirty-two SNPs defined 42 phylotypes. **Impact:** Specific SNP's have been

incorporated into a commercial assay, and can be used for source tracking and epidemiologic studies of STEC O157 in pre- and post-harvest environments.

- Six STEC non-O157 strains, O26, O111, O103, O121, O45 and O145 account for 70% of the non-O157 STEC human infection cases. USDA-FSIS requested ARS to develop methods for detecting these non-O157 serotypes. FSIS is focusing more attention on these non-O157 strains and trying to understand their role in human infections. **Impact:** Research resulted in the first draft genomic sequences for each of the serotypes. Hopefully, these sequences will lead to new DNA-based tests specific for pathogenic O26, O111, O103, O121, O45 and O145 serotypes.
- Campylobacter species are more difficult to isolate than many other foodborne organisms. Methods for detecting and identifying Campylobacter are time-consuming and complicated. Research has developed a hyperspectral imaging method to discriminate Campylobacter cultures growing in agar from non-pathogenic cultures commonly found in poultry processing. The imaging system will be used to automatically locate and identify Campylobacter grown on agar plates from non-pathogenic organisms. **Impact:** This method will reduce the incubation time and increase the discriminatory power of cultures for Campylobacter. Proper identification is important in evaluating Campylobacter species in food safety as opposed to a non-pathogenic strain in animals or in humans.
- Achieved the recovery of viable but non-culturable (VBNC) Campylobacter marker organisms that were subjected to a dry-atmospheric-temperature stressor environment. After 6 hours, cells were determined non-culturable by analysis of five different Campylobacter enrichment methodology procedures. A chick bioassay was utilized and the non-culturable cells (after 6 and 24 hr exposure) were inoculated intracloacally. The previous non-culturable cells were found to be viable using cultural recovery from the chicks' ceca after 7 days. **Impact:** The results emphasize the critical need to better understand Campylobacter survival mechanisms in the poultry hatchery environment and the role of the hatchery environment in transmission of Campylobacter. This method also highlights the need for improved culture methodology. This is the first report of the recovery of VBNC Campylobacter cells after a dry-atmospheric-temperature stressor.
- Colonization of newly-hatched chicks can result in Campylobacter species being passed to chickens through their production stages, and then pose a risk for contaminating meat products. Conventional culture methods were used to demonstrate for the first time that Campylobacter species can be recovered from tray-liners collected at commercial broiler hatcheries. Studies analyzed 2,000 chick paper pad tray-liners from several hatcheries and demonstrated that approximately 1% of the liners contained living Campylobacter. **Impact:** This study identifies a previously unknown source of chick exposure to Campylobacter that can quickly spread through a flock of birds. These data contribute to our knowledge about the Campylobacter in the poultry environment. The identification of this critical control point in broiler production will facilitate efforts for potential intervention and control programs.

- Demonstrated that a whole genome DNA microarray typing tool, or comparative genome indexing (CGI), proved to be more discriminatory than fla sequencing and Pulsed-field Gel Electrophoresis (PFGE) for *Campylobacter jejuni*. A reduced set of genes identified from statistical analyses of whole-genome *C. jejuni* DNA microarrays were utilized for CGI and found to be sufficient to differentiate *C. jejuni* isolates from cattle, humans, and chickens. The reduced gene set grouped all human *C. jejuni* strains in a single major cluster; cattle and chicken isolates were much less clonal by either CGI technique. **Impact:** CGI was discriminatory for *C. jejuni* and will provide a further understanding of the epidemiology and population genetics of this pathogen. The reduced gene set is a simplified, more efficient, and cost effective CGI technique for genotyping *C. jejuni*.
- Found a *Campylobacter coli* strain that is resistant to high levels of the antibiotic gentamicin. The colonization ability of this *C. coli* strain in broilers and the effectiveness of the incorporation of gentamicin into plating media on restricting the growth of background microflora were demonstrated. This *C. coli* strain's unique resistance to high levels of gentamicin now allows for research studies evaluating the ecology of *Campylobacter* within poultry flocks without regard to the variable presence of natural environmental *Campylobacter* contamination. **Impact:** In the absence of this marker, expensive and time-consuming conformational techniques must be used to determine if the recovered organism is the same one that was inoculated. This technology has been shared, and researchers at the University of Georgia and Clemson University are using this marker.
- Currently, there are over 2,500 *Salmonella* serotypes, and serotyping with anti-sera takes several days, requires a highly trained staff, is expensive, and often cannot type up to 10% of the *Salmonella* tested. Research developed a high-throughput multiplex PCR to identify the most prevalent serotypes of *Salmonella*. The multiplex can identify the top 31 serotypes isolated from animal samples which represent 75% of all *Salmonella* isolated from animals. **Impact:** The analysis can be completed in less than five hours, requires no specialized training, no specific anti-sera, uses inexpensive reagents, works in standard DNA sequencing instruments, and could replace traditional serotyping for most *Salmonella* isolates. In evaluating a cost-benefit, up to 90 isolates can be analyzed in a day with very little hands on time at a cost of \$1.50/sample as compared to several days and about \$40.00 for traditional serotyping.
- Validated the use of pAK1-lux (*S. typh*-lux) to visualize *S. typhimurium* movement using traditional plate counts and correlating it with photonic emissions in the duodenum, jejunum, ileum, and large intestines of pigs. These studies showed that photonic emissions were greater in small intestines than in the large intestines, and that correlations with bacterial concentrations were greatest in the duodenum and least in the large intestine at 6 hours. The greatest emissions were in the jejunum and ileum at 12 hours. **Impact:** These results demonstrate that the lux gene technology is a valid tool to study the kinetics of *S. typhimurium* movement with exposed intestine and can be modified to facilitate microbial ecology studies.

- Determined that intermittently stepping on sample material (socks, drag swabs, disposable booties) while sampling litter in broiler houses, improves the detection of Salmonella by 20% without an increase in cost or sample collection time, when compared to traditional methods of only dragging swabs. By intermittently stepping on drag swabs, studies were able to recover Salmonella in pens containing a single broiler with positive ceca (out of a pen containing 40 broilers) at six weeks of age. **Impact:** This additional step during swab sampling can increase the recovery of Salmonella from the poultry environment, and can be a useful tool in conducting studies for understanding the transmission and persistence of Salmonella in the poultry environment.
- Optimized the molecular subtype methodology of repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) for Salmonella, Campylobacter, and Clostridium perfringens. Analyses of Salmonella enterica isolates, belonging to 13 frequently isolated serotypes and one rarer serotype from poultry, revealed that the predicted serotype of isolates matched the serological typing result. **Impact:** Since serological assays can take several days to weeks to provide information, the rep-PCR system holds promise for a more rapid serotype classification for these isolates. These methods will improve tracking the pathogens through the food production chain and improve the understanding of the epidemiology of these pathogens in poultry.
- Developed methods to produce biofilms under flow conditions that mimic those of the poultry production environment. During these investigations, biofilm formation, using defined laboratory pathogens, was compared to biofilm formation by natural populations of bacteria usually present in the poultry production environment. **Impact:** This research led to the first demonstration that Campylobacter jejuni formed a biofilm when incubated either with or without other bacteria. These investigations were the first use of an epifluorescence assay for quantification of foodborne pathogens within bacterial biofilms on stainless steel. The newly developed test methods are applicable to other bacteria and substrata and have been accepted by AOAC, CDC, and EPA.
- Clostridium perfringens associated necrotic enteritis has increased in poultry when producers have stopped the use of antibiotic growth promoters. A quantitative real-time PCR assay, utilizing a fluorogenic, hydrolysis-type probe, was developed and utilized for the detection and quantification of levels of Clostridium perfringens in the chicken gastrointestinal tract. Material retrieved from the broiler chicken cecum and ileum was used for the assays. **Impact:** The results illustrated that quantitative real-time PCR correlated well with quantification via standard plate counts in samples taken from the ileal region of the gastrointestinal tract. This technology will enhance our study of C. perfringens and its role in animal and human health.
- Stress and its effect on foodborne pathogens are still unclear. Studies identified a biomarker for an infection-induced stress indicator in poultry. Previously, ARS had identified ovotransferrin (OVT) as an acute phase protein in chicken. An ELISA method was developed to determine whether OVT in blood could be used as a non-specific indicator of stress in chickens. Results showed that only inflammation and infection up-regulates blood OVT levels, whereas it is not affected by metabolic diseases such as

pulmonary hypertension syndrome or skeletal disorders. Measurement of peptides such as the OVT, under different physiological and pathological conditions, will enable study of their regulation or biological function under infection. **Impact:** Stresses may increase the potential for infections or increased pathogenic foodborne pathogens in poultry. New methods for the measurement of stress in animals are needed to better clarify the impact of stress on animal health and potentially food safety.

- Developed and tested real-time PCR methods for the detection and characterization of *Listeria monocytogenes*, *Bacillus anthracis*, pathogenic *E. coli*, and *Salmonella* in raw milk and in environmental samples (feces, milk filters, water) from dairy farms. The assays were used to detect pathogens from the National Animal Health Monitoring System surveys (conducted by USDA's APHIS) and for routine analysis of milk, fecal, and environmental samples from dairy farms. The quantitative nature of real-time PCR was shown to be useful in predicting samples that would yield successful isolation of pathogens upon culture. **Impact:** These methods applied to population samples will provide useful information on prevalence of pathogens and the potential movement in the environment and animal. One additional impact was that the assay was shown to be useful for grouping *L. monocytogenes* into clusters that are predictive of serotype.
- Developed a multiplex molecular assay that detects the major species of *Cryptosporidium* that infect humans and cattle, and differentiates these species from minor or non-infectious species. Once a specimen has been prepared, multiple species can be analyzed in this test. This is compared to the standard testing which identifies only the most prominent species present and requires multiple testing for each additional species that might be present in lower concentrations. **Impact:** This assay reduces the number of tests needed for multiple *Cryptosporidia* samples, as well as the time of analysis. The test also increases the ability to differentiate between multiple species.
- ARS scientists administer the training and quality assurance components of a *Trichinella* inspection program that allows the U.S. to export pork to the European Union (EU), Russia, and Singapore in cooperation with the Agricultural Marketing Service (AMS). Under the Program, inspectors from swine slaughter plants across the U.S. are trained and certified to perform the two artificial digestion methods approved by the USDA, EU member countries, Russia, and Singapore for detection of *Trichinella*. ARS provides quarterly quality assurance samples to certified analysts to maintain the integrity of the Program. **Impact:** This training and quality assurance program satisfies export requirements of the EU, Russia, and Singapore. Fresh pork, horsemeat, and game meats originating in the U.S. cannot be exported unless exporting meat packers participate in the Program administered by ARS. This Program preserves an export market for U.S. pork exporters exceeding \$650M annually.
- ARS planned and conducted experimental infections of horses in collaboration with the Canadian Food Inspection Agency (CFIA) and performed assays that were used to validate the U.S. and CFIA detection protocols for *Trichinella* in horsemeat. The protocol, with defined critical control points, was conducted within a quality assurance system compliant with ISO 17025 guidelines, and was found reliable, efficient, and fit for

its intended use in food safety and trade. **Impact:** This collaborative study provided the data necessary to fully validate the *Trichinella* detection protocols for horsemeat to comply with International Organization for Standardization 17025 import standards proposed for all countries with an economic impact of \$50M annually. The protocol demonstrated consistency and effectiveness at critical levels of sensitivity required by trading partners for exported pork, game, and horsemeat, and has been accepted by EU trading partners for use in inspection of meat products destined for export.

1.1.2: *Epidemiology*

The “Epidemiology” Problem Statement was prepared by Dr. Jane Robens as part of the pre harvest section of Component 1.1. This perspective as outlined in the 2006-2010 does not accurately reflect the methodology and concepts used in the field of epidemiology.

Epidemiology is “the study of the distribution and determinants of health-related states or events in specified populations, and the application of this study to control of health problems.” This concept is critical in the area of food safety, which is the interface of agriculture with public health risk. Ecology is a basic tenet of epidemiology, and is a study of the relationship of organisms to each other, as well as all other aspects of the environment. An ecological approach is necessary to explain the occurrence of disease. This Problem Statement really reflects an ecological approach rather than epidemiology in its strictest sense, and the accomplishments have to be evaluated in that light.

This problem statement is based on objectives that are focused on the determination of the origin and routes of transmission of zoonotic pathogens. This may involve the actual movement of animals in their environment or production setting, or the flow of pathogens and specific identified genes, through the production cycle from the environment (manure, water, air,) to the animal, through processing, and to the consumer. In addition, cross-contamination occurs in multiple sites as animals are transported to slaughter plants, at the plant through contact with workers and the plant environment, and during processing in plant and in retail supermarkets. Serotyping and new genomic research have resulted in more useful information for ecologic and epidemiologic research.

- What drives *S. Enteritidis* (SE) evolution contributing to human illness? Results of whole genome comparative analysis show that genetic drift can escape detection by Pulsed Field Gel Electrophoresis (PFGE) and DNA-DNA hybridization microarrays. Using SNP analysis, over 200 small scale genetic polymorphisms were discovered. The analysis confirmed that SNPs can differentiate clonally related strains of SE. The related strains vary in their ability to contaminate eggs, to grow to high cell density, and to form biofilms. **Impact:** This research reveals that genetic drift and single nucleotide polymorphisms (SNPs) are relatively unexplored components of bacterial evolution that contribute to food borne disease, pandemic potential, and persistence in the environment. Evaluating genetic drift and genetic polymorphisms is key to understanding the role of SE in animal and human health (foodborne disease).
- ARS scientists demonstrated that flies can become contaminated with *Salmonella* Enteritidis (SE). The levels of SE isolated from flies residing in the rooms containing molted infected hens was 10-100 times higher than from flies residing in the rooms containing fed infected hens. The SE isolation rate was similar for the exterior and the gut of the fly. Examination of internal organs of colonized flies found that the majority of SE was in the fly gut. Fly crops were generally not contaminated, and no fly salivary glands harbored SE. These results indicate transmission of SE from fly exteriors to other surfaces does not occur via simple contact and that other factors are necessary. Defecation, and not regurgitation, during food collection, is probably the mechanism of

SE spread. Nearly 40% of hens consuming contaminated flies became colonized with SE. **Impact:** Identifying sources of SE and mechanisms of spread in the layer house remains an important instrument in developing quality assurance protocols for reducing or eliminating SE in a flock. These data provide potential points for interventions of SE at the farm level, and provide a foundation for future strategies.

- Research identified a subset of genes within *Salmonella enterica* serotypes whose presence varies between and within serotypes. Seventeen genes were identified that have small scale polymorphisms which alter open reading frames of genes both within and between serotypes. Mutational analysis was introduced to allow exploration of these genes for causal relationships between ORF (parapox virus) disruption and the pathobiology of *Salmonella enterica*. **Impact:** This research provides insight into the combination of genes across a whole genome that in total contribute to the ability of *Salmonella enterica* to periodically emerge to challenge the safety of the food supply. These results provide genetic markers and epidemiologic tools for investigating outbreaks of food-borne *Salmonella enterica* serotypes.
- Studies compared the prevalence of *Salmonella enterica* in commercial turkey flocks during pre- (on-farm) and post- (at slaughter) transport. No statistically significant increases were found at slaughter. **Impact:** This demonstrates that commercial peri-marketing practices do not significantly increase the prevalence of *S. enterica* in market-age turkeys. This is in contrast to what has been previously reported for swine where lairage and transport have been reported to increase *Salmonella* prevalence. This study adds to our understanding of the effect of transport and other risk factors on *S. enterica* in turkeys and consequently, on contamination of carcasses.
- Research determined that *Salmonella enterica* serovars Cerro and Kentucky behave as commensal organisms in dairy cows. During a 5 year *Salmonella* outbreak on an operating dairy farm, a large percentage of the cows excreted *Salmonella* in their feces but showed no symptoms of disease. For 3 years, Cerro was the serovar most frequently detected, but with time was replaced by Kentucky. Although the milking hygiene on the farm was good, *Salmonella* was frequently detected in the milk filter indicating that high loads of undetected commensal strains of *Salmonella* may threaten US milk supplies. **Impact:** This longitudinal regional study in dairy cows mirrors the national NAHMS study and may be reflective of a national pattern. This work demonstrates the endemic nature of some *Salmonella* serotypes in cows, that serovar levels can change, and illustrates the challenge of reducing or eradicating this pathogen from dairy farms. Interestingly, Cerro was the most common serovar of *Salmonella* found in the NAHMS 2007 dairy survey. This is compared to less than 10% in the 2002 NAHMS survey. Kentucky was the second most frequent serovar at 14%.
- Demonstrated that testing in-line milk filters for the presence of zoonotic bacterial pathogens can be predictive of herd-level pathogen shedding, and is a more sensitive indicator of raw milk contamination. Intensive sampling of animals, the environment, milk and milk filters during a longitudinal study of a *Salmonella* Cerro outbreak on a dairy farm demonstrated that *Salmonella* spp. could be isolated more consistently from

in-line filters than from bulk tank milk, especially at lower levels of herd shedding.

Impact: This study provided a potential way to simply, yet effectively, monitor Salmonella species in dairy herds that could be easily implemented by industry. The method is predictive of herd-level pathogen shedding and is a sensitive indicator of raw milk contamination.

- Demonstrated that diverse commercial housing systems for turkeys may impact the prevalence of bacterial foodborne pathogens. The ceca from hens raised under two different commercial systems (non-caged and caged) were screened for Campylobacter and Salmonella. When the housing types were compared during the winter, the prevalence of Campylobacter was highest in the non-caged birds, while no differences were observed for Salmonella. In contrast, for the summer sampling, there was no difference in either Campylobacter or Salmonella prevalence for the housing types. **Impact:** This indicates that the types of commercial bird housing systems can affect the prevalence of microbial organisms in turkeys and ultimately food safety. This risk factor may be important in the study of organic versus traditional farming.
- Estimated the time of entry of Campylobacter and Salmonella into a brooder house, which is the first stage of commercial turkey production. Whereas Campylobacter was absent (0%) in 5-day old birds, it was 90% prevalent by day 20. In contrast, Salmonella was isolated early from 98% of young birds at day 5. At 138 days, Salmonella prevalence in the ceca had declined to 4.5% whereas the prevalence of Campylobacter remained elevated at 92%. Genotyping by PFGE analysis of Salmonella revealed fluctuating populations as the birds matured. **Impact:** These studies mark progress in mitigating on-farm transmission, and indicate that reductions of Campylobacter and Salmonella in commercial turkey flocks should focus on the time of its early introduction into the brooder house.
- Real-time PCR assays and standard culture techniques were used to determine the incidence of Salmonella, *L. monocytogenes*, and pathogenic *E. coli* in raw milk at the national level by participating in NAHMS dairy surveys. In cooperation with USDA, APHIS, milk samples and 523 in-line filters from farms in 17 states were examined. Although the incidence of these pathogens in bulk tank milk was fairly low, the incidence in milk filters was significantly higher, strongly suggesting that consumption of raw milk or products made from raw milk represents a significant health risk. Salmonella was cultured from 300% more filters than bulk milk. Milk filters were PCR positive for Salmonella 200% more than bulk milk, and *L. monocytogenes* was cultured 50% more in filters than milk. **Impact:** This study is important to public health and decision making agencies because a small portion of US consumers are urging State and Federal Government to allow the marketing of raw milk. This data assists the CDC, and industry (National Milk Producers Federation), who are discouraging raw milk sales.
- Assisted the California Department of Public Health (CDPH) in an environmental investigation of dairies associated with outbreaks of *Campylobacter jejuni* in milk. The second largest outbreak of *Campylobacter* illness in US history occurred as a result of contaminated milk processed at a California correctional facility dairy; two smaller

outbreaks involved raw milk from a small organic dairy. A study of both dairies in coordination with the CDPH involved sampling of cattle feces, water, milk and other samples, and isolation of *C. jejuni*. Successful isolation and novel genotyping of outbreak related strains by ARS provided crucial epidemiologic information for explaining the outbreaks. **Impact:** The results obtained in one study are critical in ongoing interactions between the dairy and the State of California on rules of acceptable levels of coliforms in raw milk for human consumption. This investigation is a good example of the collaborative expertise needed between microbiology and epidemiology. (see also Problem Statement 1.2.5)

- Demonstrated remarkable genomic diversity in *E. coli* and *L. monocytogenes* within the dairy farm. Pulsed Field Gel Electrophoresis (PFGE) was used to assess the relatedness of bacterial strains that were isolated from bulk milk, in-line milk filters, feces, and manure composites over the course of several years. Diversity in *E. coli* isolates from feces was so abundant that tracking individual genotypes through the farm system; that is from feces or the environment to the milk, was not feasible. Less diversity was observed among *L. monocytogenes* isolates, allowing this organism to be tracked within the farm environment. Detection of persistent biotypes in milk filters, collected weekly over two years, identified the milk meters as a potential problem area in the milking equipment. **Impact:** Identified a risk factor for foodborne contamination which allowed the development and suggestion of sanitation protocols as a management tool for the reduction of pathogens in bulk milk. These protocols were communicated to industry.
- A longitudinal study of *Salmonella*, *L. monocytogenes*, pathogenic *E. coli*, on dairy farms in the northeast US, conducted in collaboration with 4 universities, also resulted in finding cows shedding extremely high levels of *Mycobacterium avium* Paratuberculosis (MAP) the causative agent of Johne's disease in cattle. Culling supershedders from each of three farms resulted in significant reductions in environmental load and, more significantly, in MAP-positive animals. **Impact:** The long-term nature of the research in conjunction with intensive sampling of animals provides an opportunity for studying various foodborne pathogens. Quantifying MAP below the <300 cfu/g standard has led to a new understanding of the dynamics of MAP within the farm environment. Johne's disease is estimated to cost American dairy farmers as much as \$500M per year. The potential of MAP being a foodborne pathogen is controversial and unsolved.
- Over 1900 dairy cattle of all ages were examined in States from Vermont to Florida. On one farm, cattle were studied continuously from birth to 2 years of age. The research demonstrated that the primary zoonotic species of *Cryptosporidium* responsible for disease in humans is found almost exclusively in pre-weaned calves and rarely in older cattle. **Impact:** These findings, based on highly specific molecular testing methods, clearly established that virtually all calves less than 30 days of age became infected with this parasite and exposure to their feces carried a high risk of infection for humans. Two new species of *Cryptosporidium* infectious only for cattle were identified and named. These findings provide a scientific basis for determining risk to public health and for local, state, and federal action agencies to establish guidelines or regulations for manure management to prevent environmental contamination.

- The most prevalent type of *Giardia* found in cattle in a multi-state study was genotype E which was not infectious for other animal species tested. The less prevalent genotype was genotype A and has been reported to infect humans and other animals, such as gerbils and sheep. **Impact:** This research demonstrated that cattle can be a source of a *Giardia* genotype reported in human infections but that the predominant genotype of *Giardia* found in cattle does not appear to be zoonotic. This finding could influence State and Federal regulations regarding farm management, manure management, and proximity of livestock to surface water.
- Studies established that *Toxoplasma gondii* disseminates primarily as a few non-recombinant clones in North America and Europe, but undergoes far more frequent sexual recombination elsewhere. Researchers also established that one variant of one chromosome of *T. gondii* has become exceptionally broadly disseminated, and that foodborne spread may be promoted by this chromosomal variant. **Impact:** This research is important because it suggests that while cats (as the reservoir) contribute markedly to transmission elsewhere, transmission in the US does not typically result in the generation of new recombinants (a hallmark of feline transmission). Instead, a few strains are disseminating in North America exclusively through asexual means, including among food animals. The work also indicates that a small specific portion of the parasite genome may have disproportionately contributed to the parasite's evolutionary success. Understanding the evolution of *T. gondii* and its role in the transmission of disease, may provide suggestions for more effective future interventions customized to address regionally specific conditions.
- Determined that the proclivity of *Toxoplasma gondii* to form non-recombinant strains is unlike the reproductive biology of related parasites (including *Sarcocystis neurona* and species of *Besnoitia*), which rely on far more frequent out-crossing in their definitive hosts. Research established and employed new molecular assays to characterize the extent and pattern of genetic variation in organisms like *Sarcocystis*. The work identified only modest differences among localities, and underscored how dramatically this differs from *T. gondii*. **Impact:** The impact of these studies relate not merely to the epidemiology of bovine *Sarcocystis*, but also to the particular (anthropogenic) forces circumscribing the transmission of *T. gondii*. This research provides important comparative data on coccidian transmission which is relevant to animal health and food safety.
- Discovered that the principal agent of human trichinellosis, *Trichinella spiralis*, was disseminated to the Americas from Europe in domesticated pigs via permissive animal husbandry practices. The parasite was not present in endemic American wildlife prior to the introduction of swine. In addition, research established that the *Trichinella* Genus is more diverse than had previously been believed, and that most species are limited to particular geographic foci. **Impact:** This accomplishment sheds new light on the biogeographic history and ongoing epidemiology of *Trichinella*. The work supports the hypothesis that agricultural practices have had an influence on the evolutionary trajectory

of parasites in livestock. A continued focus on specific agricultural practices in geographical regions may provide new strategies for interventions and control.

- Reaffirmed, through new diagnostic approaches, that beef cattle are nearly always infected with *Sarcocystis cruzi*, a species of *Sarcocystis* that is acquired from dogs. However, retail meat samples (sirloin) from a representative area within the US, were infrequently, if ever, contaminated with a species known to induce human disease. **Impact:** Currently, there are no easy methods of differentiating non-pathogenic from pathogenic parasite species. The best method would be transmission electron microscopy, which is not applicable on a broad scale. The definitive impact of this research remains uncertain, but it should provide some reassurance that finding sarcocysts in beef, does not imply a human health risk. The study might suggest that industry and government reconsider the risk-based decision about the need for testing.
- Swine are the major animal reservoir for *Y. enterocolitica* strains, and *Y. enterocolitica* is one of the eight bacterial foodborne pathogens under the CDC FoodNet surveillance program. ARS screened hogs on 122 premises for the *ail* gene and identified risk factors for *Y. enterocolitica* infection. In this study, based on screening feces and tonsillar swabs four risk factors were identified, and the odds ratio (OR) for infection determined. The factors and OR were: location in a central state (OR = 0.3), vaccination for *E. coli* (OR = 3.0), percentage of deaths due to scours (OR = 3.5), and presence of meat/bone meal in grower-finisher diet (OR = 4.1). **Impact:** This research was the first attempt to identify risk factors for *Y. enterocolitica* in the US hog population. The data was provided to industry in order to lower the on-farm prevalence of *Y. enterocolitica* in swine.
- In a semi-closed fully integrated swine production operation, it was shown that certain production groups of swine (boars and nursery piglets), and certain worker populations (slaughter plant workers), were at higher risk to carry multidrug-resistant *E. coli* than other populations within the operation. **Impact:** This research suggests that certain production practices and occupational exposures may increase the risk of transfer of antibiotic resistance genes. This data shows potential points of control, provides a unique opportunity to evaluate human and animal interactions, and any link to foodborne disease.

1.1.3: Ecology, Host Pathogen Relationships and Chemical Contaminants

The challenge for future food safety research is to define and understand microbial populations, whether the communities are in the animal or in the environment, and to study the ecology of the microbial communities. Some potential foodborne pathogens are normal flora in animals or exist in a delicate balance with other pathogens or commensals. Challenges in the microbial balance, due to either disease or even an intervention, may create a niche for new or existing pathogens. The role of protozoa and their interaction with microbial organisms is being studied. New findings indicate that protozoa may “protect” foodborne pathogens in animals and soil, which can later emerge, be transmitted, or cause disease. Information about the ecology of microbial organisms and the host-pathogen relationship can assist researchers in identifying potential interventions or prevention and control strategies. For example, current research has provided data on sites and mechanisms of colonization in animals. The identification and characterization of virulence attributes and immunogenic responses are useful in vaccine strategies.

This section also includes chemical and heavy metal residues found in the environment and/or in food animals. Since many of these residues are toxic it is important to know the metabolism, transport, sites of deposition, and excretion of chemical residues. These residues are still a critical threat to food safety.

- ARS scientists were the first to identify the ubiquitous and prevalent human pathogen *Blastocystis* sp. in feces of cattle in North America. This microbe is widespread and infects humans, pigs, birds, rodents, amphibians, reptiles, and fish. The only previous report of *Blastocystis* in cattle has been from Japan involving a few animals. This ARS finding is being evaluated through genetic analysis to determine the range of genotypes present in cattle and their relationship to pathogenic and nonpathogenic genotypes found in humans. **Impact:** This study is the first report of human *Blastocystis* in cattle in North America. This report validates our development of new primers used for detection of *Blastocystis* and indicates a new and unexpected potential source of this parasite for humans. Current research may identify the range of genotypes in cattle and their relationship to humans, and as a public health risk.
- Demonstrated that rumen protozoa induced virulence gene expression by *Salmonella enterica* serovar Typhimurium, and facilitated horizontal (antibiotic resistance) gene transfer into this foodborne pathogen. **Impact:** This research highlights a new area of focus involving protozoa, and their role in bacterial pathogenesis. This is significant because it suggests the potential usefulness of defaunating agents (i.e. saponins and other plant products) in animal (ruminant) diets to remove protozoa. Removing the protozoa may eliminate a carriage element of *Salmonella*, a site for antibiotic resistance transfer, and reduce the virulence of this pathogen.
- Identified the most prevalent microsporidian parasite infectious for humans, *Enterocytozoon bienersi*, in North America. The parasite was found in cattle, and in several wildlife species including beaver, fox, muskrat, otter and raccoons. **Impact:** This

study was the first to identify the human parasite, *E. bienersi*, in cattle and wildlife. Identification of these animals as hosts for genotypes of *E. bienersi* provides a basis for understanding possible routes of transmission between livestock and wild animals, and between both of these groups of animals and humans.

- Handling of the large amounts of animal waste produced by concentrated animal feeding operations (CAFO) may aerosolize foodborne pathogens that can be transported via wind and fog. ARS evaluated the transport of bacterial communities via aerosols from two dairies, with different manure management practices. The studies found that that no known foodborne pathogens were detectable from air samples. The predominant bacteria found were from distinct classes (Betaproteobacteria and Clostridia) and were specific to each dairy. **Impact:** Bacteria specific to a dairy may be used to trace the source of pathogens that are transported to crops grown in proximity. These data are useful for further ecological and epidemiologic studies, and may aid in targeted on-farm foodborne pathogen control strategies.
- Demonstrated enhanced susceptibility of dexamethasone-treated weaned calves to *E. coli* O157:H7 colonization. This strategy allowed identification of the colon and cecum as the major sites, and the ileum and gall bladder as the secondary sites for colonization. **Impact:** ARS scientists were the first to demonstrate enhanced susceptibility of dexamethasone-treated weaned calves to *E. coli* colonization. Recognition of initial colonization sites will facilitate identification of bacterial and host factors that promote O157:H7 colonization and provide the basis for designing and evaluating strategies for reducing STEC in cattle.
- Identified intestinal sites where O157:H7 bacteria are most likely to attach initially in experimentally inoculated calves. The rectal anal junction is a prominent O157:H7 colonization site during the carrier shedder state in cattle. Using experimentally inoculated weaned calves, discovered that squamous epithelial cells and enterocytes at the rectal-anal junction represent a prominent niche for early O157:H7 colonization in cattle. **Impact:** These results increase our understanding of O157:H7 adherence in cattle and will facilitate elucidation of the bacterial and host factors that promote colonization of bovine intestines, development of techniques for identifying infected cattle, and identification of targets for intervention strategies.
- Conducted the first study to examine microbial communities of both the primary and secondary habitats of STEC O157, and the largest study to date of the bovine fecal microbiota using near full-length 16S rDNA sequencing. **Impact:** This study demonstrates that the primary and secondary habitats of STEC O157 have separate and distinct microbial communities. Consequently, a list of core bovine taxa has been developed. This work is the first to provide information on applied bacterial ecology in beef production agriculture settings.
- Demonstrated that feeding distillers grains to feedlot cattle affects the level and prevalence of *E. coli* O157:H7 and generic *E. coli* in feces and on hides. Studies examined the survival of generic *E. coli* and *E. coli* O157:H7 in manure collected from

steers that were fed a finishing diet that contained 0, 20, 40, or 60% distiller's grains. Feeding higher levels of distiller's grains was associated with a greater persistence of naturally occurring *E. coli* and inoculated *E. coli* O157:H7 in manure. In contrast, lower persistence of these organisms was associated with more lactate and lower pH in manure from cattle fed higher levels of corn. In the finishing phase, cattle that received 40% distiller's grains in their diet had a greater prevalence of the pathogen on hides and in feces compared to 0% distillers grains. **Impact:** Higher prevalence of *E. coli* O157:H7 associated with cattle fed high levels of distillers grains could result in a greater pathogen load at time of slaughter. Greater persistence of the pathogen in the manure and production environment of cattle fed distillers grains could result in a greater prevalence of *E. coli* O157:H7 by increasing risk for re-colonization of animals.

- Demonstrated that heat and handling stresses of feedlot cattle does not increase *E. coli* O157:H7 fecal shedding. Studies examined individual feedlot cattle for signs of heat stress on days when the temperature humidity index was in the "high danger" or "emergency" categories. In addition, the handling stress of individual animals was assessed by scoring temperament on a normal 28-day weighing schedule. Correlations were tested on individual animal heat stress level, the handling stress level, and the level of generic *E. coli* in their feces. In addition, analyses were conducted to test for changes in prevalence of *E. coli* O157:H7 as a result of experiencing different levels of heat stress or handling stress. **Impact:** No relationship was detected between either handling stress or heat stress and generic *E. coli* or *E. coli* O157:H7 prevalence in feces. This study provides critical information for industry and producers to ensure non-stressed cattle at slaughter. Logically this should lead to less shedding of foodborne pathogens, but further research is needed. The research has implications for industry and regulatory agencies in their role of providing healthy cattle at slaughter. The work raises potential research questions on stress, and its effect on the presence and persistence of foodborne pathogens.
- Demonstrated that livestock stress responses activate *E. coli* O157:H7 virulence genes. Using the ligated ileal loop surgical model, researchers evaluated the genetic response of *E. coli* O157:H7 during early stages of infection, when the stress associated catecholamine, norepinephrine, is present. The results in the in-vivo model showed repression of genes, including a set of phage shock genes. Alternately, induced transcripts included Shiga-like toxins 1 and 2, and genes associated with LPS biosynthesis and iron transport. **Impact:** The work concluded that norepinephrine-bacterial interactions occur in vivo affecting a number of key *E. coli* O157:H7 virulence properties. This confirms the results from previous in vitro studies. In vivo model systems, such as the porcine ligated ileal loop model, may be valuable in studying the microbial endocrinology of *E. coli* O157:H7 pathogenesis.
- Demonstrated that the sensing of specific environmental signals is important for *Salmonella enterica* serovar Typhimurium colonization of swine. The ability of *Salmonella* to sense and respond to a changing environment is critical for survival and host colonization. Research demonstrated that *S. Typhimurium* responds to the host stress-hormone, norepinephrine, by increasing bacterial motility. In addition, the

inactivation of a membrane sensor protein decreased bacterial motility and colonization of the swine gastrointestinal tract of the bacterial mutant compared to the wild-type *S. Typhimurium*. **Impact:** This research suggests that inhibitors that target this bacterial sensing system may decrease/prevent colonization of animal hosts by this bacterial pathogen. This research is important in developing intervention or prevention strategies.

- The mammalian host has developed strategies to limit iron availability within the body to prevent bacterial pathogen growth and infection. Research identified and characterized genes required by *Salmonella enterica* serovar *Typhimurium* for norepinephrine-enhanced growth. **Impact:** This work demonstrated that *Salmonella* can utilize the mammalian hormone norepinephrine as a siderophore to acquire iron for growth during infection. This research contributes information concerning the mechanisms that *Salmonella* utilizes to circumvent the iron-restricted environment of the host. These data identify potential targets for pathogen intervention.
- Using microarray analysis, research established and quantified the genetic differences between a line of chickens that are resistant and non-resistant to colonization by *Salmonella*. In addition, work identified differential immune responses in genetically distinct lines of commercial chickens and turkeys. The work demonstrated that multiple intracellular protein tyrosin kinases are phosphorylated following engagement of different protein receptors. **Impact:** This research helps explain why sub-resistant chickens avoid being colonized. The studies also provide a foundation for ongoing research to establish the genetic basis (and immune response) for bird resistance to pathogenic bacteria. Research in collaboration with industry will facilitate the development of lines of birds that meet all productivity and quality requirements while being highly resistant to colonization by food-poisoning bacteria.
- Identified the lesser mealworm (darkling beetle) as a vector for *Salmonella*. Darkling beetles are a common insect pest of broiler facilities, and commercial farms can suffer significant financial losses due to high population density. Darkling beetles can also transmit pathogens to the flock. Using a method developed by ARS to externally disinfect the beetle, research demonstrated that relatively short exposures to low concentrations of *Salmonella* result in the rapid acquisition of viable bacteria into the alimentary canal of the beetle. **Impact:** Current farm management practices can perpetuate infestations and contribute to the dispersal of beetles, and any pathogens they may harbor. The beetle model will be used to develop biosecurity procedures to control the propagation and dissemination of *Salmonella* of colonized birds at processing. (see also next accomplishment)
- Determined that ingestion of only four adult and/or larval beetles that carried *Salmonella* would result in the colonization of gavaged day-of-age chicks and that this “seeder chick” would spread *Salmonella* to non-gavaged pen-mate chicks. *Salmonella* contamination of the pen environment and pen-mate chicks persisted through grow-out at six weeks of age. **Impact:** These results stress the importance of darkling beetle control programs in the overall goal to control *Salmonella* spread within and between broiler flocks.

- Evaluated the role of biofilm and planktonic communities on the control of Salmonella in vitro. The composition of gastrointestinal bacterial communities from young birds was studied using an in-vitro continuous-flow culture technique, to test the ability of these cultures to resist colonization by Salmonella. The cultures, initiated from 14-day old chicks, were able to restrict colonization by Salmonella. **Impact:** These studies suggest that in-vitro bacterial mixtures, or cultures within the birds that are established prior to exposure to a pathogen, are effective in resisting colonization by that pathogen and will reduce the spread of pathogens.
- Quorum sensing is made possible by the production and sensing of small, extracellular chemical signals called autoinducers (AI). These autoinducers accumulate as the population density increases, and thereby help bacteria to regulate their behavior by promoting or repressing gene expression. DNA microarray experiments were conducted on a luxS mutant of *S. Typhimurium* to study its gene regulation in the presence of condition media (CM) of its own wild type, and CM of *E. coli*. In the presence of CM of *S. Typhimurium*, 1143 genes were differentially expressed (504 repressed and 639 expressed). In the presence of CM of *E. coli*, 392 genes were observed to be differentially expressed (133 repressed and 259 expressed). It was found that all the genes in the pathogenicity island of luxS mutant of *S. Typhimurium* were turned down in the presence of CM of *E. coli*, and CM of *S. Typhimurium*. **Impact:** These experiments not only suggest that AI-2 acts as a master controller of genes in the pathogenicity island of *S. Typhimurium*, but also that *S. Typhimurium* could sense the AI-2 molecules produced by *E. coli* in a multi-species environment.
- Demonstrated that hens that are molted by feed withdrawal, and also infected with *S. enteritidis* (SE), exhibited substantially higher crop levels of SE for longer periods and substantial damage to the crop wall when compared to similarly infected hens that were not molted. While crop antibody responses to SE appeared to be unaffected by feed withdrawal, the protective ability of phagocytic white blood cells, appeared to be impaired. Feed withdrawal also caused reduced weights and intestinal lengths within 48 hours without altering intestinal dry weights. This suggests that the altered measurements were not merely due to change in fluid in the tissues. It was also observed that intestinal weights and lengths returned to pre-feed withdrawal levels within 24 hours of re-administration of feed. Alterations in stomach and intestinal pH were also observed during feed. **Impact:** This work provides further evidence for increased SE problems in hens molted via feed withdrawal and establishes baseline information regarding the effect of stress on poultry health. Additional research will help elucidate the role of these stresses on foodborne pathogens and on food safety risk.
- Determined that the ability of Salmonella strains to colonize the intestinal tract and to contaminate eggs do not appear to be directly related. Salmonella strains re-isolated after passage through infected chickens in prior studies were found in feces for longer intervals than were the original parent strains. Passaged strains caused more contaminated eggs to be produced than did the parent strains. Results showed that infection of older hens with *S. Enteritidis* correlated with a decrease in shell quality that could facilitate entry of *S. Enteritidis* and other spoilage bacteria into the egg supply. **Impact:** This research helps

explain why the presence of *S. Enteritidis* in the farm environment does not always result in production of contaminated eggs by exposed hens. This research supports ongoing post-harvest research that focuses on improvement of egg quality as a general strategy to protect the safety of the food supply.

- Identified *Campylobacter* intestinal community changes that correlate to fluctuations in the intestinal microbiota. Different *Campylobacter* species and subspecies presence in the intestine were shown to correlate to intestinal bacterial community fluctuations and host signals. The results of studies applying this novel molecular approach to profiling microbial communities indicated that, *C. coli* is a commensal colonizer, while *C. jejuni* is an opportunistic colonizer. **Impact:** By understanding the microbial community of *Campylobacter* in the poultry intestine, studies demonstrated that different intervention strategies may be required to exclude the two species from the poultry intestine. This research also indicates that a single intervention may shift the balance of these subspecies in the intestine, and have an effect on animal health and/or food safety.
- Described the microbial community composition in the turkey cecum. A baseline examination of the community differences between wild and commercially raised turkeys indicated significant differences between the two as well as differences in *Campylobacter* carriage. **Impact:** Understanding microbial communities in wild versus commercially-raised turkeys will help in the understanding of colonization of foodborne pathogens and developing potential interventions. This research may help in the design of organic versus conventional production.
- Six commercial flocks were examined on the farm and at slaughter to monitor the effects of peri-marketing events (feed withdrawal, transport, and holding at the slaughterhouse) on the prevalence of *Campylobacter* in market weight turkeys. At slaughter, there was a statistically significant increase in *Campylobacter* spp. isolated from the gall bladder and crop when compared to on-farm levels. The increased prevalence of *Campylobacter* in the crop was associated with a decline in lactic acid in the emptied crop. **Impact:** These findings identify several critical control points at the pre-harvest level that may be used and studied for intervention strategies. The isolation of *Campylobacter* in the gallbladder and crop identifies sites for testing when FSIS considers performance standard measurements.
- DNA: DNA microarray and suppressive subtractive hybridization analyses were employed to facilitate the identification of potential factors involved in the colonization of poultry by *C. jejuni*. Additionally, differential gene and protein expression was monitored using RNA: DNA microarray analysis and 2-Dimensional Gel Electrophoresis respectively. Several genes and proteins, such as a fibronectin binding protein (*cadF*) and an outer membrane protein (*omp85*), were identified as factors potentially involved in colonization of poultry. **Impact:** Studies identified genes and gene products that are currently under development as vaccine targets, since these genes appear to affect colonization. Additionally, 98 isogenic mutants of *C. jejuni* were obtained for use in chicken challenge investigations and eukaryotic cell invasion assays. These mutants may improve future testing for *C. jejuni* in poultry.

- The relationship between the levels of *Campylobacter* on contaminated poultry products and human disease was investigated. Analyses showed that implicated broiler product lots (*Campylobacter* recovered from contaminated chicken flocks that are genetically similar to *Campylobacter* isolates recovered from humans with campylobacteriosis) had significantly higher median levels of *Campylobacter* contamination (~3,600 cells/carcass) than did non-implicated lots (~525 cells/carcass). **Impact:** The results suggested that foodborne campylobacteriosis linked to broilers may occur in clusters and that the differences in mean contamination levels may provide a basis for regulatory action by FSIS beyond that of a presence/absence standard.
- Determined that *Campylobacter* are naturally present in the reproductive and lymphoid systems of both female and male commercial broiler breeders, as well as caged Leghorn hens. Most importantly, *Campylobacter* are naturally present in the circulating blood of commercial broilers upon arrival at the processing plant. In the broilers tested, *Campylobacter* isolated from blood and ceca, and determined to be similar by *flaA* SVR DNA sequencing, differed in their ability to invade Caco-2 cells. Inoculation studies demonstrated that when day-of-hatch broiler chicks are inoculated (regardless of route oral, cloaca, or eye), the organism rapidly (within 1 hour) disseminated to lymphoid organs and established a reservoir. **Impact:** This research significantly adds to the knowledge of *Campylobacter* ecology in poultry. The findings indicate that interventions must be implemented early in poultry production or that mitigations are needed at the processing plant.
- There is a need to identify turkeys with an unfavorable response to the stressors associated with production, since logically this would affect foodborne pathogen prevalence. Male and female turkeys from different genetic backgrounds were compared for their response to a respiratory bacterial challenge and transport stress. An important change was that the levels of creatine kinase, an indicator of muscle growth and damage, were over 6-fold higher in challenged and transported birds from a commercial line compared to controls. Iron levels of the transported commercial line males were 3-fold lower than non-challenged male controls, and the levels of both iron and alkaline phosphatase were lower in the fast growing lines as compared to the slow growing line. These two blood chemicals were also the only parameters influenced by gender, with males having higher levels of both compared to females. **Impact:** The highly significant differences seen in the commercial turkey line for these blood parameters suggests that the genetic differences may be useful for selection of turkeys with a favorable response to production stress. Research is still needed to understand how production stress affects pathogen levels in birds and the consequential effect in food safety.
- Demonstrated that the thymidine analog bromodeoxyuridine (BrdU), when supplied in the drinking water of turkey poults, will be incorporated into the DNA of actively dividing intestinal microbes. BrdU can be used for the identification of turkey intestinal microbes that grow in response to in vivo environmental changes resulting from feed withdrawal or other stressors experienced by the fowl host. **Impact:** This novel

molecular method will allow examination of microbial and host-microbe interactions affecting animal health, nutrition and food safety.

- Compared lymphocyte changes in chicken gut lymphoid tissues Peyer's patches (PP) and cecal tonsils (CT) following SE infection. The CD4/CD8 ratio, a numeric comparison of helper vs cytotoxic T cells used for observing changes in immune function, was decreased one week post challenge in the CT and lower PP then rebounded back to control levels one week later. Decreases in the upper PP CD4/CD8 also occurred but were delayed a week. **Impact:** These results indicate that immune responses to infection differ in the various tissues and may be a reflection of location. The lower PP is close to the colon and cecum where the infection predominates, and therefore may be affected more readily than the PP residing higher in the bowel. This research provides a baseline for evaluating mucosal responses to infection and is important in future immunization strategies to set priorities as to which organs to target the vaccine.
- Research defined critical parameters in poultry that are related to the production of nitric oxide which is a mechanism used by the body's immune system to defend against bacterial invasion. The work identified components of the nitric oxide pathway in chicken macrophages and those components affected by chemicals that inhibit signals which normally result from macrophage interactions with bacteria or viruses. **Impact:** This accomplishment demonstrated that the signaling process in birds is similar to that in mammals. The full body of research on the nitric oxide signaling pathway, across higher animal types, may be used to develop effective pathogen management strategies.
- In *L. monocytogenes*, SigB activation has been shown to occur through a common pathway during both environmental and energy stress conditions. However, little is known about the role of SigB when sudden interruptions in energy supply occur during active growth. The effects of an inhibitor of proton motive force (PMF) and an inhibitor of pyruvate dehydrogenase (PD) on transcriptional changes in *L. monocytogenes* and its sigB mutant were compared. DNP or 2, 4-dinitrophenol was used to inhibit PMF while sodium arsenite (SAs) was used to inhibit PD. During growth in the presence of DNP 193 and 223, genes were differentially expressed for the wild type and sigB mutant, respectively. The data show that cellular processes involving cell division or DNA metabolism appear to be PMF-dependent while those involving the phosphotransferase system appear to be PMF-independent. Repression of genes related to cell division or synthesis/degradation of cellular components occurred following PMF loss. During growth in the presence of SAs, differential expression of 742 and 134 genes were exhibited by the wild type and sigB mutant, respectively. **Impact:** The results indicate that most of the genes involved in regulation during growth of *L. monocytogenes* may be sigB-dependent. Induction of protein synthesis genes also occurred following the addition of SAs. Interestingly, the reaction catalyzed by SAs may provide an energy source for protein synthesis. Energy stress induced by SAs led to induction of the fur and prfA genes. This data suggest that metabolic pathways involving PD may be linked to pathogenesis in *Listeria*.

- Demonstrated that stress increases *L. monocytogenes* colonization in turkeys and may be an overlooked source of contamination of poultry processing plants and products. *L. monocytogenes* colonization of turkey knee synovial tissue was increased by early cold stress and by concurrent infection with *E. coli*. **Impact:** This research was the first to demonstrate that the stressors associated with turkey production may impact colonization with this environmentally ubiquitous pathogen, and contribute to processing plant contamination.
- Demonstrated that respiratory exposure of turkeys to *L. monocytogenes* causes more disease than oral exposure. Research compared the ability of this bacterium to cause disease when fed orally to turkeys, compared to placing it in the eye and nose (oculo-nasal route). Studies also compared the level of disease under different housing conditions. The oculo-nasal route resulted in higher mortality and lower body weights as compared to both non-challenged controls and to those challenged by the oral route. Birds closely contained in battery brooder cages for 1 week after challenge had higher mortality and higher body weights as compared to floor pen reared birds. **Impact:** These results suggest that day old turkeys are susceptible to respiratory exposure with *L. monocytogenes*, and that ventilation systems and housing systems may be an important management strategy to consider in the control and prevention of disease (and potential foodborne contamination). Respiratory exposure with *L. monocytogenes* may result in subclinical infection and carcass contamination. More research is needed on battery cage versus pen rearing, and the effect on animal disease and foodborne risk.
- Demonstrated systemic infection by *L. monocytogenes* in dexamethasone (Dex) treated and transport stressed turkeys. Studies compared conventional culture methods and Taqman® real time PCR (RTi PCR) for isolation of *L. monocytogenes* from the joints of challenged turkeys. There was no statistical difference in *L. monocytogenes* levels between dexamethasone treated and transport stressed birds. No *L. monocytogenes* was isolated upon direct plating at one and two weeks post-challenge or by any method two weeks post-challenge. **Impact:** RTi PCR can be used for detection of sub-clinical *L. monocytogenes* infection of turkey carcasses which may not be detected by direct plating and culture. Detection of sub-clinical infections can provide producers the ability to intervene and help reduce the prevalence of foodborne pathogens.
- Research found a potential MAP (*Mycobacterium avian paratuberculosis*) virulence gene, and constructed a mutant strain with a deletion in this gene. The mutant strain shows decreased viability in cultured bovine macrophages. **Impact:** Although the importance of this organism to food safety is still unclear, the research findings are significant to an important animal disease in cattle, Johne's disease. These data have a potential to lead to development of treatments and preventives (vaccine) for Johne's Disease.
- Perchlorate is a strong oxidizing agent that has contaminated several locations within the US either naturally or as a result of industrial use. High doses of perchlorate can inhibit processes within the human thyroid gland potentially affecting development; therefore, finding perchlorate residues in commercial milk samples caused some concern about health risks. Studies demonstrated the low transfer rate of perchlorate into milk. Using

dairy goats as a model for lactating animals, studies showed that ingested perchlorate was efficiently converted to chloride (a component of table salt) and excreted. **Impact:** Less than 5% of the dosed perchlorate was present in the milk, indicating that perchlorate does not accumulate in lactating ruminants and is minimally transferred into milk. The study suggests that lactating animals grazing in perchlorate-contaminated areas may have low perchlorate residues in their milk, but these residues can be cleared in a few days if animals are moved to clean pastures.

- The fate of the military explosive TNT was determined in live sheep. Sheep rapidly detoxified TNT into bound residues that were rapidly excreted in feces. **Impact:** The use of grazing animals on ground with low levels of TNT contamination has the potential to speed the reclamation of TNT-contaminated lands at a fairly low cost. Over 1 million tons of soils in the US are estimated to be contaminated with TNT with remediation costs of \$200-500 per ton. Total costs for remediation of this land are prohibitive using existing technologies.
- An ultra-sensitive, automated, flow-fluorescent immunoassay was developed and validated for the quantitative analysis of thiamethoxam in foods. Thiamethoxam is a relatively new class of insecticide (neonicotinoid) that has broad use in produce and seeds. Neonicotinoid insecticides have been implicated with Colony Collapse Disorder of honey bees. Food animals grazing thiamethoxam treated areas may accumulate the insecticide in tissues or secrete it into milk. **Impact:** ARS has licensed the immunoassay technology for commercialization. The use of this immunoassay by regulatory organizations will help to ensure the safety of agricultural products, such as milk. In addition, this assay will help in further research into the fate and clearance of this insecticide in tissues and milk.
- In a study in rats, research showed that a widely used brominated flame retardant, Deca-BDE, is minimally bioavailable; however, it is more persistent than previously reported and can be debrominated to more persistent brominated compounds. Deca-BDE is a flame retardant incorporated into numerous consumer goods ranging from upholsteries to electronics. While other polybrominated diphenyl ether formulations (BDE-types of flame retardants) have been withdrawn from production due to persistence and toxicity issues, the persistence and toxicity of Deca-BDE is currently being re-evaluated. **Impact:** The data showing Deca-BDE's higher persistence and metabolic formation of more persistent and toxic compounds are of interest to regulatory agencies and the scientific community. The work was cited in "State of the Science Report on Bioaccumulation and Transformation of Decabromodiphenyl Ether" (Environment Canada, 2007) and "Toxicological Review of BDE-209" (US EPA, 2008).
- Characterized the absorption, disposition, metabolism, and excretion of polybrominated diphenyl ethers (PBDEs) in laboratory and food-producing animals. PBDEs are a class of flame retardants used in many household goods that are shown to spread and persist in the environment, bioaccumulate in the food chain, have potential health effects, and are increasingly monitored in humans, wildlife, and food producing animals to estimate exposures. In laboratory animals, studies found that most PBDEs are well absorbed from

the diet, distributed through the body by equilibrating into lipids, are not readily metabolized, but can induce certain metabolizing enzyme systems. In chickens, PBDEs mainly distribute to adipose tissue and skin. This suggests a simple means to reduce exposure from chicken consumption is to remove the skin and fat. Uniform distribution into body lipids implies that serum lipid or adipose tissue concentrations can be used to estimate exposure by predicting body burdens or, in food producing animals, the edible tissue residues. **Impact:** This research provides information on the metabolism and accumulation of PBDEs which is important for regulatory agencies, and for developing potential mitigation strategies. This study was cited by US EPA in Toxicological Reviews of BDEs-47, 99, and 209.

- Demonstrated that house dust can be a source of polybrominated diphenyl ethers (PBDEs) exposure. Like other persistent environmental contaminants, human exposure to PBDEs is thought to occur mainly through the food supply; however, recent studies have also implicated the ingestion of indoor dust as a potential exposure pathway. Our study showed that when ingested PBDEs in dust were as readily absorbed by rats and stored in their tissues as were PBDEs in fatty foods (i.e. a corn oil dose). **Impact:** The results support the hypothesis that, in addition to dietary exposure, ingestion of indoor dusts may be a significant route of exposure to PBDEs for humans. This work was cited in "An Exposure Assessment of Polybrominated Diphenyl Ethers" drafted by US EPA, 2008.
- Demonstrated that tissue residues of chlorate in treated food animals do not exceed safe tissue levels established by the FDA-CVM. Chlorate residues can cause a significant amount of beef to be eliminated from the food supply. **Impact:** USDA has subsequently granted an exclusive license to a US company for the development of chlorate as a commercial feed additive. This study demonstrates the safe tissue levels of chlorate in treated food animals. Future research could be useful to both industry and the regulatory agencies.
- Demonstrated that trace levels of a commercially available chemical, Fe-TAML, in combination with hydrogen peroxide, are capable of degrading natural and synthetic reproductive hormones that may be present in water. Both synthetic and natural estrogens found in human and animal waste are incompletely removed by municipal sewage treatment plants and some agricultural waste handling systems. **Impact:** Fe-TAML and hydrogen peroxide provide an environmentally-friendly alternative for wastewater treatment for intensive agricultural systems and municipalities. Estrogens or other hormones in water are a significant public health issue.
- Established baseline environmental estrogenic activities in a survey of municipal and agricultural surface waters and showed that a constructed wetland could eliminate estrogenic activity from swine wastes to concentrations below the proposed No Observable Effect Concentrations. Studies determined that steroid hormones such as testosterone and estradiol are converted to carbon dioxide by bacteria present in native soils and that each hormone has the ability to tightly bind to soil particles, even under sterile conditions. **Impact:** These findings are extremely important to animal agriculture

because they demonstrate that waste handling procedures used in livestock production, are sufficient to prevent contaminating the environment with steroid hormones. This information is also valuable to EPA which considers endocrine disruptors a priority.

- Sulfonamide antibacterial agents have been found in effluent from wastewater treatment plants and waterways worldwide. Sulfamethazine (used in veterinary medicine) and sulfamethoxazole (used in human medicine) are two sulfonamides of particular interest because of their extensive use. Immunoassays for sulfamethazine and sulfamethoxazole worked well in the analyses of wastewater from swine-rearing facilities and municipal wastewater treatment plants. The immunoassay results were confirmed by liquid chromatography-mass spectrometry-based analyses. **Impact:** The immunoassays are sensitive, selective, have a relatively low cost, and are commercially available. These immunoassays may be valuable in studying the presence of antibiotics and the development of antimicrobial resistance in the environment.

1.1.4: Intervention Strategies

Interventions, whether they are management strategies or specific products, are needed to reduce colonization and shedding of zoonotic pathogens by food producing animals. Ultimately, the goal is to reduce the prevalence of the zoonotic or foodborne pathogen in the animal and the environment, and to minimize the risk of human infections.

It is clear from the research to date, that there is no single intervention or prevention strategy that will eliminate the pathogens in food animals. The most successful approach will include a number of strategies focused at the most critical or most likely stage in production. At the same time, the cost-benefit ratio must be considered by producers, since feed efficiency and weight gain are important to food animal production. Interventions that can be used just prior to slaughter may be particularly useful since they limit the potential effect on weight gain or feed efficiency while also providing a very limited opportunity to acquire additional infection prior to slaughter. Research is still needed to better understand the epidemiology and ecology of these organisms in the environment, animals, and humans.

Bacteriophage and bacteriocins both hold a great deal of theoretical promise, but there remains significant barriers in approval and in their mass production. As examples of these challenges are the past development of competitive exclusion cultures (CEC), and the bacteriocin nisin. Bacteriophages are a newer form of intervention but their high level of specificity may limit their broad application, and their production is still in its infancy. “Natural” products are also being evaluated as feed additives to reduce the shedding of pathogens and as an alternative to antibiotics.

It is paramount that specific research and methods are created and implemented to determine if interventions work, and that the outcome is measured. This will require innovative ideas about measurement, collection of data, and detection. For example, it is still unclear whether the use of probiotics has a significant effect on foodborne disease or on antimicrobial resistance.

- Identified the progression of Salmonella through the upper intestine, gut associated lymphoid tissue and peripheral immune site responses, and cortisol, catecholamine, and behavior changes following an intra-nasally administered experimental infection. Salmonella counts increase within 2 hours and remained stable (under log 5) until after 4 weeks when Salmonella became undetectable. Behavior indicated discomfort and restlessness in infected pigs. Immune data demonstrated the site-specific immune changes occurred first and that more peripheral responses were altered later. An exception was changes in several immune measures that occurred at week 3 post-challenge, suggesting a sequestering of bacteria in the spleen and mesenteric lymph nodes. **Impact:** These data provide a summary of the response of pigs to a sub-clinical Salmonella infection. These results will enable the development of targeted measures for intervention strategies used to reduce *S. Typhimurium* infections and carriage in pigs.
- Research identified Salmonella genes required for survival in the swine stomach environment. The hostile environment of the porcine stomach is a first line of defense

encountered by gastrointestinal foodborne pathogens. These genes allow *Salmonella* to persist in swine intestines and provide an opportunity for contamination of the environment and at slaughter. **Impact:** With swine production losses due to *Salmonella* estimated at \$100M per year for producers, this investigation of *Salmonella* survival systems in the gastric environment has broad implications for developing novel interventions against the veterinary and human pathogen. For example, this knowledge provides specific *Salmonella* genes to target for controlling the initial stages of colonization and infection, and the inhibitory components identified in the swine stomach provide the foundation for designing analogs to inhibit *Salmonella*. This research demonstrates a new concept in *Salmonella* colonization whereby *Salmonella* surviving passage through the stomach are primed for intestinal colonization and invasion.

- Identified the effects of feed withdrawal, transportation, and their interaction on *Salmonella* concentrations and location in market-age pigs. During this study, we demonstrated that feed withdrawal created greater changes in *Salmonella* concentrations from ileal contents than transport stress, but only an interaction of the effects raised the concentration of *Salmonella* in cecal contents. The rectal contents contained very low concentrations of *Salmonella* with greater variability. **Impact:** This study provides information about those phases of typical transport practices that may lead to greater intestinal *Salmonella* concentrations, and potential pathogen contamination of the food supply. This research also highlights the challenges in sampling for *Salmonella* in pigs, and in interpreting what the numbers indicate as they relate to food safety. This research also identifies specific control factors that can be targeted for intervention.
- Orange peel and pulp that are currently fed to cattle were shown to exhibit anti-pathogenic activity against *E. coli* O157:H7 and *Salmonella* in pure and mixed cultures in vitro. Further, extracted specific oils from these byproducts were shown to contain the highest anti-pathogen activities. **Impact:** These GRAS dietary additions may be alternatives to antibiotic treatments and provide another potential tool to help reduce the carriage of foodborne pathogens in their animals. Although not a primary impact, this research enables the citrus industry to use citrus byproducts in a valuable way.
- Molting is a stressful process for poultry that can result in the birds becoming susceptible to *Salmonella* infections and producing *Salmonella*-contaminated eggs. New protocols are needed that will allow molting to be accomplished without the resulting stress and egg-contamination potential. Research developed an alfalfa diet for use during a forced molt that reduces *Salmonella* levels in the birds during and after the molt, and that greatly reduces the likelihood *Salmonella*-contaminated eggs. **Impact:** The implementation of the alfalfa molting diet will enhance the microbiological safety of raw egg products reaching the consumer, and provides producers with a method for accomplishing a necessary bird management practice with reduced risk to animal health and food safety.
- In collaboration with researchers and producers in Japan, ARS demonstrated, in a commercial operation, that feeding hens wheat middlings, a by-product of wheat-processing, successfully stimulated hens to molt. A comparison study was done between hen flocks molted traditionally using feed withdrawal, and flocks molted using wheat middlings. Increased recovery of environmental *Salmonella*, observed in hens molted via

feed withdrawal, was not detected in the wheat middlings hens. **Impact:** Scientists were the first to demonstrate, in a field situation, that producers can molt their hens just as profitably via methods other than feed withdrawal, and reduce the incidence of potential Salmonella problems. The study also showed better production and greater profits for those hens molted using wheat middlings, than hens molted via feed withdrawal. This strategy is also important to animal health and animal welfare.

- Demonstrated that controlling ambient temperatures during pre-refrigeration storage is an important adjunct to prompt refrigeration for limiting Salmonella growth in eggs, and thereby for preventing egg-transmitted human illness. **Impact:** This research shows how on-farm and packing plant management of eggs could be improved in a cost-effective manner, on a large scale, to solve a national and international food safety problem.
- Demonstrated the bactericidal effects of polymers in combination with biocides as a means to sanitize broiler hatching eggs. Breeder flocks and commercial hatcheries represent an early contamination point for Salmonella entry into commercial integrated poultry operations. Several of the commercial antimicrobial chemicals were found to significantly reduce or eliminate Salmonella on fertile hatching eggs in laboratory experiments. No significant differences were observed in hatchability from eggs sprayed with or without the chemical polymers in combination with biocides, indicating no adverse effects on livability of developing broiler chicks. **Impact:** The chemicals which provided efficacious results are now being evaluated in a commercial hatchery. Using effective antimicrobial treatments for hatching eggs is a critical part of reducing on farm incidence of Salmonella.
- Developed a novel probiotic targeting Salmonella and Campylobacter in poultry after screening more than 8 million individual enteric bacteria for efficacy against these food borne pathogens in vitro. A pre-selection process eliminated strict anaerobes and fragile organisms through a freeze-thaw process prior to screening, factors greatly reducing the cost of production compared to other commercial products. **Impact:** Product developed from this research has been field tested in millions of turkeys from Arkansas, Missouri and Virginia resulting in notable reduction in pathogen contamination, mortality, and improved health of young poults. In 2007, 2 billion doses were sold by the company which licensed the technology from ARS and the University of Arkansas. The product is being marketed in 3 countries, and 6 additional countries are in the final stages of acquiring import permits. Extrapolating current data, translates to a greater than \$6M increase in production yields for every 300 million birds treated in the US per year.
- Demonstrated that treatment of poultry litter with aluminum sulfate or sodium bisulfite would lead to significantly reduced levels of Campylobacter in chickens reared on treated litter. Cooperative research with two chemical manufacturers and a major poultry producer demonstrated efficacy of the treatments in small-scale experiments and large-scale field trials involving numerous commercial farms and broiler houses. Highly significant ($P < 0.05$) reductions (approximately 5 logs) were observed in levels of Campylobacter associated with market-age broilers raised on acidified litter. **Impact:** The compounds investigated are currently utilized for ammonia control in broiler houses

which encourages application by growers and simultaneously accomplishes a reduction in human pathogen carriage by poultry. The major US poultry producers in Arkansas, Georgia and Maryland are utilizing this technology.

- Identified a compound with novel antimicrobial activity which could potentially be used in cattle to reduce shedding of E. coli O157:H7. This compound was shown to inhibit the growth of E. coli O157:H7 and Salmonella DT104 in vitro. In neonatal pigs infected with pathogenic Enterotoxigenic E. coli (ETEC), treatment with this compound reduced the incidence and severity of diarrhea caused by ETEC. In preliminary experiments, this antimicrobial compound reduced fecal shedding of E. coli O157:H7 in experimentally infected calves. **Impact:** This novel antimicrobial is a potential intervention against E. coli and Salmonella. Plans are for an invention disclosure to be submitted following additional experiments in calves with E. coli O157:H7.
- Demonstrated that essential oils and related compounds can control pathogens on cattle feedlot pen surfaces. Field studies showed that E. coli O157:H7 prevalence on the pen surface could be reduced by 99.9% with linalool containing thymol. **Impact:** This study shows that pen feedlot surfaces can be treated to reduce the pathogen load in the cattle production environment, thereby removing pathogens for contamination of additional cattle, cattle hides, and runoff.
- The pharmaceutical product ractopamine, recently approved for use in feedlot cattle and finishing swine, was found to play a role in the population and shedding dynamics of E. coli O157:H7 and to a lesser extent Salmonella, in cattle. Studies on fecal shedding of Salmonella and E. coli O157:H7 in naturally infected cattle administered ractopamine showed that the product decreased gut colonization and fecal shedding of E. coli O157:H7 (but not Salmonella). **Impact:** This research demonstrates a potential new intervention for E. coli O157:H7 in cattle and swine.
- Identified nucleolin and Beta1 integrin as possible eucaryotic receptors for O157:H7 in the intestines of cattle and pigs. Intimin, an important bacterial adhesion, mediates adherence of O157:H7 to intestinal tissues. Intimin-producing O157:H7, produce and attach to a receptor for intimin, called Tir, which is inserted in the host membrane (Tir). In collaboration with scientists at The University of the Uniformed Health Services, Bethesda, MD, ARS showed that tissues from experimentally inoculated animals carry on their cell surface two proteins (nucleolin and Beta1 integrin) that co-localize with adherent bacteria. **Impact:** The identification of these host proteins as potential receptors for O157:H7 increases our understanding of how O157:H7 adheres in cattle and provides a basis for investigating nucleolin and Beta1 integrins as potential targets for interventions.
- Epidemiologic studies using audits of swine management practices demonstrated that a significant risk of pig infection with Toxoplasma correlated with the presence of cats on farms and transport of oocysts into confinement barns on contaminated footwear. A series of good production practices (GPP) were established and tested by producers encompassing rodent control, boot hygiene, biosecurity, and feed security. Follow-up

audits showed that participating farms became *Toxoplasma* negative over the course of 2 finishing cycles following the producer education effort, demonstrating that adhering to GPP is a practical method for eliminating *T. gondii* infection in confinement raised swine. **Impact:** The developed technology consisting of a pre-harvest food safety plan was delivered to the cooperator (Farmland Foods), and the information was made available for use by the entire swine industry through the National Pork Board. Interventions have been put into place on farms nationwide and are monitored by participating packers.

- Developed a pre-harvest certification program for food safety in livestock. Working with the National Pork Board, APHIS, and FSIS, a national program was developed to certify the safety of pigs with respect to Trichinae when they leave the farm for slaughter. A series of good production practices were established for farmers. Periodic serological testing of certified pigs is conducted using second generation diagnostic tests developed and commercialized through ARS. Educational materials detailing best management practices have been produced and widely distributed to farmers, accredited veterinarians, and industry. **Impact:** This work represents the official launch of the first animal production food safety program in the U.S. and meets or exceeds new EU standards for imported pork labeled Trichinae-free. The Final Rule for the Certification Program was published in the Federal Register on October 10, 2008.
- Detected virus-like particles in *Cryptosporidium* that may affect the severity of disease. Cryptosporidiosis is a severe gastrointestinal disease with no treatment for infected food animals and only one drug (with limited and often ineffective application) for treatment of humans. Differences were detected in the concentration of virus-like particles within the (oocyst) environmental stage of two strains (genotypes) of *Cryptosporidium* infecting calves. One strain, containing a higher concentration of virus-like particles, resulted in more severe diarrhea than the strain with a lower concentration of virus-like particles. **Impact:** These differences suggest that the virus-like particles play a role in the infectivity and pathology of these isolates. This information provides a possible means of treatment of cryptosporidiosis by targeting drugs or biologicals against viral proteins.
- *Giardia*, one of the most prevalent causes of human diarrheal disease worldwide, was found to have a family of unique proteins that are essential for locomotion and attachment to host cells. Antibodies produced against one of these proteins, designated as delta-giardin, blocked the attachment of the parasite to cultured host cells. **Impact:** Studies are underway to determine if this protein can stimulate immunity that provides protection against infection. This protein may be a focus for future interventions.
- Discovered that a yeast extract supplement stimulates gastrointestinal tract development, protects poults from cold stress, modulates transport stress, and may provide an alternative to antibiotics for both the prevention of disease and reduction of pathogen contamination of poultry. Yeast extract supplementation improved intestinal morphological characteristics of poults in two studies and increased the body weights and the feed/gain ratio of cold-stressed, *E. coli* challenged turkey poults that were the progeny of young hens in their 2nd wk of lay. Turkeys that were the progeny of older hens were

not affected by cold stress and challenge, and did not benefit from treatment. **Impact:** This research provides an explanation for the inconsistent effects of antibiotic alternatives in poultry production and suggests that the ability of yeast extracts to improve gut development may make them effective as antibiotic alternatives in stressed birds.

- Hop (*Humulus lupulus*) bitter acids are known to possess potent antimicrobial activity. Lupulone extracted from hops plants was evaluated for in vivo antimicrobial activity to inhibit *Clostridium perfringens* in a chick gastrointestinal colonization model. Lupulone administered through water inhibited gastrointestinal levels of inoculated pathogenic clostridia within the chicken gastrointestinal tract. **Impact:** Lupulone could potentially be utilized as a valid alternative to antibiotics in animal feeds.

1.1.5: Antibiotic Resistance

The emergence of antimicrobial resistance (AR) among food-borne and commensal bacteria associated with food animal production has become an important global issue. In 2006, Codex Alimentarius created an Ad Hoc Intergovernmental Task Force on Antimicrobial Resistance, and a mandate to create recommendations for Codex about antimicrobial resistance and food safety. Research on the development, prevalence, spread, and persistence of AR in foodborne and commensal bacteria is unfinished. Although the contribution of each is unclear, it is recognized that the use of antimicrobials in animals, plants, and humans, creates the development of antimicrobial resistance. Antimicrobial resistance and its impact on animal and human health continue to be studied.

Antimicrobial resistance is an integral component of food safety, since resistance genes or resistant pathogens may be transferred from food animals through food to humans. There is little focused research on the extent of this phenomenon. To aid in making policy decisions, regulatory agencies are using risk assessments in an attempt to estimate human risk. More data are needed to fill essential gaps in our knowledge.

Research is on-going and involves understanding the development, persistence and transference of resistant organisms and resistance genes in the environment, animals, and humans. There is a need to incorporate more evolutionary genetics in the study of resistance in order to infer the source, the rate of spread, and the future distribution of resistance. Understanding the spread of resistance either through clonal dissemination or horizontal transfer will provide necessary data and clues for prevention, control, and interventions. Surveillance will provide prevalence data and potential trends, and more importantly, provide hypotheses that can be explored through designed studies.

The European Union has banned all sub-therapeutic uses of antimicrobials and requires prescriptive use in animals. Although this has not occurred in the US, there are consumer, regulatory, and public health pressures to implement this requirement. Research on alternatives to antibiotics or on the proper use of antimicrobials is critical.

- *Salmonella* species resistant to multiple antibiotics frequently harbor plasmids or other mobile genetic elements that carry the resistance genes that can be spread to non-resistant bacteria. The DNA sequence of an 84.5-kb MR plasmid, pU302L, from *S. Typhimurium* U302, revealed 12 antibiotic resistance genes and 6 genes involved in mercury resistance, as well as regions suggestive of multiple gene rearrangement events occurring during the evolution of the plasmid. Moreover, the DNA sequence of 4 small plasmids conferring antibiotic resistance in *Salmonella* strains were compared and were shown to be closely related to plasmids from *E. coli* and other pathogens that cause gastrointestinal illness. **Impact:** This sequence information was used to design probes for a microarray chip for typing and characterizing multi-resistance genes/plasmids. The study underscores the importance of having a detection method for the resistance determinants and the value of continued research in this area.

- IncA/C was the predominant plasmid replicon detected in 205 Salmonella serovars isolated from beef and dairy cattle in the US. This replicon was not horizontally transmissible unless aided by a second co-resident conjugative plasmid which was absent in most of the serovars. **Impact:** This study suggests that dissemination of resistant clones, rather than horizontal transfer of resistance genes, may be the mechanism of how antimicrobial resistant Salmonella spreads through dairy populations. Therefore, management practices that control dissemination of this pathogen in manure or the environment should be considered.
- Salmonella spp. have a 44 gene integron structure, designated Salmonella Genomic Island 1 (SGI1), that are multi-drug resistant. Research showed that SGI1 also promotes a hypervirulent phenotype of Salmonella residing in protozoa of the bovine rumen. Furthermore, studies showed that Salmonella can acquire antibiotic resistance from co-existing bacteria in rumen protozoa. Elevated pathogenicity and acquisition of antibiotic resistance by multi-resistant Salmonella, such as DT104, are significant since DT104 is associated with increased cattle mortality and human morbidity. **Impact:** This research provides new knowledge about the potential interaction between antimicrobial resistance and virulence in Salmonella in cattle. These findings also begin to elucidate the role of protozoa in the increased pathogenicity of Salmonella and their potential role in antimicrobial resistance transference. Interventions focused on eliminating rumen protozoa may have an effect on Salmonella prevalence, virulence, and antimicrobial resistance.
- Research has revealed that bacterial stress in Salmonella induces mutations resulting in resistance to 5 different antibiotics. Studies were performed to evaluate the genetic expression patterns of a multiple antibiotic resistant isolate of Salmonella enterica derived during antibiotic exposure from a wild type strain. Many important genetic systems were modified in the mutant isolate compared to its parent strain. These included porins, lipopolysaccharides, efflux pumps, and global regulatory mechanisms. **Impact:** Elucidation of these genetic mechanisms that may contribute to antibiotic resistance will allow researchers to design and test better treatments, as well as determine alternative drugs or adjuvants, such as the efflux pump inhibitory chemicals. Further research is needed on the role of efflux pumps and antimicrobial resistance. This information will be useful to industry and in international trade issues.
- Determined that subpopulations of S. Enteritidis, which vary in the ability to contaminate eggs, have different antibiotic resistance profiles. Resistance patterns of S. Enteritidis emerged from small scale genetic polymorphisms that were unrelated to the antibiotic resistance problems caused by acquisition of cassettes of genes en masse. **Impact:** This genetic study helps elucidate the development of resistance in S. Enteritidis. This may also help in the understanding of the epidemiology of S. Enteritidis, and consequently interventions and control.
- A study was completed in which treatment of poultry with therapeutic and sub-therapeutic levels of tylosin was simulated. The birds were then challenged with either C. jejuni or C. coli and the emergence of resistant isolates was monitored. It was found that

both species generated resistant isolates, but *C. coli* were more likely to become resistant, especially with sub-therapeutic levels of tylosin. It was also found that the most resistant isolates had changes in all three ribosomal genes, a few had changes in two copies of the gene, and none of the resistant isolates had changes in only one copy of the gene.

Impact: This information is important for understanding how antibiotic use may cause resistance development and for developing strategies for using antimicrobials in a manner that does not encourage an increase in resistance. Data on the use of antibiotics sub-therapeutically, therapeutically, or for prevention are essential for understanding the development and persistence of antimicrobial resistance and for the development and implementation of intervention strategies to reduce resistance.

- Identified and characterized two novel plasmids in a strain of *C. coli* from cattle. Sequence analysis revealed that one of the plasmids shared homology with a different species, *C. upsaliensis*. **Impact:** Understanding the genetic diversity and phylogeny of *Campylobacter* is a fundamental component in determining the capacity of this pathogen to acquire and disseminate antimicrobial resistance, and is essential for devising intervention strategies. This work adds to the limited knowledge of plasmids from *Campylobacter*, providing a foundation for future studies on the exchange and transmission of genes, including those conferring antimicrobial resistance.
- Scientists determined the capability of a poultry feed additive, known as flavophospholipol, to inhibit the spread of genetic information from one *E. coli* to another in living poultry. The studies used chicks inoculated with *E. coli*, and established that the additive did not prevent the bacteria from sharing genetic information. **Impact:** Although flavophospholipol demonstrated no useful biochemical actions, the work is important because it provides scientific data on feed additive interactions with poultry microorganisms. Ongoing work may be successful in identifying practical means to inhibit bacterial conjugation in animals and reduce the threat posed by the antibiotic resistance phenomenon.
- Conducted a small focused study of enterococci in retail foods commonly purchased and consumed from grocery stores. Findings showed that overall resistance to antimicrobials was relatively low in enterococci, and that both positive and negative associations exist between antimicrobial resistance genes and virulence in *Enterococcus faecalis* isolated from those food items. Although the highest rates of resistance were observed for lincomycin and bacitracin, lower rates of resistance were found for chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nitrofurantoin, penicillin, and tylosin. A number of virulence determinants were present in the retail food enterococci including aggregation protein, toxins, and sex pheromones which facilitate the conjugation process. **Impact:** The presence of virulence determinants in enterococci from retail food suggests that enterococci from these sources may be more pathogenic than previously believed. These findings need to be compared to larger, more national, retail surveys.
- Determined the prevalence and mechanisms of streptogramin resistance in enterococci from animal and environmental sources. Streptogramin resistance among enterococci from animals was low even with the long history of virginiamycin use. **Impact:** This is

the first report of resistance gene, *vatD*, from enterococci from animals in the U.S. and the first report of resistance genes, *vatB* and *vgaB*, in enterococci. Findings will be used to better understand the emergence and transfer of antimicrobial drug resistance and to assist the FDA in decision-making for approving safe and effective drugs for human and animals.

- Determined resistance in other *Listeria* species in comparison to *L. monocytogenes* isolated from various sources. *L. monocytogenes* isolates displayed resistance to ciprofloxacin, ceftriaxone, oxacillin, and clindamycin. *L. innocua* isolates demonstrated resistance to tetracycline, ceftriaxone, oxacillin, penicillin, and clindamycin. Compared to *L. monocytogenes* and *L. innocua*, *L. welshimeri* displayed resistance to the most antimicrobials tested. *L. welshimeri* isolates were resistant to quinupristin/dalfopristin, ciprofloxacin, rifampin, oxacillin, penicillin, trimethoprim/sulfamethoxazole, clindamycin, and streptomycin. Tetracycline resistance in *L. innocua* was due to the presence of a tetracycline resistance gene, *tetM*. **Impact:** These data demonstrate the variability in resistance among *Listeria* species, and that the human pathogen, *L. monocytogenes*, appears to be the least resistant among the tested species.
- Research determined the antimicrobial resistance of *L. monocytogenes* isolates detected in a poultry further processing facility. This large group of isolates is unique because they had been collected during one year in a commercial plant. Most of the *L. monocytogenes* isolates were susceptible to all antimicrobials. However, some were resistant to ceftriaxone, oxacillin, ciprofloxacin, clindamycin, tetracycline or some combination of these. **Impact:** The data are useful to determine the importance of *L. monocytogenes* in processing plants and tracking the acquisition of drug resistance in such bacteria.
- Demonstrated a collateral effect of a growth performance antibiotic widely used in the US, the quinoxaline antibiotic, carbadox. Sub-inhibitory carbadox concentrations in bacterial cultures induce both prophage-like Gene Transfer Agents carrying antimicrobial resistance genes and traditional prophages. **Impact:** This research provides important data on the use of sub-inhibitory concentrations of carbadox and its role in antimicrobial resistance induction. This study has also generated new knowledge about phage induction and may be useful for future interventions.
- Demonstrated that a common swine intestinal commensal, *Megasphaera elsdenii*, carries hybrid tetracycline resistance genes. This was the first report of tetracycline gene class recombination. Studies showed that established *M. elsdenii* strains from swine that were never exposed to antimicrobials are multiply drug resistant, and that resistance genes can be transferred inter-strain at very high frequency. By genome sequencing, work demonstrated that resistance mobile elements in one *M. elsdenii* strain are identical to those in *C. jejuni*. **Impact:** These findings provide new knowledge implicating swine commensal bacteria not only as reservoirs of antimicrobial resistance for pathogens such as *Campylobacter*, but also as sites for the evolution of antimicrobial resistance. This research provides new directions for research in the role of commensals and antimicrobial resistance.

- Developed a DNA microarray to identify and track 775 resistance and virulence genes in a variety of bacteria simultaneously. Resistance and virulence genes in *Campylobacter*, *Salmonella*, *E. coli*, *Enterococcus*, *Staphylococcus* (including MRSA), *Listeria* and *Clostridium* have been identified using the microarray. Analysis of bacteria collected on-farm with this technique has identified common genes in different species of bacteria co-cultured from the same animal. **Impact:** Demonstrated and validated that this microarray. This technology is used as part of ARS' involvement and responsibility in the multiagency National Antimicrobial Resistance Monitoring System. (This accomplishment can also be applied to Problem Statement 1.2.1 and 1.2.8.)

1.2 Pathogens, Toxins and Chemical Contaminants Post-Harvest

1.2.1 Detection and Validation

Detection and quantitation of pathogens, toxins and chemical contaminants are the central challenge to any food safety [and food security] system. Diagnostic tools must be developed for the entire food chain which allow the highest detection capability, and guarantee a maximum degree of prevention. Where practical detection at the earliest point possible is best, thus avoiding or preventing the need for significant processing interventions or even the recall of food products from purchase endpoints. The term “diagnostic” implies analytical methods, and denotes the method/mechanism to establish epidemiological priorities.

Numerous pathogens: *Escherichia coli* O157:H7 and related Shiga toxin (ST) producers, *Salmonella*, *Campylobacter*, *Listeria*, *Vibrio*, *Norovirus* remain a high priority in post harvest. For example, contamination of meats (ground beef); fresh produce (tomatoes, lettuce and spinach), and seafood continue to be critical issues. The program focused on developing and validating technologies that have regulatory, industry, and research use: a commonality of interests between government and stakeholders. Having reliable, cost-effective quantitative measurements of pathogen/toxicant in all food types can provide data to carry out risk assessment, to develop and validate predictive microbial models, and to identify areas where interventions are most critically needed. This information is critical to assist the implementation of HACCP programs by FSIS, FDA, and their regulated industries.

The accomplishments listed in this Problem Statement assume the need for specific detection technologies, so there is no justification sentence attached.

- Established sampling guidelines to measure the levels and prevalence of *E. coli* O157:H7, aerobic bacteria, and Enterobacteriaceae at various steps in beef processing. Using these sampling procedures, beef processors now have established benchmarks for monitoring and verifying their processes. **Impact:** In 2008, the USDA-FSIS released two directives: (1) “Compliance Guideline for Sampling Beef Trimmings for *Escherichia coli* O157:H7” and (2) “Incident Investigation Team Methodology for *Escherichia coli* (*E. coli*) O157:H7 in Beef Slaughter Establishments” based on this sampling protocol.
- Developed a method to concentrate and recover *L. monocytogenes* from large volumes (> 200 ml) into 0.1 to 1 mL samples in 45 min using hollow fiber membranes that are 0.3 mm in diameter. The fibers are assembled in a disposable device smaller than a soda straw, and can process samples, high in fat and complex molecules, while avoiding plugging of the membrane during the concentration process. **Impact:** The resulting small sample volumes of concentrated cells are compatible with the small volumes needed for electronically based micro-fluidic detection devices. This drastically reduces the time required to obtain detectable cell numbers and therefore time to result. This research was conducted in collaboration with the Center for Food Safety Engineering, Purdue University.

- Expanded the utility of FTA-filters for detection of Salmonella and L. monocytogenes by real-time PCR compatible with high throughput samples. The protocol was simpler, rapid, and eliminated plant phenolics otherwise known to possess strong PCR inhibitory activity. **Impact:** The isolation protocol will directly assist monitoring pathogens on fresh produce. The protocol was also optimized for DNA isolation especially for ‘in-field’ conditions.
- Assays for low levels of pathogens in food currently require 6-24 h enrichment prior to the detection step, slowing response time and preventing quantitation. Using a novel combination of filters, E. coli O157:H7 was isolated from 250 mL of ground beef homogenate and concentrated to a volume of 0.1 ml in 30 minutes with high efficiency. **Impact:** The approach can be applied to a variety of complex sample matrices and pathogens. When coupled with selective microbiological plating media, this approach allowed detection of 0.3 cfu/g of E. coli O157:H7 in ground beef in less than 24 hours. When coupled with an appropriate biosensor, this approach will allow quantitative detection of pathogens without enrichment in less than 4 hours.
- Determined the optimal medium and growth conditions for improving the speed at which pathogen detection can occur in ground beef/trim samples. Further work determined that the volume of media could be reduced by 2/3, thus, creating a significant cost savings for the end user. The cost reduction for these changes has reduced the cost from ~\$10 to ~\$4 per test. **Impact:** This cost reduction saved beef processing companies over \$1M for their test-and-hold programs, demonstrating that beef processors can use a low cost medium and reduce the amount of medium used to enrich for possible contaminating E. coli O157:H7 while maintaining a high level of accuracy in the testing of beef trim and ground beef.
- Developed a colorimetric bacteriophage based assay for the specific detection of E. coli O157:H7 in which the recombinant phage could be propagated in a non-pathogenic E. coli strain. The ability to propagate the phage in a non pathogenic host was a major breakthrough allowing safe large scale production. The phage was genetically modified by insertion of a gene coding for an enzyme which, when expressed in E. coli O157:H7, causes the host cell to produce a visible red pigment. A scalable protocol for the production of the recombinant phage was developed using a nonpathogenic strain of E. coli with subsequent purification using liquid chromatography. **Impact:** Three provisional patent applications were filed, and the technology platform was licensed by “Intelliphage” a small company based in West Lafayette. This research was conducted in collaboration with the Center for Food Safety Engineering, Purdue University.
- A novel cell-based assay was developed to detect Shiga Toxins (Stxs) and inhibitors of Stx activity. A Vero cell line harboring a destabilized variant of the enhanced green fluorescent protein (d2EGFP) was used to monitor the toxin-induced inhibition of protein synthesis with a sensitivity of picogram/ml for toxin detection. A panel of plant compounds was screened for anti-toxin activities, and grape seed or grape pomace extract was identified as having significant anti-toxin activity. **Impact:** The Vero-d2EGFP

fluorescence assay is an accurate and sensitive method to detect Stx activity and inhibitors of activity.

- Developed methods for isolating and identifying Shiga toxin-producing *E. coli* (STEC) and for rapidly differentiating strains by both immunochemical and sequence-based approaches. DNA oligonucleotide microarrays were produced. Multiplex PCR products from environmental sample enrichment cultures were hybridized to the array for rapid detection and identification of pathogenic *E. coli* and *Salmonella* from diverse agricultural sources (water, feces, soil, and plants). **Impact:** The data from the environmental studies will have a major impact on identifying sources of field contamination of produce.
- Studies developed methods, including PCR- and microarray-based assays for detection, identification, and differentiation of different pathogenic *E. coli* strains, including Shiga toxin-producing *E. coli* (STEC) based on unique genes/regions carried by the organisms and genes related to virulence. STEC strains isolated from swine were isolated and serotyped, and virulence factors and acid tolerance determined. Furthermore, a 168-kb virulence plasmid of an important non-O157 STEC strain was sequenced and genes annotated. **Impact:** This research provides insights into the virulence potential and evolution of pathogenic *E. coli*, gene targets for pathogen identification, and methods that can be used in epidemiological studies, and for tracking, identification, and detection of emerging pathogenic *E. coli*. (Also applies to Program Statements 1.1.1 and 1.2.8).
- Developed and validated two rapid methods for the enumeration of *Salmonella* and *E. coli* O157:H7 from ground beef, carcass, hide, and fecal samples. Filtration through a hydrophobic grid membrane has been optimized for use with ground beef and carcass samples; spiral plating is used for higher expected background microflora such as analysis of hide and fecal samples. Both techniques utilize selective indicator media to identify the target organism. **Impact:** The methods can be performed in a fraction of the time and cost of other methods. Further, they allow for more routine quantification data collection, thus providing additional information about the effectiveness of beef processing intervention strategies. The technology has been transferred to industry stakeholders such as the National Cattleman's Beef Association.
- Developed and validated two plating media for detection and enumeration of *Campylobacter* in foods (Campy-Cefex and Campy-Line agar, (CLA). Differentiation is made easier since the normally translucent *Campylobacter* colonies appear dark red against the translucent background of the new chromogenic agar. Enumeration is made less labor intensive because fewer non-*Campylobacter* contaminants are able to grow on the selective media. **Impact:** The patented media and enumeration methods have been evaluated and validated and are used by all FSIS laboratories and numerous commercial, academic and other Government laboratories nationally and internationally.
- Processes including immunomagnetic capture and subsequent detection of a pathogen and their toxins by various available biosensor methods were developed and tested in food samples. Applied detection methods included: 1) immunofluorescent latex beads; 2) a fiber optic biosensor; 3) piezoelectric-excited millimeter-sized cantilevers; and 4) time-

resolved immunofluorescence detection of *E. coli* O157:H7 and associated toxins (SLTs). **Impact:** All the developed technologies were able to detect as little as 1 *E. coli* cell per gram of ground beef after brief culture enrichment and could be completed within the standard 8-hour work shift.

- Responding to an FDA request, ARS developed a rapid and sensitive assay for the detection of *S. Enteritidis* (SE) involving the specific capture of SE by IMBs followed by time-resolved fluorescence detection. **Impact:** Demonstrated and validated that the capture of SE in whole egg, egg white, and egg yolk varied depending upon the type of IMBs used. The technology was provided to the FDA for adoption.
- The most probable (MP) non-targeted organisms from foods, before and after IMB capture, were identified and characterized. The MP organisms adhering to IMBs (e.g., *Brochothrix* and *Carnobacterium*) grow more slowly than *Salmonella* or *E. coli* but grow relatively fast at refrigeration temperatures (5 hr doubling times). The capture efficiency for these background organisms (15±8%, at 100L) necessitates a relatively high target pathogen concentration (10L) for detection. We further demonstrated organisms naturally present in food bind differently depending upon their source matrix. **Impact:** The goal to identify a universal blocking reagent for use with IMBs is not likely to produce a practical outcome. This information is important for applying IMB methods to capture targeted pathogens in actual food samples.
- Molecular methods for antibody production were used to develop immunoreagents for the detection of foodborne pathogens. An *L. monocytogenes*-specific antibody fragment (scFv) was selected from a phage display library. Genetic tools were developed to allow the synthesis of biotinylated scFvs which were coupled to streptavidin-coated magnetic beads. The anti-*L. monocytogenes* scFv-IMBs exhibited higher efficiencies and improved specificity for capture of *L. monocytogenes* relative to commercially available anti-*Listeria* IMBs. The IMB capture was combined with microbiological and PCR methods. A surface plasmon resonance biosensor was developed for specific detection of *L. monocytogenes*. **Impact:** By request, the scFv clone was provided to Kirkegaard & Perry Laboratories and The Biosensor and Nanotechnology Applications Laboratory at the University Idaho Research Park for evaluation and biosensor development through a Specific Cooperative Agreement.
- A real-time multiplex PCR assay was developed to simultaneously detect and differentiate the *Campylobacter* species most often associated with foodborne illness, *C. jejuni*, *C. coli*, and *C. lari*. In extensive testing, the assay was found to be both specific and sensitive. The assay was also evaluated using artificially and naturally contaminated chicken samples. **Impact:** The results demonstrated the feasibility of the developed PCR assay for rapid detection and differentiation of *C. jejuni*, *C. coli*, and *C. lari* in food samples. This developed PCR assay will be useful to regulatory agencies, particularly FSIS, for doing baseline studies necessary for public health-based risk assessment and establishing regulatory guidelines.

- Antibody microarrays were developed for foodborne pathogen detection. Customized software was created to print arrays of antibodies to pathogenic bacteria and toxin analogs into 96-well microtiter plates to allow the simultaneous analysis of up to 96 food samples at a time. The efficacy of the new method was demonstrated by multiplexed detection of *E. coli* O157:H7, *Salmonella*, and a proteinaceous toxin analog. Pathogen or protein concentrations as low as 10^6 cells/mL or 5 ng/mL, respectively, were detected in growth media containing ground pork samples in an assay time of less than 2.5 h. Current efforts are aimed towards developing and improving the limit of detection, and expanding the number of pathogens detected by the array. Parallel studies on the development of appropriate media for multi-pathogen enrichment are being performed for the purpose of ensuring the target pathogens reach detectable concentrations. **Impact:** This method may be applied by regulatory agencies and food producers for high-throughput and multiplex screening of foods. Further development of this technique, in combination with DNA-based microarrays, will provide a powerful and flexible tool for confirming the presence of pathogens in foods.
- A nucleic acid-based microarray was developed for the simultaneous detection of *E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes*, and *C. jejuni*. Combined with multiplex PCR amplification, the microarray unambiguously distinguished all of the pathogens with a detection sensitivity of 1×10^{-4} ng (approximately 20 copies) of each genomic DNA. Each microarray chip was fabricated to screen up to 12 samples. Future efforts will focus on evaluating the efficacy of the microarray assay for the detection of multiple pathogens from contaminated foods. **Impact:** This method greatly expands the capability to simultaneously detect multiple foodborne pathogens and the array can be easily expanded to include additional pathogens. Moreover, the microarray analysis also provided important genotypic information related to pathogen virulence. (See also Problem Statement 1.2.8)
- Successfully transferred an ARS developed extraction method, known as the GPTT procedure, for detecting hepatitis A virus, norovirus, and other enteric viruses in shellfish tissues. The virus extraction CD was used by the FDA and CDC in training courses they offered to State Health Department personnel. This CD was instrumental in training Canadian Government scientists to identify noroviruses in oysters implicated in outbreaks in British Columbia. The Canadian Government initiated a 10-laboratory validation of the extraction technique, and adopted it as the country's standard method. In 2006, the method was published in the Compendium of Analytical Methods (http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume5/index_e.html). The research was given the National ARS Technology Transfer Award for 2006.
- Developed novel enzyme-based assay for fecal coliform whose presence are frequently used as indicators of seafood safety. The Colony Overlay Procedure for Peptidases (COPP assay) is an enzyme-based test ARS previously developed to detect *Vibrionaceae* bacteria in shellfish and seawater. **Impact:** The COPP assay was modified to detect both *Vibrionaceae* and fecal coliforms, either individually or simultaneously in shellfish and marine waters. To date, the CD provided to customers has been instrumental in

establishing the use of the COPP assay in regulatory, industry, and academic research labs, particularly on the West Coast and in Hawaii

- Discovered specific ligands in the gastrointestinal tract of bivalves that are responsible for the bio-accumulation of both genotype I and II human Noroviruses. The binding of Norovirus in bivalves can be reversed by depuration in the presence of HBGA analogues. **Impact:** Demonstrates the presence of an important environmental reservoir of the viral pathogen and provides important knowledge about the epidemiology of this common illness.
- Developed and applied real-time molecular techniques to quantify noroviruses in oysters. Real-time molecular methods were developed and evaluated to quantify noroviruses in oysters. The methods involved development of oyster processing strategies to extract the viruses from shellfish tissues and to analyze the extracts by real-time testing method. In addition, potential assay inhibitors were identified and methods were developed to remove the inhibitors from shellfish extracts. **Impact:** Development of improved methods to monitor virus levels in naturally-contaminated oysters and to determine the effectiveness of processing interventions on the elimination of viruses in shellfish.
- Norovirus is the most common causal agent of foodborne illness linked to produce, but is very difficult to detect. Type A, type H1, and Lewis human histo-blood group antigens have been identified as major receptors for norovirus (NOR) binding. ARS determined that that pig gastric mucin (PGM) contains antigens that bind to a broader range of Norovirus strains than do specific antibodies. NOR in spiked food samples (oyster extract, strawberry, raspberry, and lettuce) could be captured by PGM-conjugated beads, which minimized the presence of reverse transcriptase-polymerase chain reaction (RT-PCR) inhibitors of food origin and increased sensitivity. PGM-conjugated magnetic beads were used to detect NOR from a fecal sample at a 100-fold lower concentration than with the standard RNA extraction procedure. **Impact:** The high sensitivity of this improved detection method will be of great value to public health agencies for surveillance and in outbreak investigations.
- Developed micro-fluidic biochips for the rapid electrical detection of bacterial growth in an effort to replace the Petri-dish technology. The use of this approach reduced the threshold of detection and allows us to detect bacterial growth within 1-2 doubling cycles. The use of electrical methods facilitates automated measurements. **Impact:** The accomplishment is significant since it reduces the time of detection of bacterial growth to a few hours or less. In combination with the sample concentration approaches developed, the integrated bio-chip based system has enabled the rapid electrical detection of growth of bacteria from a liter of fluid solution, in a few hours or less. The technology has been patented and licensed to a startup company BioVitesse, Inc., aimed at commercializing the technology for pharmaceutical manufacturing, drinking water, and food safety applications. This research was conducted in collaboration with the Center for Food safety Engineering, Purdue University.

- Developed nanotechnology for Salmonella detection using a gold/silicon nanorod-based biosensor. A silicon nanorod array was fabricated by the glancing-angle deposition method and a thin layer of gold was sputtered onto the tips of the silicon nanorods. Fluorescent organic dye molecules and Salmonella antibodies were selectively immobilized onto the side walls of silicon nanorods and the gold-plated tip, respectively. Strongly enhanced fluorescence signals were obtained due to the high aspect-ratio nature of the silicon nanorods. **Impact:** These biologically functionalized hetero-nanorods have been successfully used for Salmonella detection. Further, the patent pending nanotechnology received the 2008 International Innovation Nano Research Award. This provided additional impetus for requesting new nanotechnology research funds in the USDA budget.
- Developed a citrate-reduced colloidal silver Surface Enhanced Raman Spectroscopy (SERS) method for differentiating E. coli, Listeria, and Salmonella. FT-Raman and SERS spectra of both silver colloids and colloid-K₃PO₄ mixtures were analyzed to evaluate the reproducibility and stability of silver colloids fabrication. The colloids demonstrated high reproducibility and their binding effectiveness remained consistent over a 60-day storage period. Two specific SERS bands at 712 and 390 cm⁻¹ were identified and used to develop two-band ratios for differentiating E. coli, Listeria, and Salmonella-colloid mixtures with over 99% success. **Impact:** Although not implemented, the colloidal silver SERS technique may in the future be a practical alternative method suitable for routine and rapid screening.
- Fourier Transform Infrared (FTIR) techniques, spectral libraries, and sensors were developed for the identification and detection of E. coli O157:H7 in ground beef and Salmonella enterica in chicken. These techniques enable detection of target live pathogens from food samples within 8 hours followed by FTIR analysis. Detection limits were established at 10⁶ CFU/ml and 10⁴ CFU/ml, respectively, for the filtration and Dynabeads® techniques. Additionally, live cells were accurately detected at 1% concentration of the target microbe. **Impact:** This rapid protocol shows potential for the detection of different species of bacteria in complex food systems.
- Mass spectrometry-based bioinformatics software has been developed for rapid identification of protein biomarkers of foodborne pathogens, viruses and toxins by "top-down" proteomics analysis. Sequence-specific ions fragmented from intact proteins are rapidly compared against a database of in silico fragment ions from thousands of bacterial, viral and toxin proteins. Protein/microorganism identifications (IDs) require less than a minute of computation time. **Impact:** As yet, the technology is not ready for commercial application, in part due to cost. However, an invention disclosure has been filed for the software, and a patent application is currently being evaluated through the Office of Technology Transfer. This potential for this technology is very high especially for rapidly determining the pathogenicity of an isolate.

Chemical Residues: The regulation and control of veterinary drugs, residues, heavy metals, and persistent organic pollutants are an integral component of any food safety program. To protect human health and the environment, regulations are made and limits set on contaminants in edible agricultural products. Compliance and enforcement of these regulations is a role of the Program's stakeholders. The development and validation of practical detection methods is a responsibility of ARS Food Safety Program.

- Detection of melamine contaminated imported food products is a critical issue. Existing analytical procedures for detection of melamine and its derivatives rely on GC-MS and LCMS/MS methods, which are not conducive to rapid screening of food and feed products. ARS developed a rapid, nondestructive detection/identification method for melamine and its derivatives based on Raman spectroscopic techniques. **Impact:** This work will have a direct impact on any regulatory agencies and industries ability to detect melamine and related contaminants in foods. A US patent was filed. A Cooperative Research and Development Agreement (CRADA) was formed with an industry partner (Chemimage) resulting in the development of two prototype hand-held devices currently undergoing testing and validation in commercial settings. Portable system technologies have the potential to be expanded to include other biological and chemical contaminants.
- Further investigated and adapted the “quick, easy, cheap, effective, rugged, and safe” (QuEChERS) approach developed by ARS for the extraction of hundreds of pesticides and certain other types of chemical residues from many types of food. The approach typically provides 4-fold reductions in sample preparation time, labor, waste, and material expenses over previous methods. **Impact:** Major analytical supply companies such as Restek, Supelco, United Chemical Technologies, and Waters sell QuEChERS sample preparation products. Regulatory laboratories in the USDA, FDA, EPA, States in the US, and countries on 6 continents routinely use official versions or adaptations of the QuEChERS approach.
- Developed and demonstrated the feasibility of an efficient multiclass, multiresidue monitoring approach using liquid chromatography – tandem mass spectrometry (LC-MS/MS) for >100 veterinary drug residues in animal tissues. The sample preparation method, which can be used for kidney, liver, muscle, or serum, is based on the QuEChERS concepts used in pesticide residue analysis. For detection, LC-MS/MS screens and identifies targeted antibiotic and other drug residues in the sample extracts at regulatory tolerance levels. **Impact:** The USDA-FSIS has invested significant funds both internally and to ARS for implementing this effective and efficient modern approach to replace the disadvantageous 7-plate bioassay in their laboratory.
- Due to a problem with their supplier, the USDA-FSIS could no longer use the fast antibiotic screening test (FAST) in slaughterhouses, and a replacement was needed quickly. ARS evaluated and compared different screening tests for antibiotics and other drug residues in beef kidney juice and serum. This work compared the capabilities of alternative tests (Premi[®] and KIS[™]) vs. the FAST in numerous fortified samples and tissues/serum collected from 235 culled dairy cows at a slaughterhouse. The monitoring

results and detectability levels for each antibiotic residue in each matrix using each test was established and published. **Impact:** The results assisted FSIS and similar agencies worldwide choose a screening test. Further they assist regulatory agencies identify which drugs of concern are being used in the field.

- Tolerances for veterinary drug residues have often been set for muscle tissue; however, no particular muscle type is specified. In this research, ARS found that enrofloxacin residues were distributed unequally among different types of chicken muscle (breast level > thigh), suggesting that selection of a specific muscle type for monitoring purposes may be advantageous. **Impact:** ARS found that monitoring of serum rather than tissue can provide satisfactory results with less expense of time and effort, for example with fluoroquinolones in chicken. Regulatory agencies, in particular FSIS, have been informed which may lead to improvements in current residue monitoring policies and practices.
- Developed and validated a new multi-residue analytical method for anthelmintic veterinary drug residues using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method simultaneously quantifies and identifies 38 of the most widely used anthelmintic veterinary drugs (including benzimidazoles, macrocyclic lactones, and flukicides) in bovine milk and liver. A simple modification of QuEChERS was used for sample preparation. **Impact:** The new method achieves sufficiently low detection limits to meet regulatory needs for all targeted drug residues. The method was successfully validated for implementation in regulatory monitoring labs in Europe, USA, and other countries.
- Developed two portable fluorometers based on lanthanide-sensitized luminescence using light emitting diode (LED) sources used for residue analysis of tetracycline and fluoroquinolone antibiotics, which account for >50% of antibiotic usage in agriculture worldwide. Most traditional methods are slow, expensive, and limited to laboratory applications. Due to unique advantages of LEDs, these instruments achieved field deployability, improved sample throughput, and superior sensitivity and selectivity vs. commercial spectrophotometers. **Impact:** As a result, sample preparation can be simplified and chromatography eliminated. Sensitive and simple methods that take less than 30 minutes to perform were developed for drug residue determinations in shrimp, honey, salmon, fruits, and river water. The methods typically achieve high and consistent recovery factors with < 10 parts per billion detection limits. Product recalls for detected residues can be prevented if the approach is validated and implemented in the field. Several companies and institutes have expressed interest in instrument commercialization.
- Developed a novel method to detect and quantify phenolic and polyphenolic compounds in foods. The method consists of mixing different dyes and salts with the samples containing phenolic compounds followed by addition of a buffer. The reaction is completed at room temperature in < 90 minutes, and the resulting change in color is measured spectrophotometrically. **Impact:** This new method can replace the standard Folin-Ciocalteu method for measuring “total phenolics,” which measures the chemical

reducing ability of foods. Such results are beneficial to regulatory agencies, producers, and consumers interested in knowing relative anti-oxidant properties of foods.

- Due to the toxicity and persistence of certain polybrominated diphenyl ethers (PBDEs), many countries and states have banned production and use of several PBDE formulations. A magnetic particle enzyme immunoassay for PBDEs was developed and validated which is more user-friendly, inexpensive, and portable than current instrumental methods. **Impact:** There is great interest in monitoring PBDE residues in the environment and in food samples. The immunoassay kit is commercially available, used world-wide, and has been used to determine PBDE concentrations in crab, fish, milk, dust, soil, and water samples.
- Triclosan is an antimicrobial agent that has broad usage in household and agricultural products. This widespread use make it one of the most commonly detected chemicals in fish, humans, sediment, soil, and water and has raised concerns about its environmental impact, as well as, food safety problems caused by its presence in foods. A new rapid test for triclosan based on a magnetic particle enzyme immunoassay was developed. **Impact:** The triclosan immunoassay kit is commercially available, and its development has been internationally reported. The immunoassay has been used to monitor triclosan in a large number of water samples from different regions, predominately in the U.S. and Spain, and proved to be a rapid, sensitive, precise, and cost-effective monitoring tool for the chemical residue.
- Detection of illicit residues of β -agonists in livestock, competition animals, and humans is of interest to industrial and regulatory scientists worldwide. Poly- and monoclonal antibody based immunoassays were developed and validated for the β -agonists ractopamine and zilpaterol. **Impact:** These antibodies have been licensed by several companies, and commercial products incorporating the antibodies are available worldwide. A cell line was deposited in American Type Culture Collection and Confidentiality and(or) Material Transfer Agreements for the antibodies have been filed with 20 research groups in the US, Europe, and Asia (through 2008). Use of these technologies will deter illegal use of β -adrenergic agonists countries where β -agonists are banned for use in food animals.
- The microbiological quality of intact processed chicken piece types was determined by analysis of their associated volatile compounds. Studies employed digital aroma technology to analyze the effects of microbial populations on stored poultry pieces and to detect and classify the resident bacterial species. **Impact:** Methods were developed to detect differences in the number of days that poultry meat had been stored. Further, to establish profiles for bacterial isolate identification, antibiotic susceptibility, and to determine substrates and metabolites used by bacteria that exist on poultry samples. The methodology is now in use by the Canadian Food Inspection Agency for quality measurements for catfish.

1.2.2 On-line Sensing Systems

With more focus on HACCP and HIMP (HACCP-based Inspections Models Project) both the FSIS and FDA have placed more of the burden of inspection responsibility on the producers and processors. Plants are also responsible for meeting other consumer protection (OCP) issues as determined by regulatory agencies. In essence, producers/ processors assume the responsibility for inspection, and the regulatory agencies perform oversight and verification to ensure standards are met. Under HACCP, HIMP or GMP, consumers demand safe, high quality food; however, consumer demand for more food increases the need for, and pressure on inspectors. Balancing consumer needs with the capabilities of the inspection agencies and the producers/processors will not be easy. On-line, computerized sensing-systems placed or used strategically in food processing plants will assist and improve the regulatory and in-house inspection system which will minimize the problems of human error and variability, and increase commercial processing productivity and profitability.

- An optical light scattering sensor was built to [within a few seconds] detect and identify bacterial pathogens growing on a Petri-dish (lid on). This sensor allows detection and identification of multiple bacterial colonies without using any labeling reagents such as antibody, nucleic acid, dye, or fluorophor in seconds in a cost-effective manner. This technology uses laser to generate a scatter image fingerprint of colony of individual bacterial strain/species. The image is then compared against a standard from the library for identification. Reagent requirement for the light scatter meter assay is minimal, and the assay can be easily incorporated in any microbiological or analytical laboratory for rapid detection of pathogens. **Impact:** This patent pending technology is state-of-the art and the future of food safety. Currently the technology can virtually automatically detect and differentiate serotypes of E. coli, Listeria, Vibrio, and Salmonella. The technology does not destroy the sample, thus allowing for further testing and use in trace-back and attribution. This research was conducted by the Center for Food Safety Engineering, Purdue University, as part of their research collaborations with ARS.
- To enhance food safety inspection of poultry carcasses, an automated, real-time, imaging system was developed for wholesomeness inspection of chicken carcasses. Working in collaboration with an industry partner, the line-scan multispectral imaging inspection system was extensively tested online in commercial poultry processing plants, at maximum line speed, and achieved > 99% accuracy in its identification of wholesome vs. systemically diseased birds. **Impact:** A utility patent entitled “Method and System for Wholesomeness inspection of Freshly Slaughtered Chickens on a Processing Line” has been filed. The FSIS Risk Management Division approved the ARS technology for commercial implementation to pre-sort chicken carcasses online. The industry partner (Stork Gamko Inc) intends to implement the technology both nationally and internationally.
- To enhance food safety inspection of poultry carcasses, an automated, real-time multispectral imaging system was developed to detect fecal contamination on broiler carcasses. In commercial plant testing, birds were imaged after evisceration, but prior to

carcass wash at a rate of 150 birds per minute. The accuracy of testing was 99% compared with 97% by human inspection. Differentiating false positives from contaminants was challenging for the imaging application. Therefore, in addition to image processing in the spatial domain, further processing in the frequency domain helped to identify false positives through a developed textural analysis method. This method identified “true” contamination regardless of fecal sources (duodenum, ceca, and colon) and diets (corn, wheat, milo with soybean mixture) for maximizing detecting accuracy and minimizing errors. **Impact:** Several patents were given, and the technology transferred to an industry partner (Stork Gamko Inc) for implementation with the wholesomeness inspection. The two independently developed systems were combined by ARS into a single unified platform. This combination will aid commercialization by creating one imaging system that solves two separate and significant problems in the poultry industry.

- Optimization of key components, such as the optoelectronic imaging device and imaging spectrograph, is critical to successful commercialization of the online inspection system. ARS developed a prototype online multi-task inspection system for apples based on rapid line-scan multispectral imaging methods. In collaboration with an industry partner, several fast optoelectronic imaging devices were tested to optimize line-scan image resolution for rapidly moving samples. In addition, a new CRADA with an industry partner (Headwall Photonic) was initiated to facilitate the optimization of imaging spectrographs for use in harsh processing environments. **Impact:** The strategic partnerships with these industry leaders for key components of the technology were formed to enhance potential commercial implementations of the ARS multi-task inspection system for the fruit- and vegetable-processing industries. A utility patent was filed in 2008 for the methods developed.
- A visible/near-infrared three-band handheld imaging system for fecal contaminant detection was designed and constructed with improved optical design and a system calibration algorithm. This compact prototype, which is composed of beam splitter, lenses, and high resolution cameras, has easy access to the filters which allows the system to be retrofitted for a variety of applications. This is a great advantage because of the flexibility in selecting spectral wavelengths, especially when compared to other commercial multispectral imaging systems that integrate filters and sensors as a fixed module. **Impact:** This patent pending handheld multispectral imaging system can be used to detect and/or recheck fecal contamination on all poultry and other surfaces, and is ideal for small-to-medium food processing plants. A CRADA with Andor Inc has been formed to further develop the system.
- Bones in boneless poultry filets are still one of the largest food safety issues affecting the poultry industry. ARS at the request of the USDA-Agricultural Marketing Service (AMS-school lunch program) developed a method to detect clavical bones in breast filets with an imaging system and structured back lighting. The technique requires images to be taken of both sides of the filets and for the filet to be compressed to a uniform thickness, which can be accomplished with a forming machine. **Impact:** The technology

demonstrates a bone-detection alternative to using x-rays in the poultry industry. The technology was provided to the AMS; however, implementation is industry dependent.

- ARS developed an imaging system at the request of the USDA's Agricultural Marketing Service (AMS) to help egg graders identify detect tiny hairline cracks that are practically invisible to the eye. The system uses a unique vacuum chamber to open the micro cracks for the high speed digital camera to image without causing cracks in intact eggs. Special software compares the images under normal and increased pressure. In comparative tests with AMS graders, the prototype had a 99.6% accuracy compared with 85% for human identification. The system also collects and records additional data that AMS needs for their grading process such as misshapen or deformed eggs and eggs containing contaminants such as blood spots. Further enhancements to the system include a user-friendly, touch-screen database method for recording the number of egg cracks and other egg features that cause downgrades, which the AMS graders are currently documenting with pen and paper. **Impact:** The system will help the graders by increasing their accuracy, removing subjectivity, reducing data transfer errors, increasing their productivity, and dramatically changing the way eggs are currently graded. AMS is greatly encouraged and is discussing additional financial support for developments in the system. Examples for future development include a way to determine chick viability, and a mechanism to incorporate the technology into their national grading process.
- ARS developed a portable hand-held sanitation device for detecting bacterial biofilms and organic residues conducive to bacterial growth on food processing surfaces. Working with a CRADA partner for optimization and commercialization of the hand-held inspection devices, studies have been conducted to determine optimal lighting and detection wavelengths for several types of bacterial biofilms on food processing surfaces. **Impact:** Two prototype hand-held devices were built for testing and validation in commercial settings. A utility patent entitled "Hand-held Inspection Tool and Method" was filed in 2008. The technology will have a wide range of applications in all areas of food processing, for use by Federal, State and local food sanitation inspectors, and in schools and restaurants, etc.

1.2.3 Production and Processing Ecology

Exposure of animals, seafood and produce to pathogens during production, slaughter and processing operations, and transportation can be a significant source of contamination. Understanding microbial ecosystems that exist in these stages is extremely important for numerous reasons:

- As a means to utilize and validate any new, improved and innovative technologies to detect, differentiate, type, and quantify pathogens.
- When developing intervention strategies to minimize contamination, to prevent the growth of pathogens, and kill or remove pathogens
- To understand the phylogenetic relationships between and within species, and how it may explain the variability of types/strains encountered and the range of virulence characteristics expressed by the different strains under varying conditions and stresses.
- To understand how biofilms are formed by elucidating the genes and physical/chemical structures on the surface of pathogens responsible for attachment and colonization.
- To facilitate the identification of critical control points during food production and processing.
- Allow development of alternative and/or improved HACCP/GMP systems.

Microbial ecology is also one of the most contentious problems. Not unexpectedly, such studies are often seen as collecting base-line data, which is not part of ARS' mandate. However, these studies are appropriate if the data have a purpose. For example, contributing to improved assessment of the public health risk posed by the pathogen, or to form and/or implement regulation(s).

Meats

- Studies demonstrated that immersion scalding tanks may serve as a significant source of cross contamination of broiler carcasses during processing. Samples taken from the scalding water, foam layer of the water, and carcasses exiting the immersion scalders at a commercial poultry processing facility were analyzed for microbial contamination. Bacteria that can survive the relatively high temperatures of scalding water (50-60°C) may contaminate other carcasses entering the scalding tank and be carried to other processing operations. Results indicated that large numbers of intestinal bacteria, including *E. coli* and *Salmonella*, can be isolated from scalding tank water, tank foam layer, and carcasses taken from the tanks. **Impact:** Understanding the bacterial ecology of the scalding tank will assist in developing intervention strategies that can be used to reduce cross contamination during processing. Additionally, molecular fingerprinting of bacteria isolated from the scalding tank will provide information for tracking bacteria associated with scalding.
- Broiler carcasses are scalded by immersion in agitated hot water for several minutes to increase the efficiency of mechanical defeathering. Since many bacteria are removed from the skin and feathers of the broilers as they pass through the tank, there is an initial buildup of bacterial numbers in the water before the number of bacteria suspended in the scalding water reaches equilibrium. There has been widespread introduction of multiple-tank

scalders into poultry processing facilities in the last 15 years, but there are few reports on the number of bacteria suspended in the water in these tanks. ARS research indicated that numbers of pathogenic and indicator bacteria are reduced in the last tank of a three-tank, counter flow scalding when compared to the first tank of a commercial multiple-tank scalding. Enumeration of bacteria in the scald water and on carcasses indicated that most bacteria were recovered from water and carcasses taken from the first tank and that the level of contamination decreased through subsequent tanks. **Impact:** These studies validated the investment that processing companies had made when they changed from single tank operations to multiple-tank, counter flow scalders.

- ARS completed a comprehensive study that examined external and internal contamination of broiler chickens. These were the first studies to systematically examine which parts of the carcass of broiler chickens are associated with contamination by pathogenic, indicator, and spoilage bacteria. External samples consisted of feathers, heads, and feet of the carcass; while internal samples consisted of portions of the digestive tract of the broiler. There was not a major difference in the number of *E. coli* found on internal and external samples. One pathogen, *Campylobacter*, was found mainly on internal samples, but another pathogen, *Salmonella*, was recovered from all sampling locations. **Impact:** The findings indicate that the pattern of bacterial contamination before processing is complex and highly variable. The results provided vital information that can be used by poultry processors and regulatory agencies to establish protocols for handling and sampling broiler carcasses during processing.
- ARS demonstrated that water used in immersion chill tank systems can be recycled without causing significant increases in the number of enteric bacteria in the water or on carcasses. Broiler carcasses and chill tank water were sampled in a commercial processing facility that had installed a chiller system that conserves water by utilizing a mixture of recycled water and fresh water in carcass chiller tanks. Prechilled and chilled carcasses and water from various sections of the chiller were examined for the presence of pathogenic and intestinal bacteria. During chilling, most bacterial populations decreased in all samples; however, there was no change in the number of *Salmonella* recovered from carcasses after chilling. **Impact:** Chilling systems that can use recycled water and maintain food safety goals of processing can play an important role in conserving water during poultry processing.
- Immersion chilling of broiler carcasses in cold water remains the most popular of method for chilling in commercial operations in the US. ARS studies showed that there was no difference in the level of bacterial contamination of broiler carcasses chilled in dry air or in traditional immersion chillers. However, there is less contact between carcasses chilled in dry air; therefore, the chances for cross contamination may be reduced. Research was conducted to compare the level of bacterial contamination of carcasses chilled by either method. Results showed that both chilling methods reduced bacterial contamination by 90%. The percentage of carcasses contaminated by pathogens was similar for both methods, and was not reduced by either chilling method. **Impact:** This research provided processors with data that can be used when facilities are deciding which type of chilling method would be used in new or renovated facilities. This research has particular impact

for trade with, for example, countries in the EU, since the EU will not accept product chilled using cold water. The use of dry air chilling by processors could open opportunities for trade in poultry products.

- The HACCP inspection model program (HIMP) used by FSIS is part of a test program to validate a new inspection system. ARS conducted a collaborative study with FSIS to examine the microbiological quality of broiler carcasses processed in plants under HIMP. FSIS collected carcass rinse samples from every HIMP plant in the US and those rinses were used to measure the numbers of *Campylobacter*, *E. coli* and coliforms, as well as the presence of *Salmonella*. Samples were drawn early and late in the processing continuum to monitor the effectiveness of processing under the model inspection system. **Impact:** These data provided directly to FSIS will be useful to the agency in evaluating HIMP plants and may help regulators in future decisions relative to implementation of new inspection systems.
- Poultry processing is a harsh environment that may select for populations of *Campylobacter* that are adapted for survival. Several thousand *Campylobacter* previously collected from broiler carcasses in 20 US broiler processing plants were subtyped by DNA sequence methodology. These data show that the population of *Campylobacter* on broiler carcasses becomes less diverse as the carcass proceeds through processing plant. Some subtypes of *Campylobacter* seem to be better suited than others to survive the stresses associated with poultry processing environments. **Impact:** This finding is important in ongoing efforts to determine which characteristics may allow *Campylobacter* to survive or thrive in the poultry processing ecosystem. Intervention systems can then be designed to address these specific subtypes.
- Demonstrated that transport to and lairage at processing plants leads to increased prevalence and levels of *E. coli* O157:H7 contamination on hides and carcasses. **Impact:** A major implication of this finding is that pre-harvest intervention effects would be negated by pathogen transfer in the lairage environment. The research showed that contamination could come from any of the lairage environment spaces and as little as two hours in lairage was sufficient to contaminate cattle hides to levels that would be transferred to the carcass. Additionally, plants implementing hide wash cabinets were effectively reducing the lairage-derived hide contamination resulting in much less contamination of carcasses.
- ARS examined the prevalence and load of *Salmonella* and *E. coli* O157:H7 on the hides and carcasses of cull cattle at slaughter. Samples were collected during the summer, fall, winter and spring seasons in four geographically distant regions of the US. Pathogen prevalence on hides and carcasses was not found to be significantly affected by season; however, significant differences were observed between plants with respect to incoming pathogen load and the ability to mitigate hide to carcass transfer. In spite of these differences, antimicrobial interventions employed at all four plants significantly reduced contamination between pre-evisceration and post-intervention carcasses. **Impact:** Although these results were unexpected and are surprising, they do represent the most

comprehensive characterization of pathogen prevalence and levels in US cull cow processing plants to date.

- Recently, the FSIS launched its Strategic Implementation Plan for Strengthening Small and Very Small Plant Outreach. The contribution of small processing plants to the beef chain is significant, yet small plants have not been evaluated in regard to the transfer of pathogens from hide to carcass. It was noted that small beef processors needed to know the status of *E. coli* O157:H7 and *Salmonella* that enter their facilities and the rates at which the pathogens are transferred to carcasses. ARS organized and performed an intensive sampling at numerous small plants located across the US. **Impact:** The benchmarking data collected and provided to FSIS subsequently allowed the small processors to monitor their individual pathogen reduction efforts and provided a common sampling scheme for self-evaluation.
- ARS determined levels of generic bacteria and the prevalence of *Escherichia coli* O157:H7, non-O157 Shiga toxin-producing *E. coli* (STEC), and *Salmonella* at multiple large commercial lamb processing plants. **Impact:** The results of this study established a baseline for microbiological quality and prevalence of *Salmonella*, *E. coli* O157:H7 and STEC in US lamb processing plants. The study also provided information as to the efficacy of various antimicrobial interventions in reducing bacterial pathogens on lamb carcasses.
- ARS was requested to examine and compare U.S. beef trim to trim from foreign sources (Australia, New Zealand and Uruguay). The results also showed that trim from Uruguay was significantly more contaminated than that from other countries. **Impact:** Foreign trim does not need to be treated any differently than domestic trim with regard to pathogen intervention. The findings were communicated to collaborators and regulatory agencies. The Uruguayan processors involved were required to improve their exported products.
- Jerky is a popular snack product for consumers, and of particular interest to FSIS. ARS evaluated the effectiveness of a commercial cooking process to destroy pathogens on the surface of jerky made with beef or turkey. Mixtures of *S. Typhimurium*, *E. coli* O157:H7, and *L. monocytogenes* were applied to the surface of marinated and non-marinated strips of beef or turkey. In general, heating/drying marinated jerky at 165°F or 180°F resulted in a substantial reduction of all three pathogens. **Impact:** These data confirm that processing marinated jerky at 3.5 h at 165°F or for 2.5 h at 180°F is adequate to meet the FSIS performance standard of 7.0-log lethality established for *Salmonella* spp. in ready-to-eat red meat and poultry products.
- Listeriosis, a serious disease that can cause death in susceptible populations, is commonly associated with ready-to-eat (RTE) food products which support the growth of *Listeria monocytogenes* and are consumed without additional cooking. As such, the presence of this pathogen in these food products is strictly regulated by Federal agencies (FSIS and FDA). ARS developed and evaluated the effectiveness of the Sprayed Lethality in Container (SLIC) method in combination with the antimicrobial lauric arginate (LAE) to

lower the levels of *L. monocytogenes* on the surface of frankfurters, roast beef, and turkey breast logs. Each meat product was inoculated with *L. monocytogenes* and then added to packages containing various volumes and concentrations of lauric arginate. Each package was sealed, and stored at 4°C for 24 h. Pathogen levels were substantially decreased 2.0 to 5.0 log₁₀ CFU/package on samples treated with 5% or 10% concentrations of lauric arginate. **Impact:** This study showed that applying lauric arginate using SLIC reduced *L. monocytogenes* levels on the surfaces of vacuum-packaged roast beef, turkey breast, and frankfurters within 24 h at 4°C. The application of LAE as a post-process intervention should assist manufacturers in achieving the USDA/FSIS alternative 2 status.

- An understanding of the conditions required for *C. jejuni* and *C. coli* to form biofilms and persist in the environment will help in the development of control strategies. *C. jejuni* and *C. coli* exhibit significant strain to strain variation with regards to the ability to form biofilms. *Campylobacter* isolated from clinical disease cases compared to those isolated as environmental contaminants demonstrated no significant advantage of one group over the other with regards to biofilm forming ability. Furthermore, it was determined that salt positively influences the biofilm forming ability of *C. jejuni* and *C. coli* strains; however, variation in the concentration of salt required to enhance biofilm formation was observed among different strains. **Impact:** These results have significance given the fact that many poultry producers add salt (NaCl) to the liquid added to chickens packaged for retail sale.

Seafood

- Environmental bacteria pose a threat to shellfish and human health and have been difficult to evaluate due to the lack of testing methods. High numbers of beta-hemolytic strains of the flesh-eating pathogens *Shewanella* and *Photobacterium* (*Vibrio*-like pathogens) were identified for the first time in shellfish and seawater from the Delaware Bay. Approximately 1000 beta-hemolytic isolates/g of shellfish were detected for each bacterium during the summer months. Conventional biochemical testing and the use of the enzyme-based COPP assay failed to detect these pathogens. **Impact:** Publication of these findings was a wake-up call to health agencies, regulators, and the industry. State regulators in Delaware and New Jersey have asked for ARS assistance by identifying improved methods to detect these pathogens.

Produce

- Monitoring data from an agricultural watershed shows that stream segments affected only by wildlife may have higher concentrations of manure borne bacterial pathogen (MBPs) than agricultural segments of the watershed. Another source of uncertainty is the background concentrations of MBPs in sediments. This uncertainty does not preclude the modeling of MBP fate and transport; however, it does need to be factored into the modeling process. Using monitoring data, controlled hydrologic experiments and modeling, demonstrated that stream bed sediments can serve as a reservoir for the

pathogen indicator organism *E. coli*. Sediments, rather than the runoff from pastures and manured fields, can control the level of bacterial water impairment as determined by EPA regulations regarding fecal contamination. **Impact:** This finding stressed the need to revise existing methodology of estimating the effects of management practices on bacterial water quality, and to pursue research of sediments as pathogen sources for irrigation water used on vegetables and produce.

- Rapid and sensitive assays are required to assess the potential health risks of surface waters used for recreational purposes or for irrigation of fresh produce. The project documented the widespread prevalence and distribution of presumptive virulence markers for enterohemorrhagic *E. coli* (O157 serogroup, *stx* and *eae* genes) in surface waters via genetic and immunological assays. Monitoring studies were conducted in three different watersheds associated with agricultural, urban/suburban, and wildlife habitats. Virulence markers, assumed to be indicative of enterohemorrhagic *E. coli* (EHEC), were ubiquitous in watersheds and were relatively widely dispersed among less pathogenic or non pathogenic *E. coli*, as well as other common water-borne bacteria. **Impact:** These findings have implications for the rapid detection of EHEC in surface and ground waters; namely that genetic and immunological assays alone cannot reliably detect water-borne EHEC.
- Due to ease of measurement, generic *E. coli* are routinely used as indicators of water-borne fecal contamination. It is assumed that some relationship exists between generic *E. coli* and pathogenic *E. coli*, as well as other zoonotic pathogens. These studies documented the lack of any correlation between generic and presumptive enteropathogenic *E. coli* (EPEC) in an agricultural watershed. Concentrations of generic *E. coli* and the *eae* gene were measured in surface waters receiving fecal inputs from dairy cattle; the *eae* gene is the critical virulence factor for EPEC and is ubiquitous in surface waters. No correlation was observed. **Impact:** Although limited in scope, these findings suggest that generic *E. coli* are poor indicators of microbial water quality and hence, public health risks.
- Utilization of manures containing pathogenic microorganisms is considered to be an important factor in the occurrence of water- and food-borne diseases. Currently many of the essential pathogen fate and transport processes are not understood or correctly modeled. This project developed the first mechanistic model to evaluate the efficiency of conservation buffers in retention of manure-borne *E. coli* entering buffers from fields and pastures. The project also developed the first field-scale mechanistic model to evaluate the potential of manure borne-pathogens to leave fields with runoff and reach surface water sources. **Impact:** Given that current recommendations on the filter strip efficiency in pathogen retention are based on empirical equations developed in the 1980's, the model represents a breakthrough that can provide the necessary information for the microbial risk-informed design and evaluation of vegetated filter strips from irrigation water sources. The manure model is designed and can serve as the add-on module to ARS surface water quality models making them valuable in addressing the current concerns about co-management of water quality and food safety.

- Manure particulates serve as carriers, abode, and food source for pathogens. The project found the presence of bovine manure particulates drastically decreased the association of *E. coli*, *Cryptosporidium parvum* oocysts and *Giardia* oocysts with soil particles and facilitated the transport of these microorganisms through soils and in overland flow. **Impact:** This finding indicates that many widely used pathogen fate and transport parameter values obtained in absence of manure particulates need to be substantially corrected before being used in field conditions.
- Studies demonstrated that microbial quality of compost tea, an unheated on-farm infusion of compost used as a spray or soil drench to promote plant growth and control foliar and root diseases, could be affected by production processes and addition of nutrients. *E. coli* O157:H7 decreased below detection levels in compost tea after 36 h without supplements (aerated or unaerated). In contrast, the addition of commercially formulated mixtures or combinations of nutrient supplements resulted in growth of *E. coli* O157: H7, *Salmonella*, and fecal coliforms up to 10,000 colonies per g in both aerated and nonaerated compost tea. **Impact:** This finding suggests that nutrient supplements during compost tea production should be avoided to reduce risk of contamination of fresh produce by pathogens in this organic spray.
- A study was conducted to characterize the microbial quality of finished, marketable compost prepared from a wide range of organic residuals from 15 U.S. commercial facilities. This was done to assess the compliance with the USEPA 503 limit for Class A product: fecal coliforms not greater than 1000 MPN per g and *Salmonella* greater than 3 MPN per 4g, respectively. Fifty-three percent and 7 % of commercially available compost exceeded the limit. However, prevalence of toxigenic *E. coli* in commercial composts was very low despite the relatively high number of samples that contained generic *E. coli*. **Impact:** This study shows that commercial composts that meet the fecal coliform and *Salmonella* standards may still contain low levels of pathogenic *E. coli* demonstrating the data need to help ensure selection/use of quality composts by fresh produce growers.
- Limited data on production practices relative to microbial/pathogen content of fresh produce are currently available for either organic or conventional farms in the Mid-Atlantic region, some of which market directly to consumers. A microbiological survey of fresh produce (leafy greens, tomatoes, strawberries, green onions, and herbs) grown by farmers in the Mid-Atlantic Region was conducted and revealed the presence of generic *E. coli* in soil and commodity samples. *E. coli* O157:H7 or *Salmonella* were not found in produce, water, or soil samples. *Listeria* species were recovered from strawberry and soil samples on one farm by enrichments. *E. coli*, in excess of drinking water quality limits, was recovered from 18 of 45 (40%) irrigation water samples. **Impact:** Farm practices at each site were documented and provided information on production practices. The microbiological survey suggested needs for controlling *E. coli* in irrigation water.
- ARS was preemptive in already conducting environmental sampling in the Salinas region of California, for *E. coli* O157:H7 prior to the outbreak of 2006 associated with contaminated spinach. ARS were the first to identify the spinach outbreak strain of *E. coli*

O157:H7 from environmental sources, including dust and feral pig feces. In addition, our continued survey for the presence of E. coli O157:H7 in the Salinas Watershed showed that the pathogen persisted in the environment in proximity of produce fields. Studies demonstrated that new production practices need to be put in place that take into account the general ecology of the region where produce is grown. Consequently, several large processors no longer purchase produce grown in fields occasionally flooded by stream water, and the industry is presently identifying guidelines regarding buffer zones around agricultural fields to prevent outside sources from contaminating crops. **Impact:** The importance of this research cannot be overstated. The 2006 spinach outbreak alone cost industry over \$100 million, not including the costs for litigation. ARS provided the FDA with E. coli O157:H7 isolates for comparative genome sequencing and characterization. ARS results were presented at numerous local and international meetings attended by governmental agencies and by the produce industry, and were part of reports published by both California Department Health Services and CA-EPA.

- No studies have examined how long E. coli O157:H7 can survive under field conditions on spinach leaves. Survival of E. coli O157:H7 and non-pathogenic E. coli were evaluated on spinach plants and in organic soil in a growth chamber that simulates field conditions. Populations of E. coli O157:H7 survived for a shorter duration on spinach shoots than in soil. Non-pathogenic E. coli were detected intermittently on spinach up to 28 days. The survival of E. coli O157:H7 and non-pathogenic strains were similar on leaves and in rhizosphere soil. **Impact:** This finding will help determine potential uses of non-pathogenic E. coli as a surrogate strain in field studies.
- A study was conducted in an organically managed High Tunnel system to determine the survival of E. coli O157:H7 on 'Whale' cultivar of spinach relative to leaf age and exposure to radiation. The results demonstrated that the survival of E. coli O157:H7 depends on the leaf age. The population of E. coli declined from initial inoculated concentrations of 175,000 CFU/leaf to 71 MPN per leaf in 7 days and to very low or undetectable numbers up through 28 days. Older leaves partially shaded and sometimes in contact with moist soil had greater populations than upper (intermediate age) or crown (youngest age) leaves. Results showed a rapid population die-off of E. coli on upright leaves (upper and crown leaves which became the upper leaves as plants grew) within the high tunnel environment. **Impact:** This finding demonstrates timely harvesting of spinach as bacterial survival could be longer on older leaves.
- Identified leaf age as a risk factor in the contamination of lettuce with E. coli O157:H7. Lettuce is the leading vegetable crop in the US, with a \$2 billion production value in California alone. It is also the produce most commonly linked to infections of E. coli O157:H7. Information about specific plant factors that affect the colonization of lettuce by human pathogens, such as leaf age, is critical to the industry for the improvement of GAPs and to public health agencies for the development of efficient sampling strategies to detect contamination of lettuce. Also, because the higher colonization by the pathogens was attributable to greater nitrogen levels in and on the young lettuce leaves. **Impact:** The observations suggest the possibility of modulating nitrogen fertilization as part of a hurdle strategy to minimize contamination of lettuce in the field. The observations are

also valuable for risk assessment analysis of produce contamination and for development of efficient sampling strategies to detect contamination of produce.

- Sales of minimally processed (fresh-cut) produce have increased to \$15 billion per year in North America. Concomitant with this increase in consumption has been a dramatic increase in the number of outbreaks of *E. coli* O157:H7 infection linked to pre-washed processed leafy greens in the US. ARS research demonstrated that *E. coli* O157:H7 multiplies rapidly and can reach infectious dose concentrations in just a few hours at warm temperature in lettuce leaf lesions caused by processing. **Impact:** Our observations reveal a critical control point to minimize contamination of lettuce during processing and provide guidance to the industry for its HACCP programs.
- A 3-year longitudinal study on prevalence of pathogenic and spoilage microorganisms on organic and conventional tomato plants (petiole sap or stem prints) was conducted. The research resulted in the detection of no *E. coli*, *Salmonellae*, or *Listeria*. The numbers and types of other spoilage bacteria on roots were significantly greater than those obtained from petioles of these same plants. Bacterial populations on calyx and stem scars of fruits from these plants were significantly greater than those from petiole sap. The numbers and types of bacteria on roots were significantly greater than those obtained from petioles of these same plants. Bacterial populations on calyx and stem scars of fruits from these plants were significantly greater than those from petiole sap. The stem area of tomato fruits is a site of potential survival and growth for bacteria and subsequent entry into fruits. **Impact:** This finding demonstrates the need for cleaning and sanitation regimes as biofilms can easily develop in the stem area of the fruit when water collects there during fruit maturation.
- Extracts of fresh leaf spinach are known to support growth of *E. coli* O157:H7, but it is not known to what extent this organism will grow or survive on spinach leaves that remain in the field after harvest of the crop and soil tillage. Studies were conducted to determine the survival of *E. coli* O157:H7 in soil containing organic residue of spinach leaves, cabbage leaves or daikon as these crops are known to release a group of biologically active anti-bacterial isothiocyanic esters. *E. coli* O157:H7 populations in soils decreased with time; however, they were still detectable after 35 days. Overall *E. coli* O157:H7 detected in soil amended with cabbage manure were significantly lower soils amended with spinach or daikon manures. **Impact:** This work was the first to demonstrate the effect of green manure on survival of *E. coli* O157:H7 in soil. This finding suggests that the common field practice of leaving the remnants of crops (such as cabbage) in the field after harvest may have potential use as a biofumigant to accelerate decline *E. coli* O157:H7 in some cropping systems.
- Lettuce coring-in-field is a new practice developed by the industry to improve process efficiency and reduce shipping cost, and is widely used for harvesting lettuce destined for fresh-cut processing. Studies documented the potential risk of transference of *E. coli* O157:H7 from contaminated coring knives to the edible portions of lettuce during lettuce coring-in-field harvesting. Studies also demonstrated that *E. coli* O157:H7 can grow significantly on lettuce cored areas during product field-holding and hauling. Identified

risk mitigation approaches including frequent sanitation of the coring knives, separating coring from cutting, and rapid cooling post-coring/harvesting. **Impact:** This study raised the awareness of the potential food safety risks associated with lettuce coring-in-field harvesting and identified an important new research area. Consequently, FAO/WHO Expert Panel used these findings to prepare CODEX document.

- An increasing number of food-borne illness outbreaks have been associated with the consumption of packaged fresh-cut products. Studies demonstrated that *E. coli* O157:H7 can grow significantly on commercially packaged fresh-cut lettuce and baby spinach products despite the presence of large populations of native microflora. Further it was found that the growth of *E. coli* O157:H7 benefits more from temperature abuse than do native microorganisms, and that *E. coli* O157:H7 developed significant resistance towards synthetic gastric juice during storage under modified atmosphere packaging. **Impact:** These first-reported studies filled a data gap on commercially processed fresh-cut products, and countered the common belief that native microflora on fresh-cut vegetables can out-compete pathogens; temperature control is important for both food quality and food safety. The study was specifically requested by the FDA, and the findings provided critical scientific information to the FDA for revising the “Food Code” to include packaged fresh-cut vegetables in the “Time/Temperature Control for Safety Food” category.
- *Escherichia albertii* is a lesser known pathogen but may have the ability to cause foodborne illness, and has been found in the rhizosphere of tomato plants. Studies demonstrated slow growth of *Escherichia albertii* on fresh-cut lettuce when stored at 5C, indicating that this strain of *E. albertii* may be more persistent at low temperatures than other strains. As expected, all strains of *E. albertii* grew on all commodities at abusive temperatures. **Impact:** This is the first examination of *E. albertii* on fresh-cut produce and suggests the need for additional studies to determine its persistence on produce.
- The FDA has raised concerns about the safety of acidified vegetable products. Some acidified vegetable products cannot be heat treated and maintain desirable sensory properties. Studies determined the processing conditions needed to assure a five log reduction in cell numbers for vegetative bacterial pathogens in acidified vegetable products. The acid concentrations, holding times, and temperatures needed to assure safety for non-pasteurized products were also identified. **Impact:** The results were communicated to industry through trade association meetings and incorporated as part of an acidified foods Good Manufacturing Practice course curriculum (GMP school, required by FDA for commercial producers of acidified foods). The recommended processing conditions have been widely adopted by the acidified foods industry, and these data are now used for commercial process filings by U. S. and international producers of acidified vegetable products.
- The safety of acidified foods, which are foods that have acid or acid food ingredients added to fresh vegetables. Most acidified food products are prepared in hermetically sealed jars, resulting in an anaerobic environment. Current literature on the acid resistance of *E. coli* strains does not take into account the effects of dissolved oxygen.

Research identified and measured the effects of dissolved oxygen on the survival of E. coli O157:H7 in organic acid solutions typical of acidified food products. **Impact:** Studies indicated that E. coli O157:H7 may survive much better than previously expected in acid solutions if anaerobic conditions are used. Additional research needs to be conducted to accurately determine the survival of pathogenic bacteria in acid and acidified foods, and the presence and amount of dissolved oxygen needs to be considered.

- During these acidified food studies, a protective effect of organic acids on survival of E. coli in acid solutions was observed. By comparison of buffered acid solutions with and without organic acids (using gluconic acid as a non-inhibitory buffer) it was found that low concentrations of 5 to 10 mM of common food acids such as lactic and acetic acid, can increase the survival of E. coli strains. This effect was particularly pronounced for D-lactic acid, but not L-lactic acid. **Impact:** A computer software program was developed to determine the effects of ionic strength, temperature, and other variables on protonated acid concentrations, allowing precise comparisons of organic acid in solution.

1.2.4 Processing and Intervention Strategies

New/alternative food processing technologies all have the ability to inactivate microorganisms to varying degrees. However, the high treatment intensities required for inactivation usually result in adverse functional and/or sensory properties, significantly reducing food quality. Quality and safety are intimately associated, especially considering the change in consumer demands for more fresh (minimally processed) and natural food products. Apart from irradiation, all of the new technologies (used alone) are too costly, too energy expensive, and cannot guarantee safety to be of practical use.

Postharvest operations of all sizes (large to very small) provide an opportunity to remove or inactivate pathogens and their toxins acquired during the production and processing phases. Pathogens may develop resistance to antimicrobials from traditional measures used for pathogen control. Successful technologies and strategies to eliminate, reduce, or suppress human pathogens are needed for foods and food types associated with foodborne illnesses, or at risk of becoming vehicles for human pathogens. Development of individual or combinations for new or innovative intervention technologies for (minimal) processing will be developed based on an understanding of their modes of action and effects on the microbial ecology of a food product. For example, inadequate suppression of spoilage could create an opportunity for human pathogen growth and toxin production.

Meat

- Harborage of *Escherichia coli* O157:H7 and *Salmonella* on animal hides at slaughter is the main source of beef carcass contamination during processing. Given this finding, interventions have been designed and implemented to target the hides of cattle following entry into beef processing plants. Previous interventions targeting hides have not been suitable for all beef processing plants to implement due to cost and space restrictions. Studies evaluated a hide wash cabinet design that was smaller and more economical and, therefore, might be more amenable to widespread use in the beef processing industry. The results showed large reductions in *E. coli* O157:H7 and *Salmonella* levels after hide washing in the test cabinet. **Impact:** Based on these results, the hide wash cabinet described in this study was effective and should provide beef processors, especially small and medium-sized processing plants, with an affordable hide wash intervention strategy.
- In recent negotiations, trading partners agreed to accept frozen pork from the U.S. as part of the WTO agreement. This was in spite of objections raised by international veterinary experts regarding a risk posed by cold tolerant species of *Trichinella* recently found in the U.S. and Canada, with the provision that studies be conducted to close this knowledge gap. In collaboration with the Canadian Food Inspection Agency, ARS determined the freezing parameters for destruction of all North American species of *Trichinella* larvae in pork. This research forms the basis for upgrading the current cold treatment requirements for pork (9CFR 318.10; EU CR 2075/2005) that was conducted in 1990 and determined the time/temperature combinations that inactivated *T. spiralis* in pork; other *Trichinella* species were not considered. **Impact:** The studies demonstrated that the time/temperature combinations known to render pork safe for *T. spiralis* (T-1) are sufficient to inactivate

all North American species and genotypes of *Trichinella*, eliminating concern about the safety of frozen pork products and securing an annual market worth \$400 million.

- At the request of the National Pork Board and FSIS, ARS established thermal death curves for *Trichinella spiralis* and *Toxoplasma gondii* in pork. Further, at the request of FSIS, ARS reviewed and proposed changes to assure that suggested internal temperature and holding times meet minimum requirements for inactivation of *T. gondii* and *T. spiralis* to assure food safety. **Impact:** End point freezing and cooking temperatures were determined and are the benchmark for published FSIS recommendations to pork packers and consumers for safe preparation of fresh pork products.
- Frankfurters can occasionally become contaminated by the pathogen *L. monocytogenes* following cooking and prior to packaging, leading to product recalls and foodborne illness outbreaks. The Flash (Steam) Pasteurization (FP) process developed by ARS for poultry and certain fruits was further developed to inactivate *Listeria* on the surfaces of frankfurters that contained the commonly used antimicrobials potassium lactate and sodium diacetate. **Impact:** The FP process, in combination with potassium lactate and sodium diacetate, is now being used by 4 major manufacturers of pre-cooked sausages located in North, Central and South America to provide safer ready-to-eat meat products to consumers. Over \$1 billion of product will be treated using the FP process in 2009.
- Meat processors have adopted the use of infrared for RTE meats followed by in package hot-water pasteurization as a hurdle process for inactivation of *L. monocytogenes*. Over \$1 billion of product is treated annually in the U.S. using this process. In order to improve the energy efficiency of the process, ARS developed a computer-controlled infrared heating and microwave system with automatic feedback to control temperature. In addition, new models were developed to more accurately predict thermal inactivation kinetics for *L. monocytogenes* using in-package hot water pasteurization. **Impact:** This upgraded technology if adopted, will provide meat processors a more efficient and effective hurdle in RTE meat processing.
- Ultraviolet Light (UVC) is used as an FDA approved technology for surface decontamination of foods and food contact surfaces. In studies UVC was used to inactivate foodborne pathogens on frankfurters, bratwurst, boneless skinless chicken breasts, chicken drumsticks, pork chops, tomatoes, eggs, and stainless steel. Research indicated that UVC inactivated 5 log of pathogens on stainless steel and in ice; 3.5 log on tomatoes; 1 log on eggs; 2 log on frankfurters and bratwurst; 2 log on raw fish; and 0.5 log on chicken and pork. Furthermore, the ability of pathogens to invade human cells after being subjected to multiple rounds of UVC exposure when inoculated on stainless steel was greatly reduced. **Impact:** This data will be used by food processors to provide safer products for consumers and by regulatory agencies to evaluate emerging food safety intervention technologies.
- There is considerable interest in the use of irradiation as an intervention in meat processing. ARS demonstrated that low-dose, low-penetration electron-beam irradiation has great potential for use as an antimicrobial intervention on beef carcasses during

processing and minimally impacts the quality of the treated beef products. **Impact:** The American Meat Institute used this research as a basis for their submission of “Citizens Petition to Recognize the Use of E-Beam on Carcasses as a Processing Aid” to the FSIS in July, 2005. This research was a main topic point in a 2008 FSIS hosted public meeting to discuss the application of low dose irradiation as a processing aid.

- Furan and acrylamide are two possible carcinogens found in many thermally processed foods. Low dose ionizing radiation (2-3.5 kGy) completely destroyed furan in water, and reduced furan levels by 25 to 40% in ready-to-eat meats. Irradiation completely destroyed acrylamide in water, but had a limited effect on the inactivation of acrylamide in oil and potato chips. **Impact:** These studies showed that ionizing radiation significantly reduced the levels of the human carcinogen furan in ready-to-eat meats using radiation doses that would be used to inactivate the human pathogen *L. monocytogenes*. These data will help the FDA evaluate a petition, currently under review, to allow irradiation of ready-to-eat foods.
- Chlorine is currently the most widely used sanitizer in commercial poultry processing facilities in the US. However, use of chlorine in processing water has been linked to the formation of carcinogens in foods, therefore some countries will not import carcasses processed in the US. ARS studies have shown that washing carcasses solutions of alkaline salts of naturally occurring fatty acids can significantly reduce microbial contamination of broiler skin and carcasses. The fatty acid solutions decrease contamination by killing microorganisms and washing them away from surfaces. **Impact:** The development and utilization of these alternative sanitizers may open new markets for US poultry processors, in particular the EU. ARS is a partner in a major grant application to the EU to evaluate new processing interventions.
- Chlorine is currently the most widely used sanitizer in commercial poultry processing facilities in the US. Research determined that acidic electrolyzed (EO) water can be used to kill microorganisms associated with poultry processing. The microbicidal activity of EO water is due to a combination of low pH, high oxidation-reduction potential, and elevated concentrations of hypochlorous acid produced during the electrolysis of sodium chloride solutions in EO water generators. In vitro tests performed using bacteria and yeast associated with poultry processing indicated that EO water possessed bactericidal and fungicidal properties. Other experiments were conducted that used inside-outside bird carcass washers to wash whole broiler carcasses with EO water or chlorine solutions. **Impact:** These experiments indicated that EO water was as effective as traditional chlorine solutions in reducing carcass contamination. Since EO water can be prepared onsite at processing facilities, the use of this sanitizer by poultry processors would eliminate the need for storing potentially hazardous quantities of chlorine on the premises.
- The greatest source of contamination of further-processing plants by *Listeria* is from raw meat. ARS completed a study to measure the effectiveness of germicidal UV light to kill *L. monocytogenes* on raw poultry meat prior to simulated shipment. The UV treatment significantly lowered the numbers of four subtypes on raw meat. Numbers were still low

after simulated shipment at 4 C. **Impact:** The data show that UV light has potential to be used by processors to break the chain of continuous re-contamination of a poultry cooking plant with *L. monocytogenes* from raw meat shipments.

- *C. jejuni* counts on broiler carcasses increase due to the escape of highly contaminated gut contents. ARS completed an intervention study whereby food grade organic acids (acetic, lactic, and propionic acids) were placed in the vent of carcasses prior to feather removal. The study showed that this technique was effective in killing *Campylobacter* in the cloaca and lessened the escape of viable cells during defeathering. **Impact:** This study provides poultry processors with a cost effective and simple method to significantly lower the levels of *Campylobacter* during feather removal with concomitant lowering of the numbers of this pathogen on fully processed broiler carcasses.
- It is believed that the anatomy of the whole egg contributes to the overall problem of human illness from contaminated eggs. ARS studies determined that immediate refrigeration prevented *S. Enteritidis* from penetrating through the yolk membrane to reach the nutrient-rich yolk contents in an in vitro egg contamination model. The studies also established that the whole yolk is a more favorable environment than yolk contents for growth of *S. Enteritidis* to high cell density. **Impact:** This research supports the regulation of prompt refrigeration of eggs in *Salmonella* control programs to minimize the risk that pathogens will grow to higher (and more dangerous) levels after penetrating into the yolks of contaminated eggs.
- Vacuum loader cups and brushes in shell egg processing facilities have been identified as reservoirs of bacterial populations. Research determined the level or frequency of aerobic organisms, *Enterobacteriaceae*, *Salmonella*, *Campylobacter*, and *Listeria* on vacuum loader suction cups and packer head brushes in shell egg processing facilities. *Salmonella* and *Campylobacter* were found on 3% and 1%, respectively, of the suction cups sampled. *Listeria* was isolated on 72% of the suction cups samples. All of the populations were recovered from packer heads though pathogens were only encountered 1-2 times. No pathogens were recovered from eggs. Higher numbers of aerobic microorganisms and *Enterobacteriaceae* were recovered from washed eggs when > 4.0 log cfu aerobes/ml or > 2.0 log cfu *Enterobacteriaceae*/ml per sample were recovered from packer head brushes. **Impact:** Based on the research, Federal and State regulatory groups, and egg processors, began to reevaluate shell egg processing facility sanitation procedures to address the microbial populations identified in this research.
- Post-processing shell surface sanitizers have been required by USDA-AMS to help reduce the presence of spoilage organisms, not pathogens, on the shell surface. The recommended sanitizer approved by USDA-AMS has been 100-200 ppm chlorine. Four compounds were compared in water-rinsed and unwashed eggs for their ability to reduce microbial populations associated with the shell and egg contents. Total aerobic organisms, *Enterobacteriaceae*, *Salmonella*, yeasts, and molds were monitored within the shell matrix and in the egg contents during extended cold storage. The various sanitizing compounds did not exhibit a great change in bacterial load compared to water-rinsed washed eggs. No significant differences in physical quality were seen between the

treatments during the course of the study. **Impact:** The act of washing the eggs had the greatest affect on reducing microbial populations associated with the shell matrix. Removing the sanitizing rinse requirement would reduce processing costs and would also decrease the environmental impact of shell egg processing.

- Vacuum loader cups in shell egg processing facilities have been identified as reservoirs of bacterial populations. Examined sanitation compounds to determine their ability to reduce bacterial populations on vacuum loader cups in shell egg processing facilities. Five sanitizing compounds were compared for their ability to reduce bacterial loads. The 200 ppm. sodium hypochlorite and 200 ppm. calcium hypochlorite treatments reduced bacterial levels to those comparable with the uninoculated controls. **Impact:** Both of these compounds could be easily applied in the processing facility with low potential of harm to employees or processing equipment and are affordable sanitizing options.
- Liquid egg may contain harmful bacteria such as Salmonella. Processing technologies are needed to inactivate pathogens while maintaining the quality of the food. Combining pulsed electric field (PEF) and mild heat at 55C for 3.5 minutes resulted in inactivation of *S. typhimurium* comparable to the heat treatment at 60C for 3.5 min and maintained quality and functionality of liquid whole egg. A 5 log reduction to the Salmonella population without protein coagulation was achieved by consecutive application of 4 repeated pressure treatment cycles from 0 to 350 MPa with 2 min hold time at 50C. **Impact:** The mechanism of bacteria inactivation using radio frequency electric fields (RFEF) was due to membrane damage of the bacteria leading to the efflux of intracellular ATP, nucleic acid, and protein in the juice matrix. This finding will help developing RFEF process to its potential.

Produce

- A meeting of industry experts at the International Lettuce and Leafy Greens Research Conference indicated that a kill-step for fresh produce was of the highest priority of the industry. The effectiveness of a mixture of bacteriophages, which are viruses that can kill pathogenic foodborne bacteria, was examined to kill *E. coli* O157:H7 on cut pieces of iceberg lettuce and cantaloupe stored at 4C. The bacteriophage treatment reduced populations of *E. coli* O157:H7 immediately upon application to lettuce, and reduced populations on cantaloupes over 7 days. In another study in collaboration with North Carolina A&T University, procedures were developed to use a bacteriophage mixture to kill *E. coli* O157:H7 on the surface of lettuce leaves. A 2-log reduction was achieved on the surface of lettuce pieces. **Impact:** The importance of this research cannot be overstated. The study was the first to show the effectiveness of a bacteriophage mixture to kill *E. coli* O157:H7 on fresh-cut lettuce and cantaloupes at refrigerated temperatures. These methods will be standardized for the application of bacteriophage to other produce commodities.
- The industry currently lacks an effective “kill step” for produce. With losses from the 2006 leafy greens outbreaks in excess of \$100M, developing an effective intervention is

critical. ARS established the efficacy of irradiation in killing E. coli O157:H7 internalized in leafy greens, which are protected from surface treatments such as chlorine-based washes. Research demonstrated that irradiation reduces internalized pathogen populations by 99.9 to 99.999% on spinach and five different types of lettuce. Previous studies by ARS researchers determined irradiation efficacy in eliminating Salmonella and L. monocytogenes from produce. **Impact:** These important data has been requested repeatedly by processors, retailers and regulators. Knowledge and methodologies were broadly disseminated to industry and consumers through national and international media coverage. This valuable and impactful research was cited extensively in the 2008 policy/regulatory action by FDA to amend 21CFR-179.26 to allow the commercial irradiation of lettuce and spinach.

- The use of irradiation as an intervention raises a concern for the production of carcinogens and the effects on product quality. Studies examined the appearance, texture, and aroma of 13 types of produce (including lettuce, spinach, and tomato) and showed that quality is preserved or enhanced following microbiologically efficacious doses of irradiation. **Impact:** This research was the first to demonstrate that no detectable amount of furan was produced from irradiation of the vegetables, validating the safety of the process. The work was cited extensively in the 2008 policy/regulatory action by US-FDA to amend 21CFR-179.26 to allow the commercial irradiation of lettuce and spinach. The information has been widely requested by and disseminated to producers, processors and retailers. Tanimura & Antle, a major CA grower/shipper, recognized the importance of the work and is collaborating in new irradiation research.
- Cantaloupe food safety alerts and product recalls have resulted in > \$50 million in lost revenue since 1990. ARS developed a commercially viable chemical-free thermal sanitation method to kill Salmonella on cantaloupe melons. Pathogens reductions of 99.999% were obtained under conditions that preserved the quality and appearance of treated produce. Thermal penetration models and antimicrobial efficacy models were developed to optimize the process for cantaloupe melons. Fresh-cut cantaloupe pieces prepared from treated melons had a commercially acceptable shelf-life in excess of 28 days, more than 14 days longer than melons processed with chlorine-based treatments. **Impact:** This technology has been transferred to industry through cooperative research agreements. This novel surface pasteurization process enhances the microbiological safety of whole cantaloupes and extends the shelf-life and commercial value of cut fruit pieces.
- New processes to improve the microbial safety and quality of fresh and fresh-cut produce are an industry priority. ARS developed and optimized precision chemical sanitizing processes for cantaloupes. These treatments, based on acidified calcium sulfate and acidified sodium chlorite, inactivated pathogens and reduced the surface microbial load of treated cantaloupes. In conjunction with industrial partners, ARS optimized the use of advanced in-package, gas-phase chlorine dioxide treatments. Determined that polyvinyl chloride plastic films were superior to low density polyethylene for use with in-package sanitation processes. **Impact:** This new knowledge is of high significance to processors and retailers in reducing risk of food borne pathogens on fresh and fresh-cut pieces.

- The produce industry has a critical need for effective antimicrobial treatments suitable for application to fresh and fresh-cut fruits and vegetables. Cold plasma is a novel food processing technology that uses energetic gases to kill pathogens on fragile surfaces. ARS developed a novel technology, nonthermal (“cold”) plasma, as an effective antimicrobial treatment for produce. When applied to inoculated apples, cold plasma generated in an ERRC prototype system reduced *E. coli* O157:H7 and Salmonella by 99.98%, and reduced *L. monocytogenes* by 98.74% with minimal sensory impact. Mechanistic studies to optimize the plasma chemistry showed nitrogen increases UV-light production by 80% over that of air. The research determined that active cold plasma is more efficacious than conventional negative air ion treatments to reduce *E. coli* on apples, apple slices, and mung bean seeds. **Impact:** New collaborative research agreements are being drafted with industry partners to pursue the entirely new research areas illuminated by this work. Advances in this emerging field provide the potential to develop an effective new technology to kill human pathogens on produce.
- Three non-thermal processing technologies were developed and evaluated by ARS in pilot plant operations: radio frequency electric fields (RFEF), pulsed electric field (PEF) and super critical carbon dioxide (SCCO₂). Non-thermally processed fruit juice and other liquid foods may be continuously processed at a flow rate of 100 L/h and packaged into aseptic multi-layer aluminum-foil bags for quality, shelf life and microbiological studies. **Impact:** All three processes demonstrated ability to achieve 5 log reduction of *E. coli* in apple cider and orange juice. PEF process also showed its versatility in handling products with particles such as strawberry puree. Side-by-side comparisons with traditional thermal processing were conducted using the integrated pilot plant processing system which enabled our stakeholders to evaluate novel processing technologies.
- Antimicrobial food packaging provides an additional and final intervention hurdle for controlling food borne pathogens in packaged foods. The generally regarded as safe antimicrobials, such as nisin, lactic acid, and nanoscale zinc oxide were integrated into biodegradable packaging materials (polylactic acid and/or pectin) to develop antimicrobial packaging systems used for liquid or solid foods. These antimicrobial packaging systems significantly inactivated or inhibited pathogenic *L. monocytogenes*, *E. coli* O157:H7, *S. enteritidis* in orange juice, apple cider, apple juice and liquid egg. **Impact:** The antimicrobial food packaging has a great potential to ensure food safety and extend the shelf life of many food products.
- Conventional washing and sanitization treatments cannot completely eliminate human pathogens from produce. Used in combination with these conventional chemical treatments, a biological-based intervention may be used to effectively inhibit the re-growth of surviving pathogens. Using bell pepper disks as a model system, plant-associated bacterial antagonists were evaluated for their potential as biological agents for control of foodborne pathogens and spoilage bacteria. Studies isolated and characterized a select biocontrol organism, *Pseudomonas fluorescens* #2-79, and complex bioactive cultures with unique bio-suppressive and bio-antagonist properties. The biocontrol Pf#2-79 strain effectively suppressed the growth of Salmonella on sprouting seeds by 99–

99.9%. The bioactive multispecies cultures prevented the growth of human pathogens (*Salmonella*, *Y. enterocolitica*, *E. coli* O157:H7, *L. monocytogenes*) and spoilage bacteria on surfaces of produce. Greater understanding of pathogen microbial ecology improved the application methodologies of the biocontrol agents, enhancing the efficacy of the treatment. Optimized treatments in scaled-up model systems yield pathogen suppression of 99-99.999%. **Impact:** Application of Pf 2-79 and other bacterial antagonists show promise as a new tool to restrict the proliferation of human pathogens and spoilage bacteria on produce.

- The California almond industry, which is the largest supplier of almonds in the world, has been affected by two international and one national outbreak of salmonellosis linked to raw almonds. Propylene oxide (PPO) is the only effective dry treatment to decontaminate raw kernels, but these treated kernels cannot be exported to foreign countries due to a lack of standards about PPO residue levels in the product. ARS developed an infrared heat-based technology that is at least as efficacious as fumigation in decontamination of raw kernels involves a one-hour instead of a five-day process, and achieves a reduction of over 7.5-log₁₀ cfu *Salmonella*/g kernel. **Impact:** The California annual almond crop is valued at \$4B. The Almond Board of California presently mandates a 4-log reduction and has indicated critical interest in implementing this nonchemical process. Therefore, the Board has provided ARS with funding to further develop this technology for almonds at a commercial level.
- Sanitation products and methods that kill bacteria or reduce contamination in response to governmental regulations were complemented by alternative interventions for control and prevention of contamination. Three types of compounds, with activity based on silver, iodine, or quaternary ammonium were most effective for control of pathogens and spoilage organisms. Previous studies showed that new picker-finger rubber inhibited the growth of bacteria, and electropolished stainless steel showed significantly fewer bacteria and biofilm formations than other treated surfaces. Prevention of attachment would allow bacteria dislodged from carcasses to be washed away during processing. Combining a surface material resistant to bacterial attachment with cleaning by an effective compound enabled the clearance of *Salmonella* Enteritidis from rubber picker fingers. In addition, the electrostatic space charge system was tested against pathogens and bacterial spores. The system reduced bacterial contamination and bacterial spores on stainless steel surfaces up to 99.9 %. A combination of surface modification, chemical control, or alternative interventions that are tailored to site-specific problem areas will be needed to eliminate bacterial contamination by the end of the processing line. **Impact:** ARS researchers reported bacterial resistance to chemical and alternative interventions used in the poultry environment, and found an effective treatment for bacterial spores on surfaces. The strategies increase sanitation efficacy and reduce environmental impact.
- The efficacy of sanitizers in food processing is a critical issue. ARS developed a simulated food processing system for determining how cells of *L. monocytogenes* in biofilms become resistant to commercial sanitizing agents. After repeated sanitizing, starvation, and growth cycles, it was found that the efficacy of sanitizing agents decreases with time. These conditions caused an initial reduction in cell numbers, followed by an

increase in the cell count. The sensitivity of the embedded cells (when extracted from the matrix) to the sanitizing agents did not change. **Impact:** The developing resistance to the harsh conditions was found to be due to changes in the biofilm matrix and not resistance of the cells themselves.

1.2.5 Omics

Genome and proteome analysis are critical to the development of rapid diagnostic tests/methods used by regulatory agencies, industry, and research agencies. Methods development subsequently allows for the detection and enumeration of pathogens during processing and storage (microbial ecology and epidemiology) which leads to the development of predictive models and risk assessments, both of which are critical in assisting regulatory agencies in making food safety decisions that impact public health, and industry for the development of HACCP plans. Microbial models and risk assessment, combined with genomics and proteomics will lead to the development of innovative intervention strategies, and therapeutic agents. These in turn will allow development of better HACCP plans which directly improve the safety of the food supply. Genomics and proteomics will also allow a greater understanding of beneficial genes and their products, which can be utilized for the development of new agriculturally based products. These products could be used for the development of alternate/innovative intervention strategies, particularly in ready-to-eat foods.

- *Arcobacter butzleri* and *Campylobacter lari* are known agents of human bacterial gastroenteritis. *A. butzleri* is transmitted via contaminated food and water and *C. lari* is associated with water and shellfish. ARS closed and completed the genomes of the human clinical strains *A. butzleri* RM4018 and *C. lari* RM2100. Both genomes were fully annotated, with the sequence and annotation data deposited in the NCBI GenBank database. **Impact:** Analysis of the two genomes identified novel genes and pathways, putative niche-specific genes, and potential pathogenicity genes. Completion of the genomes assisted in development of novel *A. butzleri*, *Campylobacter*, and *C. lari* DNA microarrays. Additionally, the *A. butzleri* genome sequence is critical for current development of novel *Arcobacter* typing methods (e.g. MLST).
- *Campylobacter* species, especially emerging species, remain underappreciated as food pathogens and causes of serious illness. The objective addresses fundamental research to develop high-resolution genotyping methods for characterizing and tracking the pathogen in food. ARS assisted in the annotation and analysis of the JCVI *Campylobacter* genomes: *C. jejuni* subsp. *doylei*, *C. fetus* subsp. *fetus*, *C. concisus*, *C. curvus* and *C. hominis*; participated in JCVI oral *Campylobacter* genome sequencing project to sequence *C. rectus*, *C. showae* and *C. gracilis* strains; and characterized 300+ *C. jejuni* strains, by multilocus sequence typing (MLST). ARS developed MLST methods to type *C. concisus*/*C. curvus* and *Arcobacter* strains and typed ~75 human clinical *C. concisus* isolates and 400+ *Arcobacter* strains of human, food, animal or environmental origin. **Impact:** The MLST method was used to elucidate several foodborne illness investigations, including the second largest outbreak of *Campylobacter* illness in US history as a result of contaminated milk processed at a California correctional facility dairy and an outbreak of *C. jejuni* illness that occurred due to raw milk from a small organic dairy. These investigations were conducted at the request of, and in coordination, with the California Department of Public Health.

- The objective was to develop DNA microarrays, to detect and analyze multiple food-borne pathogens and validate assays with food samples. Microarray-based comparative gene indexing (CGI) of seven different species of pathogenic bacteria (*E. coli*, *S. Typhimurium*, *C. jejuni*, *C. coli*, *C. lari*, *A. butzleri* and *Enterobacter sakazakii*) has been completed. Nineteen classes of lipooligosaccharide (LOS) loci from different *C. jejuni* strains were mapped, and mechanisms linked to changes in LOS structures were identified, including indels, gene cassettes, and variable gene inactivation by the deletion or insertion of bases. The role of the bile acid, deoxycholate, in triggering the virulence potential of *C. jejuni* was elucidated. **Impact:** Developed microarrays (panarrays) for speciating and gene-indexing numerous food pathogen strains in a single experiment. The microarrays provided data for identifying species-specific regions and some virulence determinants of interest, thus discriminating isolates for better epidemiology.
- *Clostridium perfringens* causes more than 250,000 cases of foodborne illness in the US, and is one of the bacterial pathogens of concern for regulatory agencies. Multilocus deoxyribonucleic acid (DNA) sequence datasets were developed for a diverse collection of strains from food, veterinary, clinical and environmental sources. These data were combined with data from complete genome sequences and analyzed in order to provide an evolutionary framework for understanding the ecology, virulence, and epidemiology of pathogenic and toxigenic species. **Impact:** *C. perfringens* was previously viewed as a genetically homogeneous Genus. This was shown to be incorrect. The research was the first to demonstrate that the Genus is composed of multiple lineages associated with different disease presentations. The research has significantly enhanced the surveillance capabilities of regulatory agencies and public health agencies (CDC) monitoring *C. perfringens* infections and intoxications. In addition, this information enables veterinary and public health professionals and researchers by providing them the information necessary to develop lineage-specific therapeutics and intervention strategies.
- The bacterium *L. monocytogenes* is an important food-borne pathogen that causes disease in humans and animals. This bacterium is able to grow and survive at food storage conditions such as refrigeration temperatures, low pH and high salt. However, the factors contributing to the survival and growth of this bacterium in food remain unclear. ARS developed a custom-designed commercial microarray containing genes representative of the whole genome of *L. monocytogenes*. The microarray procedure was optimized and excellent images were obtained. **Impact:** Understanding what genes are up and down regulated will assist us to develop strategies to control this bacterium in food. (see following accomplishment).
- In model systems, genes that were identified by the microarray assay were verified by another molecular-based technology, the real-time reverse transcriptase- polymerase chain reaction (RT-PCR) assay. Protein expression experiments were completed and the data analyses revealed proteins expressed by *L. monocytogenes* in the purge from vacuum-packaged frankfurters stored at refrigeration temperature. These findings will lead to the identification of the genes and proteins that are responsible for conferring growth or survival advantages to pathogens in foods, food environments, and under conditions associated with food processing or storage. **Impact:** Ultimately the

information gained on these survival processes will be used to develop targeted interventions that inhibit, control, or otherwise destroy pathogens associated with foods, which will better protect consumers against food borne disease.

- In response to a direct request from the USDA-FSIS, ARS generated baseline data on the prevalence and distribution of *Listeria* epidemic clones and virulence-attenuated strains in foods and food processing environments. Research developed a phylogenetic framework and molecular technologies to define and distinguish *L. monocytogenes* lineages and clones differing in their virulence potential or their ability to persist in food processing environments. **Impact:** This enabled identification of virulence-attenuating mutations and rapid classification of isolates by relative public health risk. This led to a new initiative within FSIS to utilize subtype data in the design and implementation of risk-based inspection programs, described in a recent FSIS report on the future of regulatory subtyping. In addition, this research enabled clone-based risk assessment and led to collaborations with the Grocery Manufacturers Association to develop subtype-specific dose response models for *L. monocytogenes*. (See also next accomplishment).
- As a result of limitations in current subtyping capabilities, the CDC PulseNet Task Force called for the development and validation of DNA sequence-based methods for subtyping *L. monocytogenes* as part of outbreak detection and epidemiological investigations. In response, ARS completed the development and validation of a panel of single nucleotide polymorphism-based subtyping assay (MLGT) for comprehensive subtyping of *L. monocytogenes*. ARS have utilized these assays to determine the prevalence and distribution of strains associated with an increased public health risk as well as strains that harbor specific mutations resulting in a reduced ability to cause infection. **Impact:** This work developed the first single nucleotide polymorphism-based technology for high-through-put subtyping of *L. monocytogenes*. After publishing the methodology, ARS transferred this novel technology to CDC, which funded a special project to acquire the equipment and training (conducted by ARS in 2008) to perform MLGT in their *Listeria* reference laboratory as a replacement or complement to existing subtyping methods. The resulting subtype data and associated strain collection (>1,500 isolates) have been requested and utilized by scientists from around the world. CDC has also asked ARS to evaluate the potential for this technology to be adapted for use with other pathogenic microorganisms (*Salmonella*). (Also applies to 1.2.1).
- The development of novel intervention strategies for the decontamination of produce requires new knowledge of pathogen factors that are involved in plant colonization. Research demonstrated that *Salmonella* has an arsenal of adhesions for plant colonization, including cellulose and O-antigen, and that certain *L. monocytogenes* strains require the sigma factor (SigB), a regulator of response to stress, for efficient colonization of sprouts. **Impact:** During the studies, researchers discovered that two lines of inquiry commonly followed for the identification of plant colonization factors were incorrect. The incorrect assumptions were that genes involved in biofilm formation in abiotic systems also have a role in plant colonization. Additionally, the incorrect assumption was that genes involved in animal cell colonization are also necessary for plant colonization. With this revised knowledge current research will help in

understanding the colonization of plants and animal cells by *S. enterica* and *L. monocytogenes*.

1.2.6 Safety and Health

This Problem Statement was included in the 2006-2010 Action Plan, based on the premise of new funding in the 2006 Federal budget. However, due to changes in research direction within the Department the program was not implemented. Collaborative research between scientists at Beltsville and Wyndmoor that was initially dual coded NP107 Human Nutrition and NP108 Food Safety was redirected solely to Beltsville. The work now supports NP107 Component 6, Prevention of Obesity and Disease: Relationship between Diet, Genetics, and Lifestyle. The project is “Contributing” coded to NP108, that is, it supports the food safety objectives but any accomplishments are credited to NP107. The accomplishments below are simply provided to show that progress has been made.

Probiotics are a class of microorganisms that can establish and grow in a compartment in the host after consumption and provide some positive health benefit. Benefits claimed include protection against pathogens, stimulation of the gut immune system, correcting some bowel diseases, reduced allergic disease, and protection against carcinogens. However, the basis of such claims is often confounded by a lack of demonstrable growth and function of the probiotic in the gut. Scientific validation of many of the claims of probiotics activity is missing. Consumer concern about probiotic reliability, efficacy, and safety can be addressed by hypothesis-based and statistically validated testing of probiotics under controlled experimental conditions. Therefore, the need for this research is to test if dietary probiotics can enhance immune function against infectious agents, prevent the onset of allergenic disease, and improve the function of the GI tract. Studies will provide information on the relationship between probiotics, diet, and immune function and identify biomarkers of nutritional status. Sound scientific evidence for probiotic efficacy will provide the food industry and regulatory agencies with relevant information for concerned consumers. Those at risk of chronic nutrition-related diseases will directly benefit. Research will also impact development of value-added food products, such as those containing probiotics or plant sterols. Other areas impacted will be by increasing opportunities for initiating joint (international) programs in “omics” to develop useful bacterial strains for food use. Future development of probiotic strains to produce innovative foods with increased bioactive capabilities useful for improved food safety and [food] security.

Accomplishments for FY 2006

- The immune response of probiotic treated pigs was measured by changes in gene expression of immune mediators and indicated that there is a local activation of the innate-immune-system in the intestinal mucosa and draining lymph nodes. This response is enhanced in *Bifidobacterium lactis*-treated pigs from treated sows. The results suggested that administration of probiotics in the diet may be an alternative stimulant of the neonatal immune system improving responses to subsequent infectious agents. Other strains from the *Lactobacillus* species have been tested. The level of host innate-immune-system activation was down-regulated compared to vehicle-treated control pigs over several sites in the large and small intestinal mucosa. These results indicated that there are important differences among probiotic strains. Certain probiotic strains induced innate immune activation in intestinal sites where numbers of bacteria are high and down-regulated genes in more distal sites in the small intestine mucosa. Other strains

may have a more general pattern of down-regulation of innate-immune gene expression throughout the intestine.

- Dietary-induced oxidative stress alters the gut immune response associated with a food-borne pathogen in a mouse model. Mice fed a diet deficient in selenium and vitamin E had an altered expression of genes associated with immune function, enhanced pathology, and delayed clearance of a *Citrobacter rodentium* infection in the colon. Deficient mice also had an elevated expression of genes associated with inflammation. Other gene expression studies showed increased expression of a selenium-dependent antioxidant enzyme, glutathione peroxidase 2, in response to infection in the colon suggesting an important role for this enzyme in colonic immune function. Heme oxygenase, an antioxidant enzyme, was also increased in response to the combination of dietary antioxidant deficiency and *Citrobacter rodentium* infection.

Accomplishments for FY 2007

- Probiotic (*Bifidobacterium lactis*)-treated pigs showed changes in gene expression of immune mediators indicating local activation of the innate immune system at the intestinal mucosa and induction of changes that improved intestinal permeability; the response is enhanced in probiotic-treated pigs from probiotic-treated mothers. Thus, probiotic in the diet ameliorated inflammatory responses against intestinal parasites and bovine milk used as a food allergen. Other probiotic strains tested showed down-regulation of the host innate immune system indicating that there are important differences among probiotic strains. Some strains down-regulated genes in distal sites in the small intestine mucosa, while others have a more general pattern of down-regulation throughout the intestine.
- Food-borne Pathogen Model Demonstrates that Dietary Induced Oxidative Stress Increases Pathology: *Citrobacter rodentium* infection in a mouse model mimics many of the pathologies associated with *E. coli* infections in humans. Mice fed a diet deficient in selenium and vitamin E had enhanced pathology and delayed clearance of *C. rodentium* that appear to be related to a delayed immune response to infection demonstrated by decreased cytokine production.

Accomplishments for FY 2008

- Certain probiotic bacteria improved intestinal immune and barrier function. Pathogen- and allergen induced changes in glucose absorption and epithelial resistance in the jejunum were reversed when young pigs were fed *Bifidobacterium lactis* (Bb12-probiotic) daily from birth. The immune response of probiotic-treated pigs measured by changes in gene expression of immune mediators indicated local activation of the innate immune system. This response is enhanced in Bb12-treated pigs born to Bb12-treated

sows compared to Bb12-treated pigs born to non-treated sows. This supports studies in humans that showed feeding probiotics to pregnant women positively affected probiotic colonization and improved health in their children. Other probiotic strains from the genus *Lactobacillus* have been tested with different outcomes. The level of activation of the innate immune system at several intestinal sites was decreased by feeding *Lactobacillus* strains to pigs. An in vitro model was developed that used intestinal pig epithelial cells (IPEC-1) to evaluate interactions between probiotics and pathogenic bacteria. Exposure of IPEC-1 to *Salmonella* in culture induced a panel of inflammatory genes that were reduced when co-cultured with probiotic strains.

- Initial studies have examined the effect of selenium and/or vitamin E deficiencies on orally-acquired reovirus or *Salmonella typhimurium* infections. These results indicate that clearance of these infections was not affected by deficiencies in selenium and/or vitamin E. A new model to replace the *Salmonella* and reovirus models has been developed. *Citrobacter rodentium* is a gram-negative bacteria that shares many important structural and functional characteristics with enteropathogenic *Escherichia coli* (EPEC) strains including the ability to induce attaching and effacing lesions in the colon of mice that are indistinguishable from those produced by *E. coli* in humans. Thus *Citrobacter* can serve as a useful model to study the effect of diet on host immunity to an important foodborne pathogen.

Impact for the 3-years:

- This research promotes the concept that probiotics in the diet can differentially affect immune components that enhance resolution of infectious and chronic disease. The studies will be of great interest to physicians and other health care workers that deal with acute aspects of infectious disease and offer scientifically-based dietary recommendations for improved health.
- It was demonstrated that a deficiency in the antioxidants selenium and vitamin E resulted in enhanced pathology and reduced ability to clear *C. rodentium* infections. Since the levels of these antioxidants vary in different foods, this information will be useful to all individuals that alter their diet to maximize inclusion of these important food components to minimize the negative disease consequences of exposure to these pathogens.

1.2.7 Risk Assessment

ARS does not conduct risk assessments (RA) per se. The Program does, however, provide data, models, and expertise in order for the regulatory/action agencies to conduct their RA. ARS is recognized as a world leader in the development of food-related predictive models, and these models are an integral part of microbial risk assessment used to support food safety measures adopted by member countries of the WTO. Also, these models assist in identifying specific food processing steps, and associated Critical Limits that can serve as Critical Control Points in HACCP systems, as well as to facilitate food safety decisions when process deviations occur. Models underpin the establishment of Food Safety Objectives, and assist public health by determining host responses to specific challenges. Research addresses realistic processing and handling conditions that provide greater value to food industries, risk assessors, and risk managers. The validation of current models, and development and validation (including accuracy) of new models are critical since there are no universally accepted criteria for determining safe prediction zones. Models with the greatest value must couple microbial ecology with a better understanding of the physiological responses of microorganisms to stressors (interventions) used in food processing. Studies also address through creative approaches the behavior of Select Agents in foods (see Problem Statement 1.2.9) since they might not follow conventional pathogen growth and survival patterns. A quantitative database can be achieved through further expansion of Combase through Center for Excellence in Microbial Modeling and Informatics (CEMMI).

- Improper storage and/or inadequate cooling practices in retail food operations have been cited as the cause of food poisoning for 97% of *Clostridium perfringens* outbreaks. Mathematical models were developed (at the request of the USDA-FSIS) to predict the growth of *C. perfringens* at temperatures applicable to the cooling of cooked meats. The models were incorporated into the USDA-Pathogen Modeling Program (PMP) and the data were contributed to ComBase. The PMP is downloaded 8,000 times per year in over 35 countries and used by 40% of FSIS-inspected food companies. The models save a large manufacturer \$1-\$2M per year and enable the food industry to determine compliance with the regulatory performance standards. **Impact:** FSIS routinely uses the information to aid in evaluating the safety of cooked products after cooling. The research addressed data gaps identified in risk assessments. The data were used by FSIS, FDA, and Health Canada, for the microbial risk assessment for cooked meats, and served as the scientific basis for the FSIS regulations on performance standards for cooling cooked products. Results were cited in a petition to the FDA Retail Food Protection Branch for pending decisions by FDA to change the Food Code recommendations.
- *Salmonella* is a leading cause of human illness and is often found associated with the skin of chicken. However, little is known about the ability of *Salmonella* to survive and grow on chicken skin during retail sale and home storage. ARS developed methods to investigate and model survival and growth of human bacterial pathogens from a low initial dose on food with native microflora. The methods were used to develop the first validated models for survival and growth of *Salmonella* from a low initial dose on

chicken products with native microflora. Data and modeling methods from this research were provided to the FSIS Risk Assessment Division to fill an important data gap in their risk assessment for Salmonella and chicken. In addition, the models were incorporated into PMP and the data were archived in ComBase for use by other modelers. **Impact:** This research has directly impacted food safety regulatory policy of USDA and has solved a problem that has perplexed scientists for many years – how to develop models in food with native microflora and using a low and ecological dose of the pathogen.

- The use of raw meat in the manufacturing of fermented dry and semidry sausage may introduce *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* spp into the finished product. Studies were conducted to collect the inactivation data of *L. monocytogenes*, *E. coli* O157:H7 and *S. typhimurium* in sausage. Predictive models describing the rates of inactivation of *L. monocytogenes*, *E. coli* O157:H7 and *S. Typhimurium* in fermented sausage during fermentation, drying, and storage were developed. **Impact:** The models are particularly useful for small and very small fermented sausage producers to identify product formulation and processing/handling conditions that minimize the presence of these pathogens in finished products and ensure the safety of their products. Processors use the models to determine the process/product parameters to determine compliance with the regulatory requirements.
- ARS developed dynamic models to predict the growth of *Salmonella* spp. in beef and chicken under time-varying temperature conditions, and successfully tested and validated four different kinetics models. The dynamic models are being used by processors to evaluate the safety of the storage facilities and also to evaluate the safety of the product in case of temperature abuse or process deviations within their HACCP systems. Regulatory agencies use the models to develop safe holding temperatures for beef and chicken and to set critical limits in HACCP plans for beef and chicken to prevent potential growth of *Salmonella*. **Impact:** This research addressed important data gaps in risk assessment. Accordingly, the models are being used by food processors and regulatory agencies, risk assessors and risk managers, to determine food processing and handling conditions that affect the risk of foodborne disease.
- ARS explored modeling issues that impact estimates of microbial risk. Developed a predictive model that accounted for the sigmoidal shape of the *E. coli* O157:H7 survival curves and the effect of serotypes. Studies developed models to describe the growth kinetics of *Salmonella* in meat, pre- and post-thermal treatment. **Impact:** The models estimate the effectiveness of a thermal process and provide a more accurate, truly kinetic-based modeling tool for risk assessment, and allow both the food industry and the regulatory agencies (Codex Alimentarius Commission, FDA and FSIS risk assessors) to conduct more realistic and accurate assessment of the risks of pathogens in processed foods.
- The effect of lactate and diacetate on *L. monocytogenes* in ham at temperatures that the product is likely to be exposed to during manufacturing and distribution is lacking. Predictive models describing the behavior of *L. monocytogenes* in ham containing sodium lactate (1.0-4.2%) and sodium diacetate (0.05-0.2%) at storage temperatures of

0°-45°C were developed. **Impact:** These robust and validated predictive models elucidate the effects of additives, product formulation, and storage temperature on *L. monocytogenes*, and enable producers to select concentrations of additives, product formulations, and storage conditions that are able to control growth of *L. monocytogenes* in ham and salad products.

- Insufficient lethality of thermal treatments is believed to be the primary contributing factor in outbreaks associated with the consumption of meat and poultry products. Research defined heat treatments, in the presence of other inhibitory factors, required to achieve a specified lethality for *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* in meat and poultry. Research also developed predictive models for the pathogens to predict D-values. **Impact:** Using these models, food processors rapidly get accurate estimates of pathogen survival, formulate foods to include acknowledged intrinsic barriers, and design reduced thermal processes that ensure safety against pathogens. The models were incorporated as part of a user-friendly USDA-PMP, and the data were contributed to ComBase, an international microbial modeling database. The models served as the scientific basis for the regulatory performance standards/compliance guidelines for cooking of meat, in risk assessment models, and in food inspection programs.
- Food safety managers currently lack the ability to predict the microbial pathogen transfer in slicing operation for ready-to-eat foods. ARS developed microbial surface transfer models to describe and predict cross-contamination with *L. monocytogenes*, *E. coli* O157:H7 or *Salmonella* spp. during mechanical slicing of deli meats. Using Confocal Laser Scanning Microscopy, research demonstrated that the slicing surface roughness significantly affects the pathogen attachment or adhesion to the blade surface, thereby affecting the microbial surface transfer during slicing operations. **Impact:** The models are used for developing HACCP plans and in risk assessments for ready-to-eat meats. Better understanding of cross-contamination with pathogens has assisted the food industry in improving the retail equipment and operations for RTE deli meat, which will reduce the risk of food poisoning outbreaks. The models were incorporated into the USDA-PMP.

1.2.8 Pathogenicity

Microbial genomic efforts, for example, the availability of genome (and gene) sequences and microarrays have helped enhance our understanding of pathogenicity and virulence. The Program has already begun, through sequencing and annotation efforts, to compare different species and strains to determine phylogenetic relationships, especially in relation to ecological niches. Studies provide the ability to understand the nature and differences between pathogens and non-pathogens, and identify factors that encode for variations in virulence. Physiological characteristics of pathogens are known to be affected by stress. Understanding the role of intrinsic and extrinsic stress responses is critical. This work will determine the effect on virulence mechanisms which directly impacts pathogenicity and the emergence of new pathogen types. Understanding the role of quorum sensing is also important. If virulence is correlated with quorum sensing this could potentially be a mechanism to disrupt or negate pathogenicity by targeting communication pathways with novel antimicrobials. Research will allow better risk assessment and allow more efficient allocation of risk management resources by focusing only on the critical pathogens in the food supply.

- Bacteria produce signaling molecules through a process known as quorum sensing resulting in changes in gene expression. Gene expression analyses using DNA microarray technology was used to determine if quorum sensing through the production of the AI-2 signaling molecule plays a role in survival of *C. jejuni*. The presence of AI-2 increased the expression of genes involved in motility, tolerance to oxidative stress, and membrane transport, and also resulted in more rapid growth. These results suggest that cell-to-cell signaling through the production of AI-2 may play a positive role in survival, motility, and pathogenicity of *Campylobacter*. **Impact:** These findings may be applied to the design of intervention strategies to disrupt AI-2 signaling in *Campylobacter* leading to decreased survival in food and the environment and potentially decreased virulence in humans.
- The Genus *Campylobacter* comprises different species that have been associated with food- and water-borne illness. The different species form biofilms on different food processing-relevant surfaces under various conditions. An understanding of the conditions required for *C. jejuni* and *C. coli* to form biofilms and persist in the environment will help in the development of control strategies. *C. jejuni* and *C. coli* exhibit significant strain to strain variation with regards to the ability to form biofilms. *Campylobacter* isolated from clinical disease cases compared to those isolated as environmental contaminants demonstrated no significant advantage of one group over the other with regards to biofilm forming ability. Furthermore, it was determined that salt positively influences the biofilm forming ability of *C. jejuni* and *C. coli* strains; however, variation in the concentration of salt required to enhance biofilm formation was observed among different strains. **Impact:** These results have significance given the fact that many poultry producers add salt to the liquid added to chickens packaged for retail sale.
- The formation of biofilms allows pathogens to persist in food environments and renders them more resistant to many conventional methods of inactivation. In biofilms composed of more than one type of bacterium, the presence of non-harmful bacteria could play an

important role in increasing the persistence and resistance of *E. coli* O157:H7 on surfaces. The augmentation of *E. coli* O157:H7 biofilm formation by growth along with a non-O157:H7 strain of *E. coli* isolated from the same outbreak was shown. Genes and proteins involved in biofilm formation and the mechanisms for cooperative biofilm formation and for transforming *E. coli* O157:H7 into a more virulent and resistant variant were revealed. **Impact:** This research begins to explain how strains of *E. coli* O157:H7 can persist in pre- and post-harvest environments and provides new perspectives on the environmental niches of this pathogen. (Also applies to Program Statement 1.2.3)

- Studies demonstrated that *E. coli* O157:H7 strains incorporated in biofilms are as much as 10,000 times more resistant to hydrogen peroxide than non-biofilm cells. Proteomic comparisons showed that resistance was mediated not only by exposure but also by increased production of specific resistance proteins. Through construction of mutant strains and gene expression analyses, a comprehensive characterization of all of the existing serotype O157:H7 peroxide-stress-genes were performed. Comparative studies were also done on planktonic (non-biofilm) versus biofilm bacteria. **Impact:** This research will define the mechanism of serotype O157:H7 peroxide resistance in biofilms and provide researchers with critical information for designing intervention strategies to eliminate *E. coli* from foods and processing surfaces.
- *L. monocytogenes* is frequently isolated from ready-to-eat food and aquaculture products. Interestingly, *L. monocytogenes* strains demonstrate varied pathogenic potential. Studies have demonstrated that many *L. monocytogenes* isolates from channel catfish are nonpathogenic, and research has identified genes that can be used to distinguish pathogenic and nonpathogenic isolates. Studies also demonstrated that the internalin gene family, which includes key virulence factors of *L. monocytogenes*, diversifies according to a birth-and-death model, and is subject to lateral gene transfer occurrences. As a result, this research characterized the mechanisms leading to diversification in these virulence-associated genes and provided the first example of birth-and-death evolution within a bacterial multigene family. **Impact:** The information generated from this research is critical to understanding the genetic basis for differences in pathogenicity and the genetic mechanisms leading to changes in pathogenicity. (This work was conducted in collaboration with Mississippi State University).
- Pathogenic strains of *Vibrio vulnificus* are natural inhabitants of estuarine environments worldwide and can be transmitted to humans to cause illness and death through consumption of raw shellfish. Studies determined that a *V. vulnificus* enzyme discovered by ARS cleaves lysine from the amino-terminal ends of human-derived (kinin-like) peptides. This process may trigger inflammation, increase permeability of blood vessels, and dilate blood vessels - conditions that would favor enhanced invasiveness of the bacterium. To measure this activity, ARS identified and published the first rapid, simple, inexpensive, sensitive, and quantitative biochemical test (fluorescent ninhydrin assay) to detect the enzymatic release of lysine from proteins and peptides. **Impact:** As insights are gained into the proteolytic mechanisms involving bacterial infections, it seems likely that non-antibiotic-related interventions directed toward blocking the enzymes' activities or the development of new receptor blockers may provide the key for preventing or controlling illness and death from *Vibrios* and other bacterial pathogens.

- A new area of produce research is to study the interaction between enteric pathogens that are linked to contamination of fresh produce and ciliated protozoa that are commonly isolated from crop production environments. Research established the identity of a soil-dwelling ciliate (in the genus *Tetrahymena*) which enteropathogenic *E. coli* use to synthesize protective vesicles which render them less susceptible to disinfectants. This accomplishment opened new avenues for research on the “Trojan Horse” hypothesis and describes unexpected benefits, to bacterial food pathogens, and of their interactions with bacteriophagous eukaryotic protists. **Impact:** This discovery helped ARS garner external financial support from the USDA-NRI. This accomplishment was built on a successful test of the hypothesis that the carriage of the Stx-encoding prophage increased the survival of *E. coli* O157:H7, *E. coli* K-12, and other acutely virulent bacterial isolates when incubated with grazing *Tetrahymena pyriformis*. Thus, natural interactions between protists and the bacteria upon which they feed may explain why some bacteria develop virulence factors responsible for clinical disease.
- *Toxoplasma gondii* is a leading cause of foodborne illness worldwide. However, little genetic evidence exists linking human infection with *Toxoplasma* strains infecting food animals. Research isolated a single, secreted kinase that serves as a major virulence factor in *T. gondii*. Strains engineered to abundantly express this protein are exceptionally pathogenic to mice and that naturally virulent parasite strains express this protein do so. This represents one of just a few virulence factors validated in a eukaryotic pathogen, and provides a mechanistic understanding for pathogenesis. **Impact:** The finding’s ultimate impact cannot as yet be determined

1.2.9 Food Security

Agencies within the Federal government have begun to implement strategic action plans to further ensure the safety of the Nation's food supply. It is apparent that even if a small number of contaminants were intentionally introduced into some part of the food chain, such an action would have the potential to seriously damage public confidence. Apart from the impact on health and safety, bioterrorism against the food supply would also directly harm the nation's economy. U.S. agriculture employs nearly one quarter of the workforce, and annually contributes over one trillion dollars to the gross domestic product. The food production industry annually exceeds two hundred billion, with exports over fifty-five billion. To enhance the effectiveness of food security, a layered approach was taken. The Program was limited to areas of expertise, and often the use of surrogate organisms, since BL-3 facilities were not available. The majority of research conducted was in response to specific requests from defense/security related agencies (DHS/FBI/DoD). Some projects were specifically funded by the Department of Homeland Security. The details of research considered "classified" has not been included within this document.

- In order to perform a risk assessment for potential toxins in food matrices, it is necessary to elucidate the toxicity of crude toxins, in comparison to the better studied purified toxins. ARS investigated the effects of heating crude ricin toxin spiked into food matrices on its bioavailability and oral mean lethal doses, and compared with results for purified ricin. **Impact:** The results of the studies, funded by the DHS, were used to complete a risk assessment that helped guide government agencies and food industry scientists to develop policies and procedures to maintain a safe and secure food supply. The information was also transferred to other requesting defense agencies.
- There is a need for rapid, sensitive assays for detection of castor bean's toxic protein, ricin, in intentionally contaminated foods, without the use of animal bioassay. A new quantitative polymerase chain reaction (QPCR) assay was developed for detection of castor plant material in foods. Although ricin is a protein toxin, crude preparations contain castor DNA that can serve as a marker for this bioterrorism toxin. **Impact:** The new QPCR test can detect the minute amounts of castor plant DNA that would be present in a food, if adulterated with a crude preparation of ricin. The technology was transferred to the appropriate agencies.
- To avoid false positives from heat-killed toxin, detection of bioactive ricin requires a sensitive test for its characteristic enzymatic activity, in which the toxin inactivates the ribosome, the cell's protein-making apparatus. ARS research assessed the utility of a recently developed cell-free ribosomal translation assay, and optimized conditions for detecting biologically active ricin in food samples. **Impact:** The results suggested that some components in these food matrices protect the activity of ricin against heat. This information was transferred to the appropriate agency, and will help to provide effective food defense.

- ARS researchers developed a panel of monoclonal antibodies to ricin. Antibodies were identified that were effective for detection of ricin in milk, egg, and ground beef in various immunochemical formats such as sandwich ELISA. **Impact:** Developed a rapid and reliable assay for ricin that will protect the food supply. The technology was transferred to the appropriate agencies.
- Along with potent neurotoxins (BoNT), the poisonous “cocktail” produced by foodborne Clostridium botulinum bacteria includes other non-toxic proteins. ARS studies compared the toxicity of crude versus purified BoNT preparations presented to mice in food, and showed that these accessory proteins protect BoNT from destruction and inactivation in the digestive tract, significantly increasing oral bioavailability. **Impact:** Understanding the stability of these biothreat toxins will help government agencies and food processors develop strategies for maintaining a safe and secure food supply. The information was also transferred to other requesting defense agencies.
- Clostridium botulinum neurotoxins (BoNT), produced by the anaerobic bacterium C. botulinum, are the most toxic agents known. Therefore, tests must be extremely sensitive to be practical. Most testing for BoNT is currently performed using live mice, the gold standard. ARS research developed four new monoclonal antibodies (mAbs) that bind specifically and unusually strongly to BoNT’s (types A and E). The mAbs were used to develop a new test for BoNT, called a sandwich ELISA, which represents a realistic alternative to the mouse bioassay. **Impact:** Since the assay requires both the heavy and light chain to be intact, a positive result can be considered presumptive positive for biological activity. The new, high-affinity monoclonal antibodies have been used to develop a new assay method, under a CRADA with Safeguard Biosystems Corporation, a company with expertise in developing rapid, field-deployable assays. A lateral flow device (“dipstick”) has been prepared for detecting toxin in liquid matrices such as milk. Simple devices like this prototype can be used by farmers, dairies, regulatory agencies, and even consumers to ensure food safety and security. The technology was transferred to the appropriate agencies.
- BoNT causes fatal paralysis by cutting a specific protein involved in nerve transmission. In a newly developed test, BoNT is first concentrated using antibodies and magnetic beads. Then the toxin cuts a specially designed peptide molecule that has structural features similar to the natural protein target, producing highly fluorescent fragments. **Impact:** This fast and effective assay system could be used for large-scale screening to detect BoNT, replacing widely used animal bioassays, and helping government agencies and food processors assure a safe and secure food supply. The technology was transferred to the appropriate agency.
- Food security has become more important during this time of heightened threats, and rapid detection methods are needed for biotoxins, such as microbial enterotoxins. ARS developed rapid and sensitive methods for detection of Staphylococcus aureus enterotoxins A and B (SEA and SEB) with a detection capability at or below 1 part per billion. SEA and SEB detection was accomplished in fortified liquid egg and chicken extracts, milk, ham, hot dogs, and water using novel biosensor (BIAcore) and

fluorescence microparticle immunoassay (FLMIA) approaches. **Impact:** The field-based FLMIA analysis costs <\$1 per test and takes 2.5 hours for 20 samples, whereas the lab-based BIAcore analysis takes 30 minutes for sample preparation of a batch of samples and 15 min each for sequential analysis. The biosensor analysis is fully automated and it is anticipated that this method will be utilized for multi-toxin detection of other toxins in various food matrices. A company has established a cooperative development agreement with ARS based on the FLMIA approach.

- Shiga toxins are expressed by some strains of the bacterium *E. coli* and are associated with serious, sometimes fatal foodborne disease. ARS studied whether milk pasteurization inactivates this toxin by measuring the effects of toxin on cells in tissue culture. **Impact:** The data demonstrate that the toxin is relatively heat-stable and that conventional pasteurization of milk does not eliminate its toxicity. This information will help guide government agencies and food processors to protect food safety and security.
- *Yersinia pestis* is the causative agent of bubonic plague. ARS was requested to develop and validate an immunomagnetic method for the detection of the agent in food. **Impact:** The details of the research were communicated to the requesting agency for their adoption to detect potential food security threats.
- *Yersinia pestis* is the causative agent of bubonic plague. ARS was requested to determine the behavior of *Y. pestis* to ensure the safety of liquid foods, especially liquid egg. D-values at 54°C were 1.58 min and D₆₀ values were 13.6 and 11.13 s by the addition of 0 and 965 IU of nisin, respectively. When stored at 4, 10, and 15°C, populations in untreated egg increased by 7 log within 3, 2 and 1 week, respectively. Low molecular weight chitosan (0.5%) and an activated lactoperoxidase system (2.18 U/ml) were ineffective, while 500 IU/ml nisin inhibited populations by up to 1 log CFU/ml in LWE at all temperatures, when compared to the control. **Impact:** The information was transferred to the appropriate agency.
- Pharyngeal plague in humans has been associated with consumption of meat prepared from animals infected with *Y. pestis*. The risks of contracting plague from consumption of deliberately contaminated meat were currently unknown. A predictive equation to describe the effect of ionizing radiation and temperature on the inactivation of avirulent *Y. pestis* suspended in raw ground pork was developed. **Impact:** It was found that irradiation can easily inactivate *Y. pestis* in meat at either refrigeration or subfreezing temperatures. It was also found that irradiation could also easily inactivate *Y. pestis* on frankfurters. The radiation resistance of *Y. pestis*, which is capable of growth on refrigerated frankfurters, was easily inactivated by gamma irradiation. The information was transferred to the appropriate agencies.
- The potential growth of virulence plasmid-bearing *Yersinia pseudotuberculosis* (YPST) at refrigerated temperatures could pose an increased health risk for contaminated retail ground beef if commercial and consumer storage is extended for a longer period such as 4 to 6 weeks. Therefore, a growth model of plasmid-bearing YPST in ground beef was developed. The data demonstrated differences in the phenotypic expression of the 70-kb

virulence plasmid (pYV/pCD)-associated genetic determinants in *Y. pestis* and *Y. pseudotuberculosis* (YPST). Research established that the potentially unstable virulence-associated plasmid is retained in YPST during its growth in ground beef and developed a growth kinetics model. **Impact:** The findings assist the government risk assessors and food companies to detect and predict the fate of YPST and *Y. pestis* where bulk foods could be contaminated, thereby exposing a relatively large number of individuals. This information helps improve exposure assessment and aid in designing more effective food safety controls.

- Potential breaches to the security of our Nation's food supply may arise due to a terroristic addition of threat agents such as *Bacillus anthracis* to higher risk foods. ARS was requested to develop intervention strategies using pasteurization and microfiltration technologies to eliminate *B. anthracis* spores in bulk, fluid milk. A 30-gallon capacity pilot plant scale microfiltration unit was used to establish that microfiltration of milk prior to pasteurization has the ability to remove greater than 99.9999% of BA spores while maintaining the quality of milk. **Impact:** The addition of a microfiltration step, in conjunction with an ultra pasteurization step for the retentate, will lessen the likelihood that pathogenic bacteria and/or their spores that are greater than 0.8 μm in size will contaminate the milk supply. The information was transferred to the appropriate agencies.
- T-2 toxin is a trichothecene mycotoxin produced by cereal-pathogenic *Fusarium* fungi. The mycotoxin is capable of infesting human food and animal feeds. T-2 toxin and its metabolite HT-2 are acutely toxic to animals. Sensitive detection of T-2 and HT-2 toxins was somewhat limited because these compounds do not fluoresce and do not have a strong ultraviolet or visible absorption band. Sensitive methods to detect both T-2 toxin and HT-2 toxins, by labeling them with novel fluorescent tags, were developed. **Impact:** The tagged T-2 and HT-2 toxins are very stable and the fluorescence response of the tagged toxins is an improvement over previous labeling materials, resulting in more sensitive assays for these toxins. The information was transferred to the appropriate agencies.
- The clinical condition caused by ingestion of the mycotoxin T2 is called "alimentary toxic aleukia" and a host of symptoms related to organs as diverse as the skin, airway, and stomach occur. T2 is the only mycotoxin known to have been used as a biological weapon. T-2 toxin is also acutely toxic to chickens. The scientific literature has suggested that a small portion of T-2 consumed might be transmitted into the eggs, although a method for analysis of this toxin in eggs has not been previously published. A sensitive method to detect both T-2 toxin and a related toxin (HT-2) in chicken eggs was developed. The method will be useful in studies to determine the possible transmission of T-2 into eggs and as a way to monitor for the presence of these two toxins in commercial eggs. The information was transferred to the appropriate agencies.

Component 2

2.1 Mycotoxins and Plant Toxins

The Plant Toxins section of this Component was realigned by the Office of National Programs to National Program 215 (NP215) Pasture, Forage and Rangeland Systems in late 2005. The research is now conducted in the new Poisonous Plants Research Laboratory, Logan, Utah. The research projects are only contributory coded NP108 for internal use. Although the 2006-2011 still outlines the research program, no accomplishments are included in this document.

2.1.1 Toxin Methodology and Identification

Protection of both the human food supply and animal feeds requires the rapid detection of mycotoxins in commodities, foods, and feeds. This includes both the commodities with which they are usually associated as well as those in which they have not traditionally been found, the latter as a tool in support of enhanced food security. Since mycotoxins are unevenly distributed in contaminated plant commodities, both regulatory agencies and the producing industries need methodologies to accurately determine the contamination of the entire tested lot, which may be thousands of tons, as in a loaded river barge.

Developing and validating technologies and rapid screening assays including on-line methods will aid high speed, high volume commerce, and the regulatory agencies. Fortunately, there is a commonality of interests among these groups which allow similar technologies to be used by each. Technologies will provide data to help resolve food safety and food security concerns, to carry out risk assessments, to develop and validate predictive microbial models, and to identify areas where interventions are most critically needed. This information will assist in continually assuring production and delivery of safe food and feed products.

The accomplishments listed in this Problem Statement assume the need for specific detection technologies, so there may be no justification sentence attached.

- Developed the first robust phylogenetic framework and molecular diagnostics to differentiate and identify *Fusarium* head blight (FHB) pathogens of wheat and barley and determine their trichothecene mycotoxin potential. Novel multilocus genotyping (MLGT) technology was used to conduct active molecular surveillance of FHB species and trichothecene toxin chemotype diversity within North America. Species and mycotoxin chemotypes were determined for over 3,000 North American FHB, providing the first evidence of a rapid, adaptive evolutionary shift in FHB pathogen populations. This study conclusively demonstrated that a highly toxigenic 3ADON *F. graminearum* population has been rapidly replacing the dominant 15ADON FHB pathogen in North America. The most striking finding was that isolates from the two 3ADON populations accumulated significantly more trichothecene than isolates from the 15ADON population. Specifically, our data indicate that FHB species and population-level

variation require consideration in the development of cereal cultivars with broad-based resistance to FHB pathogens. **Impact:** This research provided critical information to quarantine officials in APHIS focused on preventing the introduction of foreign pathogens into the US and to plant breeding efforts towards developing cereals with broad-based resistance to FHB.

- Developed the first multilocus DNA typing schemes and allele-specific microsphere array for typing human pathogenic fusaria. The typing schemes and microsphere array technology were developed at the request of the CDC to aid in epidemiologic investigations of the sight-threatening *Fusarium keratitis* outbreaks within the U.S. and Asia during 2005-2006. **Impact:** The results of these studies provide critical information and technology to public health scientists charged with the prevention and control of diseases caused by these pathogens.
- Studies of ecology, etiology, medical mycology, toxicology and pathology in *Aspergillus* rely on correct identification of isolates for a meaningful interpretation of the results. Prior to this study, identification of isolates was based primarily on phenotypes. A multilocus phylogenetic analysis of the genus *Aspergillus* was completed. **Impact:** This study establishes that there has been a major convergence occurring in the Genus, rendering phenotypic identification problematic for many species including the ochratoxin-A producing species. Research also established the occurrence of paralogy in the beta-tubulin gene, indicating that single locus studies often used for phylogenetic and identification purposes can be misleading. In addition, the database of nucleotide sequences determined from this study provides the necessary information for development of oligonucleotide probes for rapid species identification.
- Developed a pyrosequencing assay that allows for quantification of incidences of atoxigenic strains on treated and control crops. An assay that does not require culturing was developed and proofed against cottonseed samples from commercial fields. **Impact:** The pyrosequencing assay reduces the labor associated with evaluating the efficacy of biocontrol agents. It is currently in use in commercial field trials.
- A laboratory peanut seed assay system was developed to quickly evaluate nontoxigenic strains of *Aspergillus flavus* as potential aflatoxin biocontrol agents. With this assay, different toxigenic and nontoxigenic strains can be quantified and inoculated onto wounded peanut seeds to determine relative strain competitiveness. Thus, nontoxigenic strains with enhanced abilities to compete with toxigenic strains can be identified for further field evaluation. **Impact:** Use of this assay saves much time in the identification of strains that can be used as more effective biocontrol agents in the future. The potential impact is the development of new and improved aflatoxin biocontrol products.
- Developed and assessed various cultural methods to characterize aflatoxin production by isolates of *Aspergillus*. The use of the techniques has enabled large scale ecological studies characterizing the response of various variables on the displacement of toxigenic isolates of *Aspergillus*. **Impact:** These inexpensive screening tests can be of utility for lesser developed nations in assessing the threat for aflatoxin contamination.

- A multigene phylogenetic classification was developed for all known ascomycetous yeasts and this work permitted assignment of species to genetically defined genera. This work resulted in redescription of presently known genera and the description of 14 new genera. With these changes, genera are now assigned species that share common genetic and physiological properties. The gene sequence-based bar coding system that was developed for all known ascomycetous yeasts in the previous research cycle was utilized for development of a rapid identification system employing a flow cytometer and species-specific DNA probes attached to microbeads. **Impact:** The advances in classification and rapid identification are now being applied to food and beverage spoilage yeasts as well as biocontrol yeasts and will result in species-specific DNA probes for rapid identification of these fungi. Once correctly identified, the spoilage species will be correlated with their resistance to various food preservatives.
- Aflatoxins are potent carcinogens produced by certain *Aspergillus* fungi, and are routinely found in commodities and foods worldwide. For analysis the aflatoxins have traditionally been extracted from grains using solvents. In the early days of aflatoxin analysis this was done using chlorinated solvents, but handling of chlorinated solvents presents safety hazards, and most current analytical methods for aflatoxins use non-chlorinated solvents such as methanol or acetonitrile. ARS developed a solvent-free aqueous solution for extraction of aflatoxins from corn. The extraction is based upon the detergent sodium dodecyl sulfate (SDS), which is widely available and inexpensive. Extraction with the aqueous SDS was slightly less efficient than with a commonly used methanol-water extraction, yet recoveries of aflatoxins B1 and G1 from corn were very good. **Impact:** This was the first evidence that solvent-free extraction of aflatoxins from corn may be feasible, reducing the need for expensive solvents, improving the safety of the extraction, and reducing solvent disposal costs.
- Cyclopiazonic acid (CPA) is a mycotoxin produced by some of the same species of fungi that produce the more widely known aflatoxins. As a consequence, CPA and the aflatoxins may co-occur in commodities under certain conditions. CPA is not fluorescent, so most methods for detecting this toxin rely upon its absorbance in the ultraviolet (UV) region. Research determined that, upon exposure to strong UV light (CPA) reacts to form fluorescent products. Because fluorescence is generally less of a common phenomenon than absorbance, this effect may be used to impart an additional level of selectivity upon analyses for this toxin. **Impact:** The ability to photolyze CPA and detect this toxin by fluorescence may open up new avenues for determination of this mycotoxin alone or together with the aflatoxins.
- Some mycotoxins may be of concern in baby food since many of the formulations are cereal-based. ARS developed a protocol to analyze deoxynivalenol in commercially available baby cereals. Mixed cereals were selected for analysis because wheat was listed as a component on the box. A low level of deoxynivalenol was detected in the cereal, but the amount present was quantified to be present at a level below the level (20 ppb for processed cereal-based foods for infants and young children), regulated by the European Union. **Impact:** The methods provide for accurate detection and quantification of deoxynivalenol. This represents the first report of deoxynivalenol in baby cereals in the US.

- Developed a method for analysis of fumonisins (FB), sphingoid bases and sphingoid base-1-phosphates in plant tissues and discovered that maize resistant to *F. verticillioides*-induced seedling disease did not accumulate FB in leaf tissue, nor was sphingolipid metabolism disrupted. This was not the case in the susceptible variety indicating that both sphingolipid metabolism and FB accumulation were critical for seedling disease development. Only one form of FB (FB₁) accumulated in leaf. This was confirmed in maize seedlings watered with pure FB₁ which accumulated in root tissue but not leaf tissue. **Impact:** This is important because controlling FB entry into plant parts will reduce the levels in maize stalk and leaves used for cellulosic ethanol production. Less FB in plant parts will avert potential problems in dried distiller's grains with solubles used for animal feed. Developed polymerase chain reaction (PCR) assays to detect and distinguish between mycotoxin-producing strains and species of *Fusarium*. Assays are based on variability in sequence and genomic arrangements of mycotoxin biosynthetic genes in fumonisin and trichothecene-producing species of *Fusarium*. Also, researchers developed a quantitative PCR assay to measure the amount of *Fusarium* DNA in infected plant tissue. This research has developed knowledge that can be used to monitor and quantify mycotoxin producing species of *Fusarium* in the field.
- The fusarins are a group of mycotoxins produced by fungi that commonly infest cereal crops. They are produced by *Fusarium verticillioides*, which also produces the fumonisin mycotoxins. The fusarins have been reported to be highly mutagenic and to have relatively poor chemical stability. ARS developed seven monoclonal antibodies capable of binding two types of fusarins (A and C). In addition immunoassays based upon these antibodies were developed. **Impact:** The immunoassays are very sensitive, allowing ng/mL (part per billion) levels of these toxins to be detected in buffer; and therefore they may allow for their future use in detecting fusarins in foods.
- Nivalenol (NIV) is a mycotoxin produced by several fungi that are important pathogens of cereal grains. In certain regions of the world, NIV is a frequent contaminant of commodities. It has a chemical structure very similar to that of a related mycotoxin deoxynivalenol (DON, vomitoxin) and is regarded as having similar toxicity. ARS developed and validated a monoclonal antibody capable of recognizing both NIV and DON. However, NIV was not detected by commercial immunoassay screening methods for DON, because the antibodies upon which such assays are based did not recognize this toxin. **Impact:** This material has the potential to be used for screening for both types of toxins simultaneously in maize, wheat, and barley and is a significant advance in detection for these toxins. A rapid enzyme-linked immunosorbent assay (ELISA) was developed based upon this antibody, and it is capable of detecting these toxins at low levels.
- Moniliformin (MON) is a mycotoxin that is found in maize, wheat, rye, rice, and oats. MON is acutely toxic to both animals and plants and is found worldwide. ARS developed molecularly imprinted polymers (MIPs) that were capable of binding to MON. **Impact:** Such materials may lead to improved methods for isolating and detecting this toxin in foods. MIPs may potentially be used in intervention and control programs.

- Patulin is a mycotoxin produced by a variety of molds, particularly *Aspergillus* and *Penicillium*. It is commonly found in rotting apples, and the amount of patulin in apple products is generally viewed as a measure of the quality of the apples used in production. Research demonstrated that different fungi capable of producing the mycotoxin “patulin” can be identified by use of DNA probes specific for their respective isoeipoxydon dehydrogenase (*idh*) gene. ARS also demonstrated that all of species of *Penicillium* that had been reported to be producers of patulin possessed an isoeipoxydon dehydrogenase gene having the critical sequences necessary for having activity. **Impact:** Determined that a specific amino acid at a particular position in the protein was correlated with the ability to produce greater amount of patulin. This research is particularly important for baby food products that contain apple derived products.
- Zearalenone (ZEN) is an estrogenic mycotoxin produced by *Fusarium* fungi with broad potential to contaminate cereal crops. ARS developed a capillary electrophoresis (CE) method for detecting ZEN in maize. Certain cyclic oligosaccharides, cyclodextrins, enhanced the fluorescence of ZEN, which permitted the development of assays with improved sensitivity for this mycotoxin. An extensive study of the relationship between the structure of the cyclodextrins and the ability to enhance the fluorescence of the toxin was conducted, using cyclodextrins purchased commercially and synthesized as part of this project. Results indicated that the ability of the cyclodextrin to enhance fluorescence of the toxin was related to the size of the cyclodextrin cavity and the degree of methylation. **Impact:** The technology developed permit the detection of ZEN at 5 ppb, a level well suited for the testing of maize for compliance with the EU guideline of 50 ppb.
- Mycotoxin detection procedures are time-consuming, destructive, and costly. Therefore, the goal is to use non-invasive hyperspectral imaging techniques to detect and quantify mycotoxins produced by various molds on grains. Research developed a fluorescence hyperspectral imaging system to study and demonstrate the fluorescence hyperspectral properties of healthy and aflatoxin contaminated corn kernels. Statistical analysis is on-going to find the relationship between fluorescence hyperspectral response and aflatoxin concentration. An aflatoxin detection algorithm is also under development using significant wavelengths identified in the analysis. The algorithm is expected to be used in the prototype fluorescence multi-spectral imaging system, currently under development, in a field test. **Impact:** The fluorescence multi-spectral imaging system will have the ability to tune to specific key wavelengths for aflatoxin detection and will be suitable for rapid detection and easy deployment. The system will be tested in scaled-up experiments where groups of corn kernels will be examined simultaneously for the detection of aflatoxin. Each image will include 25 grams of corn, which is half the size of a standard sample for currently used analysis, that are widely used in grain inspection stations and ethanol plants for aflatoxin detection. This Prototype Inspection System 2 will be tested in 2 locations: USDA/GIPSA at Destrehan, LA, and in a commercial grain inspection station. A 1 kg system is also in the process of being built, and will be tested in 2009 and 2010. Field experiments are also on-going this year, testing corn that’s been inoculated at several locations. A patent for the algorithm for aflatoxin identification is being developed. A patent applied for covering mold identification is pending. This research was conducted in collaboration with the Institute for Technology Development, Stennis Space Center.

2.1.2 Crop/Fungal/Toxin Relationship

A thorough understanding of the very complex fungal/insect/crop/environment interactions during both fungal and plant growth and maturation is necessary to develop effective strategies to prevent toxin accumulation, whether of fungal or plant origin, in crop plants. For example, the factors affecting the interaction between soil and crops need to be identified so that interventions can be developed. These may include avoidance of certain soil series, use of soil amendments, crop production practices and/or development of crop varieties that do not absorb the toxic trace elements. Knowledge of the biology of the production systems will provide data to help construct predictive systems, and to determine under what conditions is it economical to implement specific management strategies, particularly for insect control. Crops will be able to be produced in areas where it is not otherwise economical or even possible to do so. We recognize that this type of research is not normally associated with, or considered as food safety. However, it was included in the 2006-2010 Food Safety Action Plan subject to potential realignment within ONP at a later date. Unfortunately this action never resulted due to unexpected activities within ONP.

- The presence of nivalenol producing strains in the US should be of concern and monitored by corn producers and breeders. Research demonstrated that strains of *Fusarium graminearum* that produce different trichothecene mycotoxins. For example, deoxynivalenol versus nivalenol, since both toxins cause corn ear rot, but in controlled experiments nivalenol producers caused less ear rot than deoxynivalenol producers. **Impact:** The studies indicate that the impact of nivalenol-producing strains on corn production in North America may be limited compared to deoxynivalenol-producing strains.
- Demonstrated that field-grown wheat grain that exhibits symptoms of the disease black point can contain low levels of the carcinogenic fumonisins as a result of infection by *Fusarium proliferatum*. **Impact:** These findings provide awareness that fumonisin contamination can occur in crops other than maize. These data are particularly important for countries that consume high levels of mycotoxins and suffer other disease states, such as AIDS.
- Knowledge of the role of culmorin production in virulence on wheat has revealed a likely target that plant biotechnologists and breeders can use to enhance resistance to *Fusarium* head blight and reduce trichothecene contamination in wheat. Research demonstrated that production of the mycotoxin culmorin, but not the mycotoxin butenolide, contributes to the ability of *Fusarium graminearum* to cause head blight of wheat. In addition, researchers identified the gene clusters that are responsible for the synthesis of these two lesser-known, but potentially harmful mycotoxins. **Impact:** Identification of the gene clusters will provide tools to study the regulation, enzymology and evolution of mycotoxin biosynthesis. These gene clusters may provide sites for development of control strategies.
- Demonstrated that a large numbers of *F. verticillioides* genes exhibit differential expression in maize tissue and, therefore, may be required for pathogenicity. The

differential expression was determined by microarray analysis of *F. verticillioides*-infected maize: 1) in response to stress, 2) with variation in disease resistance, and 3) in symptomatic versus asymptomatic tissue. The differentially expressed genes include some involved in polyketide metabolism and nutrient uptake. **Impact:** Another strategy to reduce fumonisin contamination in corn is to reduce the incidence and severity of corn ear rot caused by fumonisin-producing fungi. Genes required by the fungi to cause ear rot are potential targets for this control strategy.

- Determined functions of *F. verticillioides* genes required for fumonisin production and thereby elucidated the biochemical pathway that leads to the formation of fumonisins. **Impact:** The proteins encoded by the genes are potential targets to block fumonisin production in the field. The genes are being used by other researchers in the US, Asia, and/or Europe as genetic markers to monitor the occurrence of fumonisin-producing fungi in the field, to study gene expression related to fumonisin production, and to study the enzymology of fumonisin biosynthesis.
- Elucidated complexities of the interaction between *F. verticillioides*, fumonisins, and maize. Demonstrated that a fumonisin-producing strain of *F. verticillioides* is more effective at systemic colonization of maize seedlings than a nonproducing strain. However, fumonisin producing and nonproducing strains did not differ in ability to cause seedling blight or ear rot of maize. In addition, one of two maize lines that were highly tolerant of fumonisins was more resistant to *F. verticillioides*-induced seedling blight compared to an intolerant line. **Impact:** The research provides new information on the effect of fumonisins on the interaction of *F. verticillioides* and maize, and the fumonisin-tolerant maize lines provide a trait that can be incorporated into maize breeding programs.
- Identified and demonstrated efficacy in transgenic corn of new potential broad spectrum insect resistance genes, some of which were identified as having this function for the first time. Several of the gene products may also be active against mycotoxigenic fungi. **Impact:** This development provides alternatives to Bt genes. Although Bt corn hybrids can reduce insect damage and associated mycotoxins, they are limited in breadth of species they control. Three major U.S. corn ear insect pests (European corn borer and corn earworm) have developed resistance to Bt plants in the field. Effective combinations of these genes should provide broad spectrum, and stable resistance to the multiple species of insects that promote mycotoxin problems in corn.
- Insects have been proven to play a significant role in enhancing *Aspergillus flavus* and *Fusarium verticillioides* infection and mycotoxin contaminations in corn. Three years of field studies using several Bt- and non- Bt corn hybrids in mid-April showed a reduction in mycotoxin levels compared to mid- May plantings. Bt- corn hybrids showed more resistance to mycotoxin than non-Bt corn hybrids. **Impact:** These data further show the value of Bt-corn hybrids in the avoidance of mycotoxins. This work may provided new directions for research on the influence of season and hybrid strains.
- Demonstrated that robotic molecular evolutionary apparatus could be utilized to develop a more effective insecticidal peptide. **Impact:** This research sets the stage for understanding

critical sites and developing a multitude of more effective insecticidal proteins, defensive pathway regulatory genes, or genes involved in the biosynthetic pathways of insecticidal secondary metabolites in plants, as well as genes of value in other systems. Current interest by a company in the first modified peptide may lead to a CRADA and additional funding.

- Demonstrated through transgenic plant studies that insect resistance genes active against different classes of target sites can be combined to provide highly effective, broad spectrum insect resistance (completion anticipated 2010). **Impact:** Once all effective source-plant homologs are identified and tested, their combination should provide a durable, broad spectrum resistance to the many insect species that promote mycotoxin contamination in corn. In addition, these homologs would provide needed alternatives to the Bt genes currently being utilized in corn and other crops to which target caterpillar species have begun to develop field resistance.
- Discovered and demonstrated the efficacy of fungal and plant secondary metabolites from new sources that are active against corn insect pests associated with mycotoxin contamination. **Impact:** Research indicated pathways that are compatible with existing corn biochemistry, gene cloning and expression are anticipated to provide new structures of value or as leads, for control of insects involved in mycotoxin contamination in corn.
- Identified through array analysis putative regulatory gene(s) for a secondary metabolite pathway of maize that produces a compound responsible for broad spectrum insect and disease resistance. **Impact:** Once the actual regulatory gene is determined through expression analysis and verified through transgenic studies, this gene could be a source for additional resistance to insects involved in mycotoxin production in corn, as well as the mycotoxin-producing fungi themselves. The gene would have utility in a multigenic expression system for pest resistance and as a component to help delay insect resistance in existing commercialized Bt proteins.
- Discovered and demonstrated the efficacy of a gene from a mutant line of maize as a selectable marker for identifying newly transformed transgenic plants likely to contain the gene of interest (such as an insect resistance gene). Completion of studies on efficacy of a second selectable marker gene is anticipated by 2010. **Impact:** This research now allows seed companies to produce transgenic corn hybrids without the presence of foreign antibiotic genes, permitting a greater acceptance of this material to importers and thereby helping to speed adoption of new insect (or fungal) resistance genes likely to reduce mycotoxins in corn.
- A major insect pest of almonds, the Navel Orange Worm (NOW), has been found to be a major contributor to promoting infection of almonds by the fungus that produces aflatoxin. This insect can carry the spores of this fungus (*Aspergillus*), on hairs that cover its body, to the almond kernel. Moreover, when NOW created feeding wounds on almond kernels, these spores were transferred to these wounds, increasing the potential for aflatoxin contamination. **Impact:** This was an important finding in the continuing debate concerning the role of insect pests and aflatoxin contamination of tree nuts. This finding also allows further emphasis to be placed on the control of insects in tree nut orchards.

Insect damage is a critical concern to the almond industry in California, where the annual crop is valued at >\$4 billion.

- Major progress has been made in the control of tree nut damaging pest insects by host-plant volatiles (HPVs), also termed volatile organic compounds (VOCs). These HPVs can be used as lures to trap, or as a means of confusing or distracting, the insects from locating the host-plant. Attempts to identify HPVs that could be used against the navel orangeworm (NOW), have made substantial progress. **Impact:** Development of a lure for NOW has been elusive, to date. ARS scientists are using state of the art volatile trapping and electrophysiological tools to hone-in on the NOW lure. The lure provides an excellent strategy for control of the NOW and resulting aflatoxin contamination.
- A previously discovered by host-plant volatile (HPV) for the codling moth (CM), a major pest of walnuts and pome fruits has resulted in two unique commercial lures for adult monitoring and control. **Impact:** The lure alone, and in combination with pheromone have become the standard lures for population monitoring and spray decision making for CM (> \$1.3 million in sales). A microencapsulated formulation of the lure with a broad range of insecticides enhances control efficacy of CM larvae. This product is currently under application to the EPA for registration as a biopesticide.
- Designed and implemented small-scale and large-scale volatile organic compound (VOC) collection systems in collaboration with Paramount Farming Company, the Almond Board of California and the California Pistachio Research Board. **Impact:** These systems are capable of monitoring VOC emissions directly from orchards intact on the tree (in situ analysis) for potential background signaling volatiles which are ubiquitous and may act as obligatory cues to direct NOW towards key attractants. VOCs collected via these systems can be analyzed via a gas chromatogram coupled to a mass spectrometer, and/or used in bioassays with an electroantennogram detector, and the NOW moth. The new system will also be able to monitor pheromone/attractant release, mating disruption, herbicide/pesticide/fungicide sprays, wind-carried VOCs from neighboring industries, and monitoring other insect pests (such as the Light Brown Apple Moth).
- Developed a simple and affordable chemical method for determination of defensive, antifungal phytoalexins produced by peanut seeds. The method was used to study the interrelationship of phytoalexin production and disease resistance in different peanut genotypes. There was an association between phytoalexin production and the resistance of different genotypes to major peanut diseases. **Impact:** The phytoalexins can be used as chemical markers to assist breeding programs in the development of disease-resistant peanuts, which should improve yield and economic return for producers.
- As part of another research initiative, researchers serendipitously discovered a fungus that produces relatively large quantities of styrene - an industrially important chemical used in many plastics. **Impact:** This strain of *Fusarium oxysporum* could be used as a potential green source of a vital commercial chemical.

2.1.3 Production Practices and Expert Systems

The goal of this Problem Statement is to have effective strategies to prevent toxin accumulation, whether of fungal or plant origin, in crop plants. Optimal cultural and crop handling practices facilitated by expert systems, [where they are available], can help decrease mycotoxin accumulation. Optimized practices are generally the least expensive methods for controlling aflatoxins. For instance, rehydration procedures used to facilitate cracking of closed-shell pistachios results in exceptionally high aflatoxin levels. In the contamination of cottonseed, the second phase between boll opening and harvest is the most important factor predisposing the crop to aflatoxin contamination. This contradicts previous knowledge implicating the first phase. This knowledge assists cotton breeders target events in plant growth and development cycle for reducing aflatoxin susceptibility of their crop. For corn, computer programs provide predictions for mycotoxin occurrence. Predicting when mycotoxin problems may occur, and under what conditions, is critical for implementing specific management strategies, particularly for insect control.

- Research indicated that the application of the herbicides Round-up and Liberty to resistant corn hybrids has no adverse impact on growth, development, yield or mycotoxin incidence of those hybrids. There was no difference in these parameters when comparing the hybrids based upon their being a mid-season maturing (110 day) or a full-season (115-120 day) hybrid. **Impact:** This research has encouraged the planting of earlier maturing corn hybrids in the Mid South that are resistant to the herbicide Round-up.
- Grain processors are required to satisfy statutory levels by removing a small percentage of fungal damaged and toxin contaminated kernels instead of discarding the entire lot. ARS developed and validated that high speed optical sorting is a viable method to remove mold-infested, discolored corn kernels contaminated with aflatoxin or fumonisin in a single pass through a commercial high speed sorter (>7,000 kg/hr). The optimal pair of absorbance bands (filters) giving the lowest classification error rate for removing whole yellow or white maize grains contaminated with aflatoxin or fumonisin were (750 and 1,200 nm) and (500 and 1,200 nm), respectively. **Impact:** A neural network was trained to identify infecting fungal species on single kernels. The full near-infrared reflectance spectra of single maize grains was measured automatically and grains with multiple symptoms and mycotoxins were sorted into different fungal species categories at rates of about 1 per second using commercial instruments.
- A website was developed for a predictive computer program for mycotoxins in Midwest field corn. Data is being collected to modify (as necessary) the program for popcorn. Establishment of this site will allow more ready utilization by interested farmers. **Impact:** Results of the program aided the Central Illinois Irrigated Growers Association in management of mycotoxins. The program was utilized by grain elevators in Central Illinois as part of a process to determine whether and to what level to monitor for aflatoxins in 2005, a drought year in the area. Farmers continue to cooperate in data gathering and efforts have recently expanded to South Dakota.

- Models were developed that predict aflatoxin content of cottonseed prior to harvest based on timing of rainfall and temperature. Commercial data from both Arizona and Texas were combined to create region specific models. Rainfall on the mature crop was found to be a primary contributor to aflatoxin contamination of cottonseed. **Impact:** Geostatistics and multiple regression analyses demonstrated that both crop rotations and geography influence both fungal community composition and contamination. Results influence both biocontrol strategies and recommendations for management of crop development and off-take. (Also applies to Program Statement 2.1.5).
- Soil potassium levels were thought to play a role in aflatoxin contamination. Research investigated the use of potassium fertilizer levels above those currently recommended for corn production in the Mid South. Two potassium fertility experiments were conducted at two locations over three years. **Impact:** The studies failed to disclose any advantage to using high rates of the element as a means of reducing aflatoxin.
- A four year field study was initiated in 2005 to determine the effects of eight different corn (and soybean rotation schemes) on aflatoxin and fumonisin contamination levels in the crops and colonization of the grain by *Aspergillus flavus*. Aflatoxin levels in soybean were 2.3 and < 0.5 ppb in 2005 and 2006, respectively, while higher levels were found in corn 16.7 and 37.1 ppb in 2005 and 2006, respectively. Levels of *A. flavus* colonization were greater in corn compared to soybean. A higher frequency of aflatoxigenic *A. flavus* isolates were associated with corn compared to soybean. Significantly higher level of fumonisin contamination was found in corn compared to soybean. **Impact:** The studies showed some significant differences in mycotoxin levels and colonization; however, as the experiment has not undergone a full sequence of rotation treatments; it was not possible to completely assess the impact of rotation on mycotoxin contamination.
- Clipping of juvenal corn plants to simulate uneven emergence was studied to assess potential implications on aflatoxin contamination. The research investigated the effects of manually removing the top growth of corn at the 3-leaf and 5-leaf growth stage to induce stress encountered in fields where emergence is uneven. **Impact:** Clipping had no consistent influence on aflatoxin contamination or the agronomic characteristics of the plant.

2.1.4 Breeding Resistant Crops

Mycotoxin contamination of food crops cannot be completely prevented by improved cultural practices, insect control, and even competitive exclusion products. To economically produce crops that meet regulatory guidelines we need intrinsic crop resistance to colonization by the fungus and subsequent production of toxins. A thorough understanding and utilization of fungal genomics, including functional genomics offers an unprecedented opportunity to affect mycotoxin control through resistant crops. Genomics can help elucidate the dynamics of the fungal-crop relationship, including the role and mechanism of action of secondary metabolites in pathogenesis to the host plant, and the mechanism of transmission from one plant to the next. Fungal genomic information can help identify both the genes in the biochemical pathways that lead to mycotoxin formation, and the regulatory elements of mycotoxin production. Genomic information will also provide identification/characterization of the complex set of genes involved in fungal virulence, and the signaling pathways between the fungus and the environment, and fungal reproduction/survival which will help prevent fungal infection and mycotoxin production in crops. This genetic information in turn will identify effective control strategies for mycotoxins during crop production, and, set the stage for providing farmers with crop varieties that will not support fungal growth and toxin production. A lower amount of crop will need to be discarded because it does not meet regulatory guidance levels. We recognize that this type of research is not normally associated with, or considered as food safety. However, it was included in the 2006-2010 Food Safety Action Plan subject to potential realignment within ONP at a later date. Unfortunately this action never resulted due to unexpected activities within ONP.

- A quantitative pin – bar inoculation technique was developed to inoculate corn with *Aspergillus flavus* in field studies. Results from the studies were evaluated using the Gompertz model to characterize the dynamic and optimum colonization of non-toxicogenic *A. flavus* as biological control strains. Using this technique, it was found that non-toxicogenic strain K49 is a superior biocontrol agent for aflatoxin contamination in harvested corn, and that formulation did not reduce colonization potential. **Impact:** The development of these techniques led to many benefits, including the ability to accurately and economically screen a large numbers of *Aspergillus* strains. These techniques are being used nationally and internationally (Lithuania, Nigeria and Italy) in breeding corn for resistance for aflatoxin.
- Previously, aflatoxin-resistant inbreds used in the US were in poor agronomic backgrounds. Six new aflatoxin-resistant inbred maize lines were developed through a research collaboration between International Institute of Tropical Agriculture-Nigeria (IITA) and ARS. These lines have been registered as an Active Plant Invention. The lines have resistance to foliar diseases, southern corn leaf blight, Southern corn rust and possess good agronomic features, thereby increasing their potential value to a variety of US maize breeding programs. The six lines are the product of crosses between the most resistant US lines and ear rot resistant lines identified under severe disease pressure in Central and West Africa which demonstrated great potential for aflatoxin-resistance in the quick, lab-based Kernel Screening Assay (KSA). **Impact:** Seed of the six lines has been sent to the ARS Plant Introduction Station at Iowa State who will oversee sanitary

certification and seed increase, making these lines available to the public in late 2009. Near-isogenic corn lines varying in aflatoxin accumulation were identified among IITA-ARS program breeding materials being tested by the KSA. These will be useful in research to identify traits associated with aflatoxin-resistance.

- Transgenic cotton plants were developed that expressed a synthetic peptide (d4e1) gene. These plants demonstrated significant antifungal activity against several microbial pathogens, including the fungus, *Aspergillus* spp. that causes aflatoxin contamination of several food and feed crops. Transgenic cotton lines are currently being field tested for agronomic traits and resistance to fungal pathogens that cause seedling diseases. **Impact:** Pathogen-resistant lines will be released for breeding with commercial varieties. Availability of such resistant lines will be useful to reduce or eliminate the health risks and annual crop loss of about \$250M due to market rejection of aflatoxin contaminated food and feed commodities.
- An *Aspergillus flavus* strain was developed expressing the green fluorescent protein (GFP) gene under the control of a constitutive promoter. This fluorescent strain will be used to study the infection process by the fungus in cottonseed and bolls. **Impact:** Understanding the mode of infection and spread is essential to devise effective control strategies. This fluorescent fungal strain is also in demand by medical research scientists studying aspergillosis in humans and animals. Local and international collaborators working with other susceptible crops (corn and peanuts) have also utilized this fungal strain in their research.
- Cotton germplasm lacks resistance genes to *A. flavus* so transgenic approaches are being utilized to introduce antifungal traits into cotton. A gene was introduced into cotton from maize that encodes a trypsin inhibitor (TI) protein previously identified as a resistance factor through proteomic analysis of maize lines that were resistant to aflatoxin contamination. In vitro assays with pure TI protein showed inhibition of growth of a number of fungal pathogens including *A. flavus*. Transgenic cottons expressing the maize TI gene demonstrated increased resistance to the cotton wilt pathogen *Verticillium dahliae* whereas *A. flavus* spread in cotton bolls was not significantly inhibited in laboratory bioassays. This indicated that increased levels of expression of the TI gene in cotton will be required to achieve significant inhibition of *A. flavus*. **Impact:** These findings point to the utility of proteomics as a tool to rapidly identify fungal resistance factors that can then be used in transgenic as well as marker-assisted breeding strategies for control of aflatoxin contamination of susceptible crop plants.
- Studies elucidated the functions of genes required for trichothecene production and thereby elucidated the trichothecene biosynthetic pathway. The genes include a multifunctional oxygenase (TRI4) required for early steps of trichothecene biosynthesis and another oxygenase (TRI1), which is multifunctional in some species but not others, and is required for later steps in the biosynthesis. The latter steps are responsible for critical structural differences between different classes of trichothecenes. **Impact:** This basic information will be used to develop strategies to reduce trichothecene accumulation in grain crops. It has also provided other researchers in the US and abroad with genetic

tools to monitor trichothecene-producing fungi in the field and to assess gene expression as it relates to trichothecene production.

- Developed *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* as model systems to examine trichothecene mycotoxin toxicity in plants. Studies showed that even structurally simple trichothecenes are toxic to plants, and thus discovery of genes that confer ability to detoxify these compounds should be a high priority. Additionally developed methods for producing quantitative amounts of trichothecenes and their derivatives. **Impact:** The availability of these compounds will facilitate screening of trichothecene-degradation enzymes. This research also provides tools to provide high throughput screening for trichothecene-degradation enzymes and other factors that enhance resistance to trichothecenes and Fusarium head blight in cereal crops.
- Demonstrated that the evolutionary history of the trichothecene biosynthetic gene cluster in the genus *Fusarium* has been complex. By mining sequence databases as well as performing our own sequencing studies on a number of diverse *Fusarium* species, we determined this history has involved rearrangement of genes within the trichothecene cluster, relocation of genes into the cluster from elsewhere in the genome, and loss of other genes from the cluster. **Impact:** This research has created new knowledge in the area of genome evolution of trichothecene biosynthesis and should contribute to development of methods to combat Fusarium head blight and trichothecene contamination.
- Developed a system for quantitatively measuring the expression of trichothecene genes and subsequent discovery of the effects of xanthotoxin on trichothecene gene expression. In order to accurately measure the effects of fungicides, other antimicrobial compounds, and chemicals on selected genes, we developed a quantitative polymerase chain reaction system that measures real-time trichothecene gene activity. Addition of the antimicrobial compound xanthotoxin, at low levels to cultures of *Fusarium*, was found to increase expression of certain trichothecene biosynthetic genes yet blocked formation of the end product of the mycotoxin biosynthetic pathway. **Impact:** This research provides the knowledge for the testing of selected chemicals for the development of future fungicides and antitoxin compounds as well as showing the effects of the antimicrobial compound, xanthotoxin, on trichothecene production.
- A fungal gene previously identified by ARS, which provides self-protection to mycotoxins has been used to engineer plants (wheat and barley) to resist fungal infection. To understand the molecular basis of protein-mycotoxin interaction researchers examined the three-dimensional structures and kinetic properties of the self-protection protein from two different species of *Fusarium*. Important differences in activity of these enzymes toward mycotoxins were found. **Impact:** This research emphasizes that the choice of a mycotoxin resistance gene in transgenic crop protection strategies must take into account the kinetic profile of the selected protein. These findings are crucial to developing maize and wheat varieties that are resistant to maize ear rot and wheat head blight.

- Microarray expression analysis of the corn endophyte *Fusarium verticillioides* led to the identification of six genes similar to fungal killer protein (KP4). KP4-like genes have been identified in the maize pathogens *Ustilago maydis* and *Fusarium graminearum* as well as *Epichloe festucae*, a protective endophyte of tall fescue. The genomic organization of the three *E. festucae* KP4 genes is identical to *Fusarium* and raises the possibility that they were attained by horizontal gene transfer. Disruption vectors were created to examine function of the KP4 genes in *F. verticillioides*. Expression analysis has also identified numerous *F. verticillioides* genes that respond to diffusible metabolites produced by other fungal colonists of corn kernels. **Impact:** The goal of this research is to identify *F. verticillioides* genes that augment maize plant defenses.
- Proteins from both fungus and plant origin were identified that are involved in disease resistance in corn. Loss of resistance is due to direct modification of the plant protein by the fungal protein. Both the fungal protein and the corn disease resistance protein have been purified and the interaction can be reproduced in vitro. Polymerase chain reaction (PCR) primers that specifically recognized the FUM8 gene and green fluorescent protein (GFP) used to tag the FUM8 gene were developed and used to track *Fusarium verticillioides* in plants of several corn lines growing under ideal and under stress conditions. Fungal colonization was detected in stalk, shank, cob, tassel, silk, and kernel tissue. **Impact:** This research seeks to identify how *Fusarium* moves through a developing corn plant and determine what, if any, influence host genotype has on fungal colonization and fumonisin formation.
- Demonstrated that *Acremonium zeae*, a symptomless seed-borne endophyte of maize, produces pyrrocidines A and B, polyketide-amino acid-derived antibiotics exhibiting potent in vitro activity against major stalk- and ear-rot pathogens of maize. The great majority of *A. zeae* isolates (94%) from maize grown in regions associated with drought and temperature stress produced pyrrocidines. Pyrrocidine A also exhibited potent activity against *Clavibacter michiganense* subsp. *Nebraskense*, causal agent of Goss's bacterial wilt of maize, *Bacillus mojavense* and *Pseudomonas fluorescens*, both maize endophytes applied as biocontrol agents. *A. zeae* and other symptomless endophytes represent potential confounding variables in maize variety trials for resistance to pathogenic microbes and their mycotoxins. **Impact:** This research seeks to investigate the natural biocontrol potential of *A. zeae* antagonism to microbial endophytes and pathogens of maize.
- Endophytes of cereals are under explored sources of metabolites that may elicit plant defense responses, suppress fungal growth, or silence genes critical to mycotoxin synthesis while also being adapted to function "in planta". The research has isolated and identified numerous antifungal metabolites produced by fungal endophytes of maize that show activity against *Aspergillus* and *Fusarium*. Both fungi are important mycotoxin producers in maize and are also capable of systemically infecting cancer and HIV patients while being resistant to most clinical antifungals. **Impact:** These studies are contributing to a growing database that will be useful in evaluating the structure-function relationships and potential cellular targets in *Aspergillus* and *Fusarium*. Metabolites

recently isolated from a major pathogen of maize are capable of promoting initial symptomless infection by disrupting a plant hypersensitive response.

- Marker assisted backcrossing was used to create aflatoxin resistant corn inbreds from lines with good agronomic characteristics that can be used by seed companies to produce hybrids, or as sources of resistance. The first commercial bag quantities (80,000 kernels per bag) of aflatoxin resistant hybrids were produced using aflatoxin resistant inbreds developed in this research program. The resistant hybrids have 50 to 80% less aflatoxin than hybrids produced from susceptible inbred parents. Hybrids from crosses between two resistant parents were also shown to be low in aflatoxin in an inoculated field trial in southern Texas. **Impact:** The research seeks to greatly reduce or eliminate preharvest aflatoxin contamination of corn.
- Identified seven genes that regulate (turn up/down) fumonisin production in the fumonisin-producing fungus *Fusarium verticillioides*. One gene is located next to the fumonisin biosynthetic gene cluster and the others are dispersed throughout the genome. Identification and characterization of the genes were facilitated by the *F. verticillioides* EST database and microarray described above as well as gene deletion technology. ARS also, identified extensive sequence variability in messenger RNA transcribed from fumonisin biosynthetic genes. This variability results from alternate splicing of intron sequences from the RNA. **Impact:** These results provide knowledge on how fumonisin production is turned on and off in *F. verticillioides*. One strategy for reducing mycotoxins contamination in food and feed is to harness the fungal regulatory mechanisms that block their production. Thus, genes that regulate fumonisin production by *F. verticillioides* are potential targets for reducing fumonisin contamination.
- DNA sequencing and annotation have identified genes that suppress aflatoxin biosynthesis. Research showed that turning on certain genes involved in producing enzymes that protect the organism from exposure to oxygen stress turns off genes that make aflatoxin. Antioxidants induce the production of peroxiredoxins, enzymes that degrade certain oxygenated compounds in fungi, with a concomitant shutdown of the aflatoxin biosynthetic pathway. Therefore fungi could be treated with antioxidants to suppress aflatoxin production. **Impact:** This is a major breakthrough in attempts to solve the aflatoxin contamination problem. This has led to identification of natural compounds in crop plants that can be augmented through breeding and suppress aflatoxin production.
- Ochratoxin A is a chemical made by a number of different fungi that can infect agricultural commodities such as, barley, grapes, coffee, dried fruit and nuts. It is carcinogenic and can damage the kidneys. As such, contamination by this toxin is a food safety issue, nationally and internationally. Research identified a number of safe, natural compounds (not identified due to patent issues) that prevent ochratoxin production **Impact:** Augmentation of these compounds in food crops, through breeding, may help to lower or prevent ochratoxin contamination, improving the quality and safety of the product.

- Quantities and distributions of vegetative compatibility groups (VCGs), clonal genetic groups, of *Aspergillus flavus* in agricultural fields are highly complex and earlier studies indicate a lack of host specialization among the many *A. flavus* vegetative compatibility groups. Assays were developed for identification of individuals within complex communities and for population genetics analyses. Sixty-eight microsatellite loci were identified from *A. flavus* NRRL3357; each DNA region was sequenced across 12 isolates of three VCGs from sympatric populations. Twenty-four loci were useful, based on distribution across strains, polymorphisms within loci, and lack of interfering polymorphisms in flanking regions. **Impact:** This allows for the first time analysis of gene flow and genetic isolation among and within *A. flavus* VCGs.
- The entire *A. flavus* genome was sequenced by ARS in partnership with the J. Craig Venter Institute and North Carolina State University. The initial genomics project to sequence 7000-9000 unique expressed sequence tags (ESTs = representing gene messages), as well as all the deoxyribonucleic acid (DNA) in the *A. flavus* genome has been completed. About over 60% of the functional genes (7,218 unique ESTs out of a total of approximately 12,000 genes) have been identified. A database BLAST server containing this EST database has been established. A Gene Index has been constructed for public access. Two different format whole genome microarrays were designed based on the 12,000 genes identified, and used to identify critical genes involved in fungal response to various environmental factors favoring toxin production. These microarray resources have been used in large scale functional genomics studies to analyze which genes are affected under varying conditions. These include: nutritional (high or low carbon source), environmental (temperature), developmental (veA mutant), and during the fungus-corn interaction that affect toxin production. The long-term survival of aflatoxin-producing *A. flavus* strains in comparison with non-producing strains has been studied. Twenty genes have been selected for further study. **Impact:** Advancing the mycotoxin program on deciphering genes and their products involved in aflatoxin production in *A. flavus*. The entire *A. flavus* genome was sequenced and a public access database developed.
- In order to distinguish pathogenic *Aspergillus* species from non-pathogenic organisms, researchers have been searching for genes that contain SNPs (single nucleotide polymorphisms or variations in single bases in fungal DNA) unique to each type. DNA probes were identified for universal screening for genetic variability of *Aspergillus* group fungi. Studies on the molecular characterization of the aflatoxin biosynthetic pathway from the aflatoxigenic cousin of *A. flavus*, namely toxin-producing *A. ochraceoroseus*, *A. rambelli*, as well as non-toxigenic *A. oryzae* are on-going. **Impact:** Of the four common aflatoxin-producing *Aspergilli*: large sclerotia-producing *A. flavus*, small sclerotia-producing *A. flavus* (*A. parvisclerotigenus*), *A. parasiticus*, and (*A. minisclerotigenes*) (formerly the West African variant of *A. parasiticus*), ARS has identified usable polymorphisms in genes encoding enzymes such as an amylase, a xylanase, and a methyl transferase. These markers can be used in research to determine if aflatoxin production provides a competitive advantage to *A. flavus* for its ability to survive in field conditions.

- Elucidated the entire gene cluster that is responsible for aflatoxin biosynthesis in *A.flavus* and *A. parasiticus*. More recently, studies have been conducted to understand the roles of hypothetical genes in aflatoxin biosynthesis, as well as the importance of the protein encoded by these genes in the final steps in formation of aflatoxins. ARS is conducting comparative studies to test the importance of these genes in fungal survival under a variety of growth conditions that mimic natural field conditions to which the fungi would be subjected. **Impact:** This research assists development of rational intervention strategies to prevent preharvest aflatoxin contamination which is dependent on a full understanding of aflatoxin biosynthesis.
- Resistance-associated proteins (RAPs) have been identified through proteome analysis of US aflatoxin-resistant lines and also of near isogenic lines found among IITA-SRRC breeding materials and varying in aflatoxin accumulation. The RAPs, which were identified from kernel embryo or endosperm tissue, are in the storage protein, antifungal and stress-responsive categories. Stress responsive RAPs may facilitate the defense of plants under drought stress, a condition highly favorable to the accumulation of high levels of aflatoxin in maize kernels. Specific proteins identified as RAPs in earlier studies also were associated with resistance in these investigations. Investigations supporting/confirming a role in aflatoxin-resistance have been conducted on several RAPs such as PR-10, TI, peroxiredoxin and glyoxalase I. Proteome analysis will also be performed on the six newly released inbreds and results compared to earlier proteome studies. **Impact:** The identification of these proteins is an important step toward development of markers for use in marker-assisted breeding.
- Identified the majority of the genes involved in patulin production are present in a cluster, and elucidated the order of the genes in the *Penicillium patulin* biosynthetic pathway. This was aided by the recent availability of the complete sequence of *Aspergillus clavatus*. When compared to the genes in the patulin gene cluster in *Aspergillus clavatus*, the genes examined in *Penicillium griseofulvum* have been re-arranged and some are in different orientation. **Impact:** This information is of value for determination of genomic origins of various patulin producing fungal species. Patulin is of concern as a contaminant of baby foods containing apple derived products.
- Developed genomic resources for the fumonisin mycotoxin-producing fungus and maize ear rot pathogen *Fusarium verticillioides*. These resources are: 1) an extensive Expressed Sequence Tag (EST) database and 2) a microarray (DNA chip) that facilitates genome-wide analysis of gene expression of the fungus. **Impact:** The EST database has been incorporated into multiple genomics websites for example, Dana-Faber Cancer Institute and Broad Institute that are accessible to scientists worldwide. University and government scientists in the US and abroad are using the resources to examine *Fusarium* genes involved in mycotoxin production, pathogenicity, and other traits. In addition, scientists at the Broad Institute have used the EST data to aid in annotation of the whole genome sequences of *F. verticillioides*, *F. graminearum*, and *F. oxysporum*.
- Characterized a polyketide synthase (PKS) gene that is required for fusarin production in *F. verticillioides*, and identified a cluster of eight genes that are coordinately regulated with the PKS gene. The genes are most likely a fusarin biosynthetic gene cluster. Fusarins are mutagenic mycotoxins that can accumulate in grain and are produced by

multiple *Fusarium* species, including *F. verticillioides*. **Impact:** Demonstrated widespread occurrence of the cluster in the genus *Fusarium*. Identification of the cluster contributes to efforts aimed at examining the biosynthesis and toxicology of fusarins and will contribute to an evaluation of the impact fusarins have on food and feed safety.

2.1.5 Biocontrol Technologies

Biocontrol is the use of living organisms to control pests. The correct selection of organisms can provide a highly selective tool for controlling the pest of concern with minimal adverse effects on the environment. The simplest systems are where the organisms are amplified in the laboratory and then applied to the field; just as effective, and certainly more elegant and less expensive, are biocontrol organisms that amplify in the field to the necessary numbers to provide effective crop protection. Effective and economical biocontrol strains that are approved by regulatory agencies and readily available to the producing industries will provide control of aflatoxin in most years under most environmental conditions. There is occasionally a year with environmental conditions favorable to toxin production so that no single intervention can reduce losses from mycotoxin contamination. Long-term use of competitive exclusion products could assist producers sufficiently so that there is a financial incentive to continue crop production.

- Antioxidants have been found to inhibit aflatoxin production. Caffeic acid, an antioxidant, was found to reduce aflatoxin production by *Aspergillus flavus* by > 95% without affecting fungal growth. Microarray analysis of caffeic acid-treated *A. flavus* indicated that expression of almost all genes in the aflatoxin biosynthetic cluster were down-regulated. Surprisingly, several genes were also up-regulated, the most notable were four that encode specific enzymes such as alkyl hydroperoxide reductases that detoxify organic peroxides. **Impact:** The research discovered how to prevent aflatoxin production with safe, common natural chemicals. In addition, the research showed how these compounds work in the fungus so as to turn off the aflatoxin biosynthetic machinery of the fungus. In short, the compounds trick the fungus into "thinking" that it does not need to produce aflatoxin, which are produced by the fungi to protect them from chemical attacks from plants. This information should help in developing methods of breeding crop plants to prevent aflatoxin contamination. (Refers to research in Program Statement 2.1.4)
- In parallel to the stress response studies, researchers found a putative oxidative stress-induced signaling pathway through a comparison of *A. flavus* and yeast genomic data. This pathway was suppressed by plant antioxidants. **Impact:** This finding substantiates the correlation of highly expressed stress induced kernel proteins with a lowering of aflatoxin levels and points to solutions through breeding to enhance stress resistance traits that suppress toxin production. (Refers to research in Program Statement 2.1.4)
- Aflatoxin contamination of agricultural crops can result in serious economic losses on an annual basis. One approach that shows some promise of success in controlling contamination is the use of other microbes as biological control agents against aflatoxin producing fungi. Strains of two bacteria were discovered that reduced aflatoxin-producing fungi by 10 to 100 fold in soil from corn fields. The second approach is use of competitive micro-organisms through the yeast, *Pichia anomala*. This yeast shows a great deal of viability in tree nut orchards, has no human pathogenicity (unlike current atoxigenic strains of the fungus that produces aflatoxin that are currently being promoted as the best biological control agents), is not phytotoxic, and thus can be sprayed directly

onto the tree nut canopy. **Impact:** Research suggests that adding biocontrol microorganisms to soil environments that are reservoirs of aflatoxin-producing fungi will ultimately result in reducing aflatoxin contamination of crops planted in those soils.

- In collaboration with commodity organizations, farmers, and academia, small scale field testing was conducted to develop information for both the EPA and the California Department of Pesticide Regulation to support Experimental Use Permits for corn and pistachios. As a result, tests of atoxigenic strain efficacy in commercial agriculture on pistachios and corn were initiated in orchards (3,000 acres in CA) and corn fields (5,000 acres in TX during 2008). **Impact:** This built upon a decade of testing, filings, and meetings that resulted in the first full Section 3 Pesticide Registration for a biocontrol for limiting aflatoxins. Large-scale field studies will lay foundations for expanded use of atoxigenic strains of *A. flavus*.
- In collaboration with commodity organizations, continued the development, optimization, and use of atoxigenic strains (competitive exclusion) for prevention of aflatoxin contamination in cottonseed for central and western Arizona. Formulations to improve product residence for early applications will provide best efficacy. Sorghum based formulations with traditional seed coatings were found to be good candidates for next generation formulations. **Impact:** Technology transferred to appropriate agency and stakeholders, showing that early applications gave best displacement of aflatoxin producers but was associated with rapid loss of viable biocontrol product in the field.
- The potential for biological control of aflatoxin contamination in corn was demonstrated in a 2-year field study in which different inoculation methods were tested for application of the competitive, nontoxigenic strain of *Aspergillus flavus* used for biocontrol. Researchers observed that the previously developed and commercialized formulation, afla-guard, was the most effective inoculation method, particularly when it was applied to plant whorls prior to tasseling. **Impact:** Data from this study was included in a submission package to the Environmental Protection Agency (EPA) for an experimental use permit to allow large-scale field testing of afla-guard in corn. These data were instrumental in obtaining the experimental use permit from the EPA, which paved the way for testing afla-guard on 3000 acres of commercial corn in Texas. (see next accomplishment)
- Conducted studies under an EPA-approved 2-year experimental use permit to gather efficacy and safety data related to the use of afla-guard to control aflatoxin contamination of corn in commercial fields. The studies demonstrated that afla-guard was safe for use and that it reduced average aflatoxin levels by 85 and 88% in 2007 and 2008, respectively. **Impact:** Based on these studies, the EPA approved the use of afla-guard to control aflatoxin in corn beginning with the 2009 crop. In addition to mitigating this major agricultural problem and improving food safety, use of afla-guard should greatly reduce the cost of aflatoxin to the corn industry, which is millions of dollars per year.
- ARS had previously discovered that biocontrol strategies utilizing a species of endophytic bacterium, *Bacillus mojavensis*, are considered efficacious as a control of

Fusarium species. Research now demonstrated that all *B. mojavensis* strains are endophytic, plant friendly, and produce plant growth responses. In addition, it was determined that this bacterium is interactive with corn in enhancing the plant's natural defense system to produce a more stable and fungitoxic substance. **Impact:** This was the first demonstration that a biocontrol organism can reduce the titer of the fumonisin mycotoxin "in planta". Research identified the substance produced by this species of bacterium as surfactin, which is fungitoxic, has low to no mammalian toxicity, and is readily biodegradable. This bacterium's biocontrol spectrum has enormous potential for disease and mycotoxin control as well as other plant friendly qualities.

- Discovered that the fate of benzoxazolinones, specifically benzoxazolin-2(3H)-one (BOA), in corn relative to *Fusarium verticillioides*. BOA is an important transformation product of the benzoxazinones and is an allelochemical providing resistance to corn from pathogenic bacteria, fungi, and insects. However, maize pathogens such as *Fusarium verticillioides* are capable of detoxifying the benzoxazolinones to a nontoxic substance. The role of fumonisin production on corn plant development and foliar disease was assessed utilizing two distinct mutant phenotypes of *F. verticillioides*. **Impact:** Studies indicated pure FB1 did accumulate in the roots but accumulation in leaf tissue differed between corn genotypes. The combined results suggest the corn-fungus interaction contributes to the translocation and accumulation of fumonisins in vegetative tissues. Thus, a physiological and biochemical basis for seedling blight of some corn genotypes is suggested and a role for this mycotoxin in corn is indicated.
- Identified a number of safe, natural products that significantly enhances the effectiveness of commercial fungicides or antifungal drugs. Once these fungi are weakened by this "chemosensitization" the commercial products are anywhere from 100 to 1000 fold more effective. In collaboration with the Anderson Cancer Center, University of Texas and the Institute of Hygiene and Tropical Medicine, Lisbon, Portugal, chemosensitization was also found to be effective against a number of human pathogenic fungi that had become resistant to drugs. **Impact:** This work provided a mechanism for enhancing the activity of commercial biocontrol products. This research was awarded the 2008 Thomas J. Walsh Clinical Mycology Award. (see accomplishment below)
- In addition to the research described above, studies identified other chemical compounds related to the natural compound "ferulic acid" that have significant fungicidal activity. **Impact:** Some of these compounds show commercial promise and the structures of the compounds will help to understand how they work against the fungus.
- *A. flavus* reproduces clonally and has a vegetative incompatibility system that limits gene flow between individuals belonging to different vegetative compatibility groups (VCG). Two-hundred-twenty-one clone-corrected samples from three common VCGs in Arizona and Texas on cotton were analyzed using 24 microsatellite markers. High levels of genetic differentiation among VCGs and no evidence of recombination between VCGs was found. VCGs were found to be very old with divergence time between VCGs estimated between 34,000-49,000 and 140,000-189,000 years before present. **Impact:** This is the first study to find VCGs with high levels of genotypic diversity,

recombination via the parasexual cycle in natural populations, and migration over very large distances.

- Quantities and distributions of vegetative compatibility groups (VCGs) of *Aspergillus flavus* in agricultural fields are highly complex. Commercial cottonseed in Texas and Arizona was sampled over a four-year period and over 200,000 VCG analyses were performed to determine that certain VCGs frequently infect cottonseed in both states and may be adapted to that crop. **Impact:** The most common atoxigenic VCG was that to which the biocontrol AF36 belongs suggesting AF36 is the atoxigenic strain best adapted to cotton production. Other relatively common atoxigenics also may be useful in biocontrol.
- Demonstrated that *Trichoderma koningiopsis* IY-1 has biocontrol potential for the soil-borne state of *Fusarium verticillioides*. The use of this agent was based on basic biology of the fungus using the first transformants of *F. verticillioides*, and following the transfer of the fungus to kernels, including specific tissue types preferences, to the corn stalk and finally back to the ear of corn. ARS characterized *T. koningiopsis* for its biocontrol potential for mycotoxin reduction at the post-harvest stage and from root infections. Isolates were compared for growth rate and temperature tolerance from three continents, including: Brazil, Ecuador, North America (Georgia, and Kentucky), Canada, and Germany. Results varied among the isolates, indicating marked variations. **Impact:** This research was the first to demonstrate and define the life cycle of the fungus. The biocontrol potential of the [Georgia] isolate could be greatly enhanced by sexual manipulations with one of the isolates identified in this world-wide collection.
- Tall fescue (*Festuca arundinacea*) and its fungal endophyte *Neotyphodium coenophialum* are speculated by some mechanism to reduce plant-parasitic nematode populations. In vitro bioassays and greenhouse studies were performed to assess the effects of specifically identified compounds on *P. scribneri* motility, mortality, and chemoreception. It was determined that the major ergot alkaloid, ergovaline was nematicidal, while N-formylloline was nematicidal and the effects were reversible at low concentrations. However, the loline was also nematicidal and a repellent. **Impact:** This work identified some of the biologically active compounds produced in endophyte-infected tall fescue as nematotoxic, which should be further studied to enumerate their modes of action against other plant-parasitic nematodes. This work also demonstrated the interactive toxicities of ergot alkaloid, loline alkaloids, as well as polyphenolic compounds from endophyte-infected tall fescue on toxicity to the lesion nematode, *Pratylenchus scribneri*.
- Aflatoxin contamination of corn is a serious problem in the mid south especially in the Mississippi Delta. Studies completed four years of field trial assessing the potential for biocontrol of aflatoxin contamination in maize by non-toxicogenic isolates of *A. flavus*. These studies indicated the superiority of strain K49 in reducing aflatoxin contamination by 67-93% when co-inoculated with a toxicogenic isolate of *A. flavus*. Studies are continuing to optimize its application method and understand its mechanism for reducing aflatoxin contamination. This allows industry to test the use of non-toxicogenic strains to

develop a commercial product to control aflatoxin contamination in corn. **Impact:** The studies developed non-aflatoxigenic *A. flavus* strains for commercial use. A patent US 7,361,499 for K49 was issued (2008) and an application for licensing of this technology by a US company was filed (2008).

2.1.6 Toxicity Evaluations and Mechanisms of Action

The FDA has established guidelines for several mycotoxins in the US, but their toxicity continues to be assessed by the world community. Moreover new research, including human epidemiologic studies, continually raises new questions of cause and effect links to human disease. There are also questions about the effectiveness of certain industrial crop handling and food product preparation procedures for irreversibly reducing mycotoxins. In addition the presence of plant toxins and heavy metals may be strictly regulated by the FDA and EPA. Research needs to provide information to make solid recommendations with a scientific basis. The producing industries and the public will more likely accept regulatory decisions when they know and understand the basis of the regulatory guidance.

- Fumonisin is a family of toxic and carcinogenic mycotoxins produced by *Fusarium verticillioides*, a common fungal contaminant of maize. Fumonisin inhibits ceramide synthase, causing accumulation of bioactive intermediates of sphingolipid metabolism as well as depletion of complex sphingolipids, which interferes with the function of some membrane proteins, including the folate-binding protein. Research showed the sensitivity of rat kidney to fumonisin (FB) is a result of preferential accumulation of FB and sphingoid base 1-phosphates in kidney compared to liver. Researchers also found that FB1 was accumulated much more than other FB, and this was confirmed in human urine. **Impact:** This research is important because the rat kidney is the critical target organ used in the FB risk analysis and upon which the recommended FB tolerable daily intakes are based. The research also suggests that perhaps only a very few FB compounds are of toxicological significance. If proven correct, this finding could greatly simplify the FB risk assessment saving millions of dollars since monitoring for exposure could be focused on FB1.
- In collaboration with University of Nebraska ARS found the likely role of fumonisin (FB) induced elevation of sphingoid base 1-phosphates, ligands for S1P Receptors (S1P₁₋₅) as a risk factor for neural tube defects (NTD) in mice. The results show that sphingoid base 1-phosphates are elevated in tissues of mice exposed to FB during fetal development. An analog of sphingoid base 1-phosphates (FTY720) was a potent inducer of NTD in the model. The effects were strain-dependent and suggest that the S1PR receptors could play a role in FB-induced NTD in mice. **Impact:** Understanding the mechanistic basis for NTD development in FB-treated mice will assist in predicting the risk in humans and aid in the goal to reveal the genetic and nutritional factors that increase susceptibility to NTD in populations where maize consumption is high and diets are deficient in folate.
- In collaboration with the University of Nebraska, research demonstrated that neural tube defects (NTD) were not induced in a NTD-sensitive mouse model by hydrolyzed fumonisin B₁ (HFB₁), which is formed from fumonisin B₁ (FB₁) during alkaline cooking (nixtamalization) and occurs together with FB₁ in maize-based foods such as masa, tortillas and tortilla chips. Consumption of tortillas made from FB contaminated maize is a suspected risk factor for NTD that contributed to an NTD cluster in Texas. **Impact:**

Showing that HFB₁ was not teratogenic at doses > 5 fold higher than doses of FB₁ that cause NTD in the mouse model benefits consumers, risk assessors, and regulatory agencies by reducing concern that HFB in nixtamalized food products contributes to NTD formation.

- Demonstrated that mycotoxin-food matrix binding occurs during preparation of maize-based products by nixtamalization (cooking/steeping in alkaline liquid) and contributes to reducing fumonisin (FB) concentrations in the cooked product. ARS demonstrated using in vivo mechanism-based bioassays, that matrix-bound (and hence not measurable with routine methods) or unknown FB degradation products do not enhance toxicity. Toxicity is correlated with the amount of unbound FB measured in the product by routine methods. **Impact:** This research benefits consumers, risk assessors and regulatory agencies by reducing concern that the toxicity of food products could be underestimated due to the presence of undetectable matrix-bound FB.
- In collaboration with Texas Woman's University, ARS, the University of Nebraska and the FDA, demonstrated that reduction of fumonisins (FB) in maize products by extrusion cooking (combines high temperatures, pressure and torque) is significantly enhanced by addition of glucose to the extrusion dough. An in vitro bioassay demonstrated that extrusion alone or in combination with glucose supplementation did not enhance toxicity (e.g. by formation of unknown toxins or masking bioavailable FB), and extrusion with glucose supplementation can reduce toxicity under some conditions. **Impact:** The research shows that extrusion in combination with glucose supplementation is a feasible strategy to safely reduce FB concentrations in commercial maize-based products. This reduces concerns that the toxicity of extruded food products could be underestimated.
- In collaboration with Georgia Tech, Emory University and Health Canada, ARS found a new category of sphingolipids called 1-deoxysphinganine. Because 1-deoxysphinganine was found to preferentially accumulate in liver of mice fed fumonisin (FB) containing diets it is possible that this novel sphingoid base and its metabolites contribute to the hepatotoxicity of FB in mice. **Impact:** This is important because the target organs for FB toxicity and carcinogenicity are species-specific and the mechanism of action in rat kidney is much better understood compared to mouse liver (the most sensitive target in mice). This information could help explain an important gap in the current FB risk assessment.
- Vomitoxin, also known as deoxynivalenol (DON), is a type B trichothecene, an epoxy-sesquiterpeneoid. This mycotoxin occurs predominantly in grains such as wheat, barley, oats, rye, and maize, and less often in rice, sorghum, and triticale. In collaboration with food industry scientists, ARS demonstrated that deoxynivalenol (DON) is resistant to degradation during the preparation of wheat-based food products under commercially relevant conditions. Little to no reduction in DON occurred in cookies, crackers or pretzels made from flour or in cereal flakes prepared from wheat. Baking bread and frying donuts achieved the highest degree of DON reduction. **Impact:** Understanding the fate of DON in common commercial wheat products is necessary for the food industry to establish mycotoxin management strategies to protect consumers. The findings also

benefit regulatory bodies by contributing to the data base needed to set new or confirm current guidance for DON in commodities and foods.

Heavy Metals

Background: Cadmium (Cd) concentration in crops may inhibit marketing of crops grown in soils with high plant-available Cd levels. Previous research has shown that production of leafy vegetable crops on Cd-mineralized soils of the Salinas Valley, CA promotes high Cd accumulation in lettuce and spinach. Orchards were commonly sprayed with lead arsenate from about 1900 to 1950, and these elements accumulated in orchard soils. Although apples, pears and citrus did not have increased amounts of Pb or AS in fruit, carrot and other root and low growing leafy crops grown on these soils show significantly higher of Pb and As.

- Demonstrated that zinc fertilization plus liming of cadmium (Cd) -mineralized soils of Salinas Valley, CA, could strongly reduce cadmium accumulation by lettuce and spinach, allowing production of crops which meet international limits for crop cadmium. Previous research had reported that liming alone or zinc alone could not lower cadmium in these crops. **Impact:** Findings provide cost-effective solution to limit crop cadmium levels for natural soil high in cadmium in the US, and permit production of products that meet international regulatory limits.
- Identified an effect of low phyto-available soil zinc causing increased cadmium (Cd) accumulation by lettuce and other crops. Soils with a low ratio of zinc to cadmium then comprise a much greater risk of producing crops with excessive cadmium. Altered regulatory control of zinc transporters in roots due to absorbed cadmium may be causing this undesired effect. **Impact:** This study is important for international and domestic produce and potential intervention strategies.
- Characterized the higher bioavailability of cadmium (Cd) in polished rice, as well as its ability to accumulate cadmium from contaminated soil, compared to other crops. Rice has inherently low levels of bioavailable iron and zinc. Evidence indicates that other cropping systems are less likely to cause high incidence of proximal tubular dysfunction if soil becomes contaminated with cadmium and zinc. **Impact:** Data show that cadmium limits for rice and rice soils should not be arbitrarily applied to all crops and soils.
- Demonstrated that a strong cadmium (Cd)- adsorbing phase in biosolids (e.g. a iron-organic matter complex) persistently limits cadmium phytoavailability in amended soils and limits food-chain cadmium risk from beneficial use of biosolids on cropland. This study contradicts the assumption that cadmium would become more phytoavailable over time as added organic matter decomposed. **Impact:** This study suggests that deliberate addition of iron to soil amendments could reduce cadmium phytoavailability.
- In collaboration with Thailand, ARS tested soils, crops, and humans for cadmium (Cd). Rice can uptake cadmium from soil and is a human health risk. This study predicted areas

where zinc mining in rice production areas would likely cause adverse effects. Found extensive cadmium and zinc contamination of soil irrigated with mine waste waters, and excessive cadmium in rice, soybeans, and tobacco grown by local residents. Epidemiological survey confirmed cadmium-induced kidney disease in exposed population. **Impact:** These data convinced the Thai government to test the soil, crops, and humans to determine the extent and severity of contamination.

- Demonstrated that metallothionein (MT) played no role in cadmium absorption in rats when dietary levels of cadmium (Cd) were tested. Other researchers had concluded that MT was central to cadmium metabolism. Using mice with null genes for MT, and rice with levels of cadmium which are a risk to humans, studies found that the MT gene had no effect on cadmium absorption, or on the effect of low dietary iron, zinc, and cadmium causing 10-fold higher cadmium absorption. **Impact:** This work shows the importance of determining the exposure levels in the diet rather than conducting toxicological type studies.
- Contaminated rice soils have caused nearly all human cadmium related disease. ARS characterized the change in soil cadmium (Cd) chemical species during alternate flooding and drainage of cadmium-contaminated rice paddy soil. Studies demonstrated the formation of CdS (sulfide) during anaerobic incubation, and rapid transformation to non-sulfide forms upon drainage. **Impact:** This study clarifies how drainage allows much higher cadmium uptake during grain filling than for paddies which are kept flooded until rice grain is mature.
- Showed that lead (Pb) accumulated inside carrot roots rather than only on the peel layer because xylem elements running through the root collect lead during growth, while potatoes with no xylem connection to storage tubers are insensitive to soil lead enrichment. **Impact:** The clarification of this mechanism supports regulatory decisions to limit production of carrots on high lead soils.
- Found that long term application to Atlantic Coastal Plain soils of poultry litter containing residues of arsenic (As) feed additives did not cause accumulation of arsenic in amended soils. This likely resulted from the low levels of adsorbent iron oxides in these soils, and the high levels of phosphate which competes for arsenic sorption (the action of both absorption and adsorption taking place simultaneously) on soils. **Impact:** In the case of litter arsenic, leaching into subsoil would reduce potential risks from arsenic accumulation in topsoil.