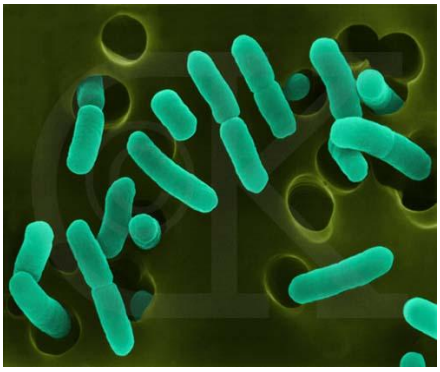
Food Safety: ARS National Program 108
Accomplishment Report
For the 2011-2015 Action Plan



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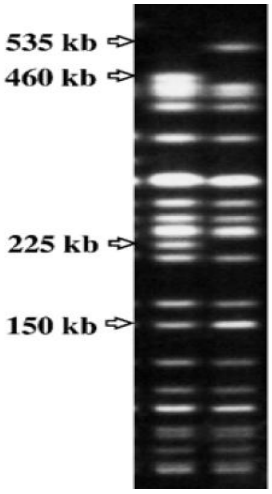

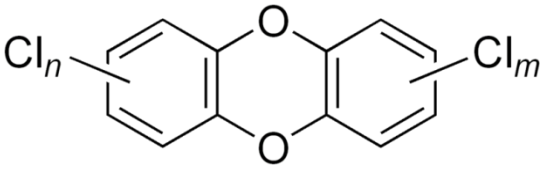

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Executive Summary

The current Administration has emphasized its commitment to change by overhauling the United States food safety system and moving towards more preventative regulatory strategies. The formation of the President's Food Safety Working Group (FSWG) and the development and implementation of the FDA-Food Safety Modernization Act (FSMA) and its various components within, are examples of initiatives that are the cornerstones of these changes. However, despite the many new initiatives, and now years of focused research activities, the safety of the food supply continues to be of critical concern. Food safety remains a highly visible agriculture and public health priority, albeit research area.

Foodborne outbreaks and consequential illnesses are a major cause of morbidity and mortality, as well as economic devastation, both nationally and internationally. The cause of many of the increased outbreaks remains unresolved but issues such as intensive food production, the globalization of the food and resulting international trade, changes in consumption habits, and travel and immigration of people are regarded as areas of significant focus. Further, despite continued evaluation by government, academia, industry and consumer organizations, the full extent, specifically the cost/burden resulting from food safety outbreaks, remains both unknown and moot.

Regardless of the cause(s) of the outbreaks, food safety research must continue to be conducted and evolve, especially as technologies become more sensitive and provide more detailed data. Continued outbreaks of major commodity-specific foods that may directly affect regulation, industry, and trade, require [our] focused attention. Food safety research poses numerous challenges because of the complexity of the production, processing and distribution processes. Food safety is and must continue to be seen a continuum, not a linear process. It is the intertwining of food animals and plants, the environment, and humans, to create opportunities for contamination through infection, intoxication or transmission, by bacterial, viral, parasitic pathogens, chemical contaminants, residues, or toxins.

Each of the ARS Food Safety Program's 5-year Strategic Action Plans since year 2000 has been considered a progressive step toward the ultimate goal of providing the necessary technological tools to increase the safety of the food supply. As technologies advance and the data increase and improve, each 5-year cycle provides further opportunity to develop, validate and implement more rapid and sensitive detection methods, study more hypotheses, and develop and evaluate more interventions and control strategies.

The ARS Food Safety Program strives to provide data [for policy change], and to develop new technologies that can be utilized by regulatory and/or defense agencies, or industry, to ensure the safety of the food supply. To be successfully conducted, scientific research requires a balance between the pursuit of an individual hypothesis and studies on a problem that will have outcomes and impact. This was and still is critically important in times of challenging fiscal and personnel resources, albeit very apparent during the past 7-years. It is also important that the Food Safety Program ensures the continued expertise and infrastructure to respond to changing issues in food safety, public health, and the Federal system.

In developing the 2011-2015 Action Plan; ongoing research activities and technology developments were reviewed, and input was gained from a variety of internal and external sources. As this report demonstrates, many of the projected research issues did emerge, and ARS was able to develop new technologies and provide more and better data to answer them. At the same time [we] also recognize that some food safety events would not be foreseen and so one of our goals were to create expertise and flexibility to respond to unknown events.

During the first 3.5 years of the 2011-2015 research cycle, the Food Safety Program realized many accomplishments. These accomplishments, their outcomes and impact(s) are reported in the following review document. Some accomplishments had a major impact, while others are still in the potential-impact phase. Unfortunately some research has so far achieved minimal or no accomplishments due to various and sometimes mitigating circumstances.

One of the strengths, evident from this report, is the Programs expertise in developing and validating various "detection technologies". This strength is being 'tapped' to assist in one of the most difficult challenges, that is, the rapid detection and unequivocal characterization of pathogens, chemical residues and toxins. These technologies are not only important to the science of food safety, but are critical for regulatory agencies, producers, and industry.

Although there were many outstanding accomplishments, there were also some major disappointments, in some-part due to mitigating circumstances: for example, loss of critical personnel, lack of continuing and/or new funds (sequestration), changes in research directions which required realignments, time expended for retraining, and finally the Federal Government Shutdown. One issue for example is of major concern. Despite food safety being a priority for both the [Administration and Congress] there was a reduction in research funding for the Program for many years. These funds were taken from discretionary allocations [monies directly for research not salaries] or through elimination of vacant research positions, combined with personnel retirements. Monies were also taken to shore-up other ARS research programs. To indicate the situation over time; the Food Safety Program went from 77 appropriated projects, with a budget of ~$105 million/250 scientists in 2005 to 63 projects a budget of ~$98 million/174 scientists in 2013. For fiscal 2014 there was an increased to $112 million but there are still less than 165 scientists. These new funds are anticipated to remain in the base budget for 2015 and the future, and these can be used to develop and implement the next 5-year research cycle due in 2016.

Productivity-wise, the Program overall has attained 90.6% of its predicted milestones [as officially reported to the Office of Management and Budget (OMB)]. If [we] exclude the mitigating circumstances of the Federal Shutdown*, loss of staff including retirements, critical vacancies, insufficient resources, redirection and milestones no longer applying, the accomplishment score increases to 97.7% Attaining 90% is considered the maximum attainable score by OMB since there is an acceptance that there will always be circumstances that preclude attaining 100%.

**It must be noted that the Federal Shutdown had severe effects, including: complete loss of some ongoing experiments, some of which can never be repeated; animals being sacrificed prematurely; graduate student career progress lost often to 6 months; opinion of a government*

research career impacted; critical delays with our international collaborative projects; and scientists unable to attend, present and interact with collaborators at international conferences, one of which is held only every 4 years.

In conclusion, the Program expects to complete the majority of its predicted milestones in the next 1.5 years, even under the current constraints. The new funding (approximately $14 million) allocated in fiscal year 2014 was implemented to negate [in-large part] the effects of sequestration and previous budgetary cuts. Scientifically, the future will bring a variety of challenges. Some are new, and are in response to external influences, such as trade issues. Others will result from a series of unexpected events inexplicable at the time they occur. These challenges will be the "drivers of change" providing a basis for constructing the 2016-2020 Program Strategic Action Plan. However, the overarching issue is the continued fundamental need for a systematic and multidisciplinary [integrated] approach to food safety research. That is, food safety is truly a continuum, and that events (positive or negative) no matter where they occur have consequences that affect both agriculture and public health.

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Introduction

Background
National Program for Food Safety (NP 108) is one of 19 National Programs (NP) within the USDA-Agricultural Research Service (ARS) Office of National Programs (ONP). The National Programs are organized within four broad program areas: Nutrition, Food Safety and Quality (NFSQ); Animal Production and Protection (APP); Natural Resources and Sustainable Agricultural Systems (NRSAS); and Crop Production and Protection (CPP). http://www.ars.usda.gov/research/programs.htm

The Food Safety National Program is part of NFSQ. Significant collaborations and/or interactions occur between researchers in food safety, both nationally within the Federal Government and academia; internationally with nearly 60 different countries; and between some other National Programs, in particular (NP 107) Human Nutrition, (NP 306) Quality and Utilization of Agricultural Products, (NP 103) Animal Health, (NP 106) Aquaculture, (NP 214) Agricultural and Industrial Byproducts, and (NP 303) Plant Diseases. The National Program structure allows ARS scientists to collaborate with researcher regionally, nationally or internationally to address food safety issues, needs or concerns.

2011-2015 NP108 Food Safety Action Plan (prepared in Fall 2009)

The safety of the food supply is an increasingly visible public health issue and a national priority for the Federal government. Outbreaks of foodborne illness are seen as a major cause of morbidity and mortality, and economic devastation both nationally and internationally. The full extent, specifically the cost/burden resulting from these outbreaks, remains unknown. The cause of the increased outbreaks also remains unresolved, but issues such as intensive food production, rapidly increasing international trade in foods, changes in consumption habits, and travel and immigration of peoples are regarded as areas of concern.

Persistent outbreaks of major commodity-specific foods that may directly affect public health, regulations, industry, and trade, require our immediate attention. Research towards improving public health requires reverse thinking on the food chain; and the food chain to be treated as a single entity, a continuum, not separated into pre-/post-harvest. Food safety research has also changed during the past decade, having moved past simple, surveillance/prevalence studies to asking more complex questions. Consequently, researchers are required to think creatively to solve problems, which means considering alternate perspectives, exploiting new opportunities and technologies, and crossing conventional boundaries. Multidisciplinary collaborations between Centers/ Institutes are an absolute necessity. Therefore the Program will draw upon relevant expertise and coordinate and effectively integrate resources to develop focused strategies for solving specific problems. In this way the Program as a whole is expected to substantially enhance the impact of its research accomplishments.

NP108 Mission Statement
To provide through research, the means to ensure that the food supply is safe for consumers; and that food and feed meet foreign and domestic regulatory requirements. Food safety research seeks ways to assess, control or eliminate potentially harmful food contaminants, including both introduced and naturally occurring pathogenic bacteria, viruses and parasites, toxins and non-biological-based chemical contaminants, mycotoxins and plant toxins. Food safety is a global issue; thus, the research program involves both national and international collaborations through formal and informal partnerships. Accomplishments and outcomes are utilized in national and international strategies delivering research results and advances to regulatory agencies, commodity organizations, industry and consumers.

NP108 Vision Statement
To enhance and protect public health and agriculture through the development of technologies, strategies, and data that safeguard food from pathogens, toxins, and chemical contaminants during production, processing, and preparation, thus increasing the safety of the food supply.

Relationship of this National Program to the ARS Strategic Plan:
The NP 108 Action Plan is aligned to the USDA-Research, Education and Economics Strategic Plan, and is articulated in the Office of the Chief Scientist Food Safety Science White Paper http://www.usda.gov/documents/food-safety-science-white-paper.pdf. Under the Office of Management and Budget (OMB) Performance Assessment Rating Tool (PART) the specific Program Performance Measure is to: Develop new technologies that assist ARS customers in detecting, identifying, and controlling foodborne diseases that affect human health. Details of the Actionable Strategies/Activities for Objective 4.1 may be found at www.ars.usda.gov.

Component 1. Foodborne Contaminants
The production, processing and distribution system for food in the United States is a diverse, extensive, and easily accessible system. This open system is vulnerable to introduction of contaminants through natural processes and global commerce, and by intentional means. Thus the food supply must be protected from pathogens, toxins, and chemical contamination that cause disease in humans. The Food Safety Research Program seeks ways to assess and control potentially harmful food contaminants. ARS will conduct research and provide scientific information and technology to producers, manufacturers, regulatory agencies, and consumers to support their efforts to provide a secure, affordable, and safe supply of food, fiber, and industrial products**.**

There are six (6) Problem Statements or Research Areas with this Strategic Plan.

- ***Population Systems***
- ***Systems Biology***
- ***Technologies for the Detection and Characterization of Contaminants***
- ***Intervention and Control Strategies***
- ***Predictive Microbiology and Data Acquisition***
- ***Chemical and Biological Contaminants: Detection Methodology, Toxicology and Toxinology***

Problem Statement 1.A Population Systems

The populations themselves may be multi-layered, for example, microbial, animal, plant, human, or environmental, or any combination of these. This approach unifies pre-and post-harvest food safety into a single entity, as it identifies and characterizes the movement, structure, and dynamics of populations throughout food production and processing; hence, the entire continuum. At a microbial level, the diversity and complexity within environments and food matrices may change with spatial and temporal influences, or with the competitive or synergistic relationships among pathogens and commensals. Microbial populations influence the safety of food, that is, the environment may determine the conditions under which a microorganism exists, and the microorganism may in turn influence the conditions prevailing in the environment. An identifiable area of study would include, for example, biofilms. Biofilms combined with quorum sensing studies could be extended to investigate polymicrobial communities found within the food chain continuum. Since interrelated they fall under both Problems Statements 1 and 2.

Research Needs

- Develop approaches that will evaluate the impact of intervention or management strategies on microbial contamination in the food continuum. These may include epidemiologic methods that will facilitate the understanding of quantitative data on pathogen load within the safety continuum, and facilitate the linking of food safety outcomes to public health outcomes.
- Develop new approaches to analyze and interpret more complex and emerging microbial methods; for example, molecular serotyping and phylogenetic analysis.
- Develop and use different approaches/designs for both microbial and population-based studies, monitoring of emerging pathogens, and supplying data for identified data gaps.
- Develop multidisciplinary approaches to understand the development, persistence, and transmission of resistant genes, and antimicrobial resistant microorganisms.
- Develop and validate best sampling collection protocols to maximize the probability of describing the exceedingly large number of diverse organisms that inhabit ecological communities.
- Determine any particular ecological niche or reservoir for a specific pathogen, and elucidate any factors, for example: environmental, nutritional, and/or biological, which enhance or reduce fitness characteristics related to persistent colonization, survival and growth.
- Determine the complex interactions among fungal/crop/environmental factors/production practices. Specifically, research will be focused on factors that elevate mycotoxins and their effects on the availability of a safe food supply.
- Determine/evaluate the role of food safety on public health outcomes, including acute and chronic sequelae.

Anticipated Products

- Epidemiologic studies will provide a scientific approach for population-based studies on new detection methods and interventions, to design and evaluate risk factors for potential control or intervention strategies, and a framework to integrate genomic data with disease in populations.
- Ecologic studies will determine the attributes and changes in the ecological communities in order to understand the transmission and dissemination of pathogens and toxins in and

among food producing animals and crops, and the interactions and relationships within the population community.
- Host-pathogen relationship studies will provide an understanding of the acquisition of genetic traits, such as the development and movement of resistance genes; traits connected with colonization and evolution of virulence; the role of protozoa in harboring or transmitting bacterial foodborne pathogens (Trojan horse concept); and the role of commensals.

Potential Benefits
- Epidemiologic studies conducted in various populations (microbial, animal, plant, or human) will help bridge the gap between agriculture and public health.
- The knowledge gained will help understand the transmission and dissemination of pathogens and toxins in and among food producing animals and crops; develop effective production practices and intervention strategies; develop and validate predictive microbial models; and provide data for risk assessments.

Problem Statement 1.B Systems Biology

The 2006-2010 Action Plan included the implementation of an "omics" research effort to sequence and annotate the genomes, develop microarrays, and construct data-bases of several critically important pathogens. Although some sequencing efforts will continue in the next research cycle, it is clearly recognized that "omic" technologies are in reality a series of methods used to examine more complex challenges that involve a systems approach to the study of pathogens. The concept of "systems biology" involves an integrated, systematic approach combining genomics, proteomics, transcriptomics, and metabolomics, as well as bioinformatics. Included within this Problem Statement is the study of pathogenicity and virulence. The pathogenicity of a specific organism is an issue at all stages of the food safety continuum. It is important to differentiate between microorganisms that are relevant to agriculture versus food safety and public health. Understanding pathogenicity is critical for pathogen intervention and control, modeling, and providing data for the development of risk assessments by regulatory agencies. Pathogens have the capacity to readily and rapidly adapt and evolve. Implemented control strategies may lose their effectiveness forcing the development of new production processes and products to maintain and improve the safety of foods. This in turn may restart the cycle of pathogen adaptation resultant from the altered environmental stresses. Risk assessment(s) conducted by our regulatory stakeholders are also predicated on understanding the pathogen, the dose response, the behavior in foods, and any positive or negative influences that may affect virulence. Assessing the virulence of foodborne organisms and differences among serotypes is critical in implementing new surveillance and intervention strategies.

Research Needs
- Assist in the development of specialized detection technologies, for example to differentiate pathogenic from non-pathogenic strains, and elucidate the differences between pathogens and non-pathogens.
- Conduct genome sequencing of specific pathogen strains to provide data for developing high resolution genotyping and molecular serotyping methods, and for identifying virulent strains.

- Expand knowledge on virulence factors: Why are some species/serotypes are highly virulent while others are less virulent; identify and characterize virulence factors and determine their interactions; determine if and/or how virulence is directly related to the infective dose.
- Understand the adaptive responses to intrinsic and extrinsic stressors, and determine any role in pathogenicity and virulence. Determine if resistance genes affect virulence or pathogenicity.
- Identify and characterize virulence attributes and responses of specific pathogens to their environment relative to changes in immunogenicity in the host.
- Determine why evolutionary shifts occur when populations system studies fail to identify the selection pressure.
- Determine whether quorum sensing is involved in regulating virulence factors or persistence. This area is appropriate for metagenomic studies to understand the fundamental composition of microbial communities and their contribution to pathogen persistence and/or toxin production, and metabolism.
- Understand the effect of environmental (extrinsic and intrinsic) conditions under which microorganisms exist, and determine how the microorganism may in turn influence the conditions prevailing in the environment.
- Develop a comprehensive database that can used to supply information on the ecological context of molecular, physiological and genetic data. For example; develop an integrated information system for risk management prevention and surveillance of foodborne diseases and/or genomic and proteomic databases specific to unique Program generated data. This should be directly coordinated and conducted within Problem Statement 5.
- Develop new or modified effective biocontrol organisms and delivery systems that do not introduce toxic factors; for example, in the control of mycotoxins.
- Delineate the role of endophytic fungi in regulating plant metabolism and in providing effective defense against predators and stresses.
- Identify unique fungal genes for specific biological and physiological functions.
- Determine how plant and environmental factors affect the mycotoxin synthesis, as it relates to food safety and public health.
- Utilize "omic" technologies in order to develop the multidisciplinary approach to address this Problem Statement.

Anticipated Products

- This approach provides a unique opportunity to understand the basic genetic components of pathogens, their expression, and directly relate this information to the microorganism's biology.
- While the tools for gene expression studies are available, there needs to be an increased focus on understanding how the studies will be performed and interpreted, and how they can be used to promote food safety.
- Establish a metagenomics approach to selected research areas which will for example, allow determination of metabolic contributions to risk.

Potential Benefits

- Implementing a "systems biologic" approach will impact various areas. For example: the generation of data for the specific development of molecular phylogenetics.
- The approach will identify genes which code for resistance to antimicrobials and disinfectants, for toxin production, or for the ability to grow in specific ecological niches.
- Regions of the genome that may have variations in the rate of nucleotide substitution or in the rate of intergenic recombination will be identified. These data can be utilized for the design and optimization of detection technologies, and to facilitate comparative genomic analyses for determining critical areas for targeting strategies for controls, and improve molecular tracking.
- This approach provides an understanding and comprehension of organisms and how they may induce disease, such as data on genes that contribute to pathogenicity; gene expression involved in virulence and/or viability in foods; and understanding population genetics and epidemiology.
- Implementation will provide better scientific data for more complete risk-based decisions.

Problem Statement 1.C Technologies for the Detection and Characterization of Microbial Contaminants

Challenges arise from either uncontrolled microbes entering the food chain through raw materials, or contamination during processing. To answer these challenges, detection and characterization is required at the earliest possible stage in the food chain, providing the necessary data for targeted interventions and reducing the need for recall of food products from purchase endpoints. Where possible, technologies must be developed for the entire food chain which allows the most effective and rapid detection and characterization capabilities. It is critical that research be focused to address the specific needs of the stakeholder, while balancing the inherent capabilities of the Program, and the need to focus on the most promising technologies (depending on the matrix) or point of use, and whether the technology is used for baseline studies, traceability and/or forensics. This requires that decisions be made relative to what should be detected, and the required level of detection and characterization. Further, this dictates that technologies which have the highest level of detection/characterization capability might not necessarily be the most practical, useful, economically viable, or be readily implemented. High-through-put analysis is important, but it may be impractical. Coordination among various agencies with similar initiatives and priorities will be critical.

Research Needs

- Develop and validate best sampling collection protocols to maximize the probability of detecting contaminants; combined with innovative approaches to sample processing [universal separation/concentration steps].
- Develop culture methods that do not bias the types of strains isolated, for example: virulence factors, resistance attributes, and serotype) food, animal/plant, and environmental samples.
- Develop and validate sample recovery methods. More attention must be paid to the initial sample preparation as various matrices present particular problems.
- Develop and validate methods that detect viable-but-non-culturable (VNC) cells.

- Developing data to assess the risk that VNC pose. This requires integration and coordination with Problem Statement 5.
- Development and validation of technologies for multiple agents in light of limited time for trace-back and attribution, and where fiscal and personnel resources are also limited.
- Develop and validate technologies that have improved speed, are cost effective, and provide most, if not all required informational detail for the determination and implementation of subsequent actions.
- Develop and validate technologies utilizing "omics" and nanotechnology where appropriate.
- Develop and validate technologies that allow uniformity of implementation both nationally and internationally [may require Codex involvement].
- Develop and validate technologies that have a critical use in food defense.
- Develop and validate integrated food safety/public health databases [national and global]. This will require significant collaborative efforts.
- Develop detection technologies that consider the needs for surveillance systems, not just technologies for monitoring the food supply.

Anticipated Products

- Promising technologies will be advanced. Technology transfer has to be done quickly, and where possible, and appropriate, will undergo validation through national or international bodies (FERN, Codex).
- Research that offers minimal outcome or impact will be terminated, and alternate approaches formulated. For example, detection methods related to serotyping and subtyping pathogens are useful; however, stronger emphasis must be placed on methods for more effective identification.
- Development of technologies must yield method(s) that are faster and yield improved resolution.
- In developing technologies decisions cannot be made in isolation. There needs to be an integration of biology, epidemiology and the physical sciences systems.

Potential Benefits

- ARS will lead in developing and specifically validating technologies that have public health, regulatory, trade, industry and research use; that is, a commonality of interests between government and stakeholders.
- Effective and efficient technologies that can be readily implemented will allow improved response times to events, and subsequently allow for the development of mechanisms for treating foods taken out of commerce.
- The technologies will provide data to identify areas where interventions are most critically needed, thus assisting the implementation of HACCP programs by FSIS, FDA, and their regulated industries.
- The data will also assist to develop and validate predictive microbial models and to help fill identified data gaps.

Problem Statement 1.D Intervention and Control Strategies

Intervention and control strategies will help to significantly decrease or eliminate pathogens in food animals and their derived products (eggs/milk), seafood and plant crops (produce/grains/ tree nuts) during critical periods of production and processing. Reduced shedding of zoonotic pathogens by food producing animals, and contamination of seafood and plant material will subsequently help reduce the pathogen load during slaughter/harvesting, and subsequent processing and storage. Recent foodborne outbreaks have shifted attention from solely animal origins to a balance of animals (meats) and plants (produce). This provides an opportunity to strengthen and increase research specifically addressing produce needs.

Many food processing/storage technologies have the ability to inactivate microorganisms to varying degrees; however, the intensities required can result in adverse functional and/or sensory properties, combined with a significant reduction in quality. Consequently, there is a continued need to develop and subsequently combine new/innovative processing technologies using the intelligent hurdle concept. In the concept, incremental changes in interventions are additive or synergistic, leading to greater control over pathogen growth without potential changes in food quality or reduction in nutrition. Unintended or unanticipated consequences of alternate technologies and processing intervention strategies such as changes in virulence, production of toxins, pathogen resistance or selection of resistant strains, and shifts in microbial ecology should certainly be considered for further investigation.

Research Needs

4.1: Animals and their Derived Products

- Develop interventions that prevent colonization or modulate pathogens from the gut; target specific metabolic endpoints; and decrease shedding of zoonotic pathogens at the time of slaughter.
- Determine the role of transportation and lairage; slaughter/processing methods and equipment on pathogen survival, transfer, post-harvest processing and storage.
- Identify and describe the critical points in production and processing that can be mitigated through the development and implementation of intervention and control strategies.
- Determine the effect of intrinsic and extrinsic parameters in production, processing and storage of eggs and milk.

4.2: Seafood

- Elucidate the mechanism(s) of pathogen introduction, persistence and survival in shellfish.
- Identify and describe the critical points in production and processing that can be mitigated through the development and implementation of intervention and control strategies for shellfish and USDA regulated finfish (Catfish).

4.3: Plant Crops/Produce

- Elucidate the mechanism(s) of pathogen introduction, persistence and survival. Determine the role of: environmental factors; seasonality, production cycles; adjacent land use, buffer zones, water sources (irrigation); epiphytic and soft rot microorganisms on pathogen internalization and/or attachment; and pathogen occurrence and movement.
- Develop practices and tools to control and predict the transport and fate of pathogens.
- Determine differences associated with the production and processing of conventional and organic grown crops.
- Determine the role of harvesting methods and equipment on pathogen transfer, post-harvest processing and storage.
- Identify and describe the critical control points in both production and processing of fresh produce, plant crops (grains/tree nuts) that can be mitigated through the development and implementation of intervention and control strategies.
- Develop methods to prevent the growth of pathogenic and spoilage microorganisms in minimally preserved, brined, and fresh-cut foods.
- Develop predictive models that describe the growth, survival and inactivation of critical pathogens, conducted in collaboration with Problems Statement 5.

4.4: General

- Develop, evaluate, and validate through laboratory, pilot-plant processing and commercial processing facilities the effect of single and combinations (parallel and serial) of intervention technologies (multi-target approach) on pathogen reduction.
- Determine whether combinations of non-thermal technologies can be incorporated in the hurdle concept; and determine whether single or combinations of non-thermal technologies are more effective if used in combination with traditional interventions.
- Increase fundamental understanding of the mechanisms, modes and sites of action at the cellular level of various intervention (inactivation) processes, and combination(s) thereof.
- Develop and evaluate the outcome/impact of post-harvest interventions options for small and very-small FSIS regulated plants.
- Use a “systems approach” to evaluate intervention and control strategies.
- Develop mechanisms and approaches to evaluate the effect of intervention and control strategies on food safety. That is, any technology transferred must have utility, have the capacity to be readily implemented, and be effective in reducing pathogen load, and/or biological/chemical contaminants.
- Use knowledge of host pathogen relationships/population systems to determine and evaluate interventions and control strategies.

<u>Anticipated Products</u>

- Intervention strategies will be developed to eliminate and/or control microorganisms in animals and their derived products, seafood and plant production, processing and storage systems. An underlying assumption is that production control interventions reduce downstream contamination which subsequently reduces disease risk.

- Efforts will focus on developing environmentally compatible technologies.
- Strategies will be developed for operations of all sizes (large to very small).
- Pathogens may develop resistance to some interventions; thus, efforts should focus on development of combinations of new or innovative intervention technologies for (minimal) processing.
- Interventions will be developed based on an understanding of their modes of action and effects on the microbial ecology of a food product, since inadequate suppression of spoilage could create an opportunity for human pathogen growth and toxin production.

Potential Benefits

- Development of intervention strategies can provide critical data to industry, commodity organizations and regulatory/action agencies that allows for the development, evaluation and implementation of Good Agricultural Practices (GAP's); Good Manufacturing Practices (GMP's) or regulations based on sound science.
- Studies will enable methods/strategies for the evaluation of any developed interventions and controls.

Problem Statement 1.E Predictive Microbiology and Data Acquisition

The basic principle of predictive microbiology is that the behavior of any microorganism is deterministic and able to be predicted from knowledge of the microorganism itself, and the microorganism's immediate environment. Behavioral predictions are [with limitations] accepted as an integral part of microbial risk assessment used to support food safety measures by both industry and regulatory bodies. It is widely accepted that predictive microbiology, in order to be effective, requires a multidisciplinary approach. This is a special challenge that necessitates coordinated efforts with other research institutes throughout the world.

Research Needs

- Develop predictive microbiology [models] that have validity and usefulness while addressing the limitations of the predictive ability. This includes the influence of challenge strain(s); assessment of a model's performance; predictive value on extrapolation; and efficacy especially in complex food matrices where the intrinsic and extrinsic parameters may change.
- Determine if growth/no-growth interface models predict the probability of growth occurring when a population faces more than one stressor/constraint.
- Develop models that have utility for risk assessment from both the producer and consumers perspective. There are distinctly different consequences of conservative (over) vs non-conservative (under) prediction of growth (or risk).
- Determine if changes in the microorganism(s) themselves occur, due to up/down regulation of genes; quorum sensing; or transfer of genetic information between species.
- Utilize the inactivation data to model pathogen and non-pathogen behavior in complex food systems. These types of studies are fundamental to developing HACCP systems and regulations.

- Provide predictive models for external examination and review through such efforts as the ARS Pathogen Modeling Program (PMP).
- Provide through national/international efforts an accumulation of data into a shared informational database. This is being done in-part through the continued development and expansion of the international collaborative project Combase, and the National Antimicrobial Resistance Monitoring System (NARMS).
- Provide relevant data to regulatory agencies for use in HACCP programs, risk assessments, labeling, persistence, and issues relative to international trade.
- In order to address the "systems" approach, develop and maintain the inherent potential underlying the data produced by the Programs sequencing efforts.
- Increase the focus on bioinformatics (computational biology) as more sequence data becomes available, and the complexity of both the data and questions being asked become more sophisticated.

Anticipated Products

- The ARS Food Safety Program does not develop or conduct risk assessments (RA), where RA is defined as the determination of a quantitative or qualitative value of risk related to a specific situation and a recognized hazard.
- The Program conduct research and provide data when requested by our regulatory stakeholders (FSIS, FDA) for their use in conducting risk assessments.
- Collaborations with regulatory and public health agencies will be strengthened regarding research for RA development efforts, so as to effectively utilize the inherent ARS expertise and modeling mechanisms.
- Methods used to identify data gaps will be described and integrated into the research project.
- Data acquisition will be an ambitious interdisciplinary research challenge that will eventually translate into improved public health.

Potential Benefits

- To generate data on the responses of microorganisms to both defined and changing environmental conditions, and to translate these data into mathematical models and user friendly software tools such as the PMP. These will be readily usable by national and international regulatory and public health agencies, and industry, to assist in ensuring the safety of the food supply.
- Internet-based database construction and development through Combase will ensure that data-mining and acquisition will continue to be coordinated. Database and bioinformatics efforts become increasingly important so that biologists have the ability to gain information that will foster technological innovation, and an understanding of the genetic basis of foodborne microorganisms.

Problem Statement 1.F Chemical and Biological Contaminants: Detection Methodology, Toxicology and Toxinology

The regulation and control of veterinary drugs, chemical residues, heavy metals, persistent organic pollutants, and biological toxins derived from bacteria, fungi and plants are an integral component of any food safety program. To protect public health and the environment, regulations [through Laws] have been passed and implemented that set limits on contaminants in edible agricultural products. Compliance and enforcement of these regulations is a critical role of the Program's stakeholders that requires the availability of practical detection and characterization methods for chemical residues (dioxins, pesticides), veterinary drugs (antibiotics, beta-agonists), heavy metals (As, Cd), and organic pollutants (polybrominated diphenyl ethers). In addition to regulatory monitoring there is a need to understand the biological effects of any inadvertent human or animal contamination. ***Toxicology*** examines the relationship between dose and its effects on the exposed organism; whereas ***Toxinology*** deals specifically with animal, plant and microbial toxins produced by or accumulated in living organisms, their properties and their biological significance for the organisms involved. This Problem Statement also includes food safety research directed towards methods for detection and identification of mycotoxins, their toxicity evaluation and mechanism of action. Research on the development of biocontrol technologies, and crop/fungal/toxin relationships is found under Problem Statement 2. Research on production practices and expert systems, and breeding resistant crops, will either be limited or where appropriate transferred to other National Programs within the 300 series.

Research Needs

- Develop and validate accurate, rapid, and easy to use analytical detection methods: single/multiclass, single/multi-contaminant analytical methods; lab and field-based (real world) methods and instruments for analytical screening.
- Develop and validate mechanism/action-based bioassays for laboratory and field use.
- Develop and validate multi-task on/in-line [field] inspection technologies [for all size processors] that detect contaminants and quality attributes simultaneously at required line speeds [functioning in real or near real-time].
- Develop and validate assays for assessing the efficacy of various processing methods to reduce or eliminate the toxicity in contaminated foods for human/animal consumption.
- Develop and validate assays that can be used for and in toxico/toxinological studies.
- Identify major sources of contaminants. Develop and validate intervention methods [for example, in bioremediation] to reduce bioavailability.
- Determine the fate and transport of contaminants and their derivatives in food systems and the environment. Provide parameters on residue depletion and withdrawal rates. Determine factors that affect fate and transport.
- Develop and validate when requested, technologies that have a critical use in food defense.
- Determine the dose-response relationships and tissue specificity of biological toxins.
- Determine the relevance of biotoxins with undetermined toxicity through the use of animal models. Determine/improve exposure assessment data.
- Determine the use of biomarkers as a measure of exposure and disease susceptibility.

Anticipated Products

- The successful implementation of technologies developed and validated through research is the major goal.
- These technologies provide tangible benefits through a more effective and efficient means of monitoring the food supply, and environment where food is grown. Better methods assist researchers conducting toxico/toxinological studies.
- Toxico/toxinological studies provide basic and applied knowledge on the effect of exposure to biological toxins.

Potential Benefits

- This research also provides data for better scientific and regulatory decision-making, reducing the likelihood of tolerance limit-errors, protection of consumers, and prevention of economic losses resulting from inappropriate regulatory actions.

Report

This Accomplishment Report was prepared by the current NP108 National Program Leaders, Dr. James Lindsay, Senior National Program Leader, and Dr. Eileen Thacker, the new National Program Leader for Food Safety and Animal Health. Since May 2013 Dr. Thacker has been the interim Food Safety Program Leader and co-lead for National Program NP 103, Animal Health until she took her new appointment in April, 2014.

The Report provides examples of research accomplishments, their outcome and impact, in the fiscal years 2010-2013, with inclusion of predicted accomplishments and their impact for the first quarter of fiscal 2014. Not all accomplishments during the period are listed since this would be too extensive a document. The report is based upon input from the field. Program scientists were requested to provide a Project Report which summarized (so far) their project accomplishments, outcomes, conclusions, and their impact and benefits. In some cases the scientist acknowledged that the accomplishment had addressed objectives within more than one Problem Statements. So as not to duplicate reporting, we determined and allocated the accomplishment(s) to only one Problem Statement, that we considered the most appropriate. Where a division could not be made or it was determined that a strong cross-cut existed, we have thus indicated that the accomplishment also addressed another Problem Statement.

The accomplishments, their outcome and impact should provide a broad picture of the research conducted in the Program. Many of the accomplishments represent a summary of several research projects conducted by multiple scientists. Some research described was conducted with partners from academia: however, individual laboratories and scientists are not identified since the purpose of the review is to assess the overall National Program. The Accomplishment Report is organized by Problem Statement with a brief introduction followed by accomplishments and discussion.

We asked the scientists to address the impact of the research noting that any assessment of an accomplishment's impact and benefit is inherently a qualitative and imprecise science. A series of criteria were identified in order to impartially conduct the evaluation, these were:

- Did the research advance the knowledge of food safety?
- Was the research innovative?
- Was there technology transfer?
- Was there regulation and policy development?
- Was there academic, industry and/or consumer relevance?

Further, it was critical that "actual impact" as described by the project was likely to be far less that the "potential impact". There is always a considerable gap between real and perceived impact; the practice of doing science and its transfer into usefulness.

Return on Investment
As noted previously; the Action Plan and the Project are dynamic and flexible, and could be modified at any time due to changing priorities. An example of a major change that occurred during this research cycle is described below, and some changing priorities:

Major changes:

- Realignment: The project "Alternative Processing Technologies" where the objective was to address USDA FSIS retail catfish. The rationale for the project was that in 2008, the Farm Bill transferred responsibility for catfish inspection from the FDA to USDA-FSIS. To assist FSIS evaluate appropriate assignment of resources a collaborative study was conducted with USDA-FSIS, USDA-ARS, Delaware State University, and Cheyney University to evaluate the presence of spoilage microorganisms, Salmonella, heavy metals and banned antibiotic residues. This study was completed and all results were transferred to FSIS. Catfish inspection was transferred back to FDA (in new Farm Bill transferred back to FSIS); hence the project objectives were realigned to determine the inactivation kinetics for foodborne pathogens suspended in foods treated using non-thermal process interventions (*e.g.*, ionizing radiation and high pressure processing).

Changing priorities: The following are examples of changing priorities during the time period which required program/project realignment.

- Assist FDA and industry regarding manure use for fresh produce production.
- Assist Department of Homeland Security on biosecurity issues.
- Assist FSIS and FDA in developing detection methods for new chemical residues of concern.

Collaborations
The Program has numerous international collaborations. Listed below are [58]countries where collaborative research with NP108 is conducted: Argentina; Austria; Australia; Azerbaijan; Belgium; Brazil; Burkina-Faso; Canada; China; Colombia; Czech Republic; Denmark; Egypt; England; Estonia; Finland; France; Germany; Ghana; Greece; Guatemala; Hungary; Iceland; India; Israel; Italy; Japan; Kenya; South Korea, North Korea; Mali; Mexico; Mozambique; Nepal; Netherlands; New Zealand; Nigeria; Northern Ireland; Norway; Peru; Portugal; Republic of Ireland; Romania; Russia; Saudi Arabia; Scotland; Senegal; Singapore; South Africa; Spain; Sweden; Taiwan; Tajikistan; Tanzania; Thailand; Turkey; Uruguay, and Zambia.

The following are examples of new Official [research] Memorandums of Understanding implemented during the past 3 years.

- University of Aberdeen, Scotland
- University of Sterling, Scotland
- Illinois Institute Technology (IIT), Chicago, IL
- Food Environment Research Agency (Fera) - UK
- Environmental Science Research - NZ
- NAPQMS - Research Institute, Republic Korea
- Cranfield University, UK
- RIKILT, University of Wageningen, the Netherlands (Food Safety Institute)

Existing collaborations that were expanded/ Example of new collaborations and studies done

Shanghai Jai Tong University, China

- Biosensor research. The initiative now included the Center for Food Safety Engineering (funded in part by NP 108) at Purdue University. This initiative falls under the Ministry of Science and Technology (MoST) Agreement, Annex V111.

UK-Food Standards Agency

- Collaborated on an International workshop on E. coli STEC's in cattle at the UK-FSA, Scotland.
- Study on the behavior of E. coli STEC's in foods. Data incorporated into Combase, and risk-pathogen models developed for international use
- Developed E. coli O104 growth model for FSA, data to PMP and Combase
- Conducted review of Risk Assessment for Toxoplasma in UK food chain indicating that disease is a major concern due to poor monitoring.
- Initiating research on STEC super-shedders in cattle (international priority)

NAPQMS-Research Institute, Korea

- Sensing technologies. Joint grant application to Food, Agriculture, Forestry & Fisheries (Korea). Funded $1.0 M

Food Environment Research Agency/DEFRA (UK) (New)

- International Workshops: Novel Field Based Diagnostics; & New Developments in Food Science, and Potential of Omic Technologies.
- Collaborative research implemented on: Pathogen genomics; Pathogen detection technology; C. botulinum neurotoxin (BNtox) detection;
- Incumbent (Lindsay- Advisory Board Chair)/Program (participant) in €12 M Food Integrity grant from DG-Research/Sanco, EC

Publications

As of May 2014 research conducted by NP108 during the 2011-2015 Cycle had produced an extensive list of peer reviewed research publications (> 1500). The list is available in a Publication Appendix. Due to the extensive nature of the data, information on Conference Proceedings, Abstracts and Presentations etc., was not collated.

Extramural (Incoming) Funds

There are several mechanisms for funding CRADA, Reimbursable, Trust and [Grant]. The funding information in the Table below was provided by the ARS Office of Technology Transfer. The Food Safety Program and NPL's were aggressive in encouraging scientists to obtain extramural funds. This was in-part the result of increased international collaborations and the need for scientists in the Program to understand and appreciate that: "No single individual, project, Department, or Center can possibly encompass the breadth of skills or competencies need to deliver results against the challenges and issues that confront us now or in the future. [That] we must continue to increase collaborations both nationally and internationally [where

appropriate], increasing the capability to deliver results through creative science and innovative solutions, in a timely manner."

Extramural Funds

Fiscal Year	*2010*	*2011*	*2012*	*2013*	*2014#*
Total $	**4,518,020**	**7,100,571**	**7,204,819**	**9,927,330**	**5,825,476**

Technical Transfer

The information was provided from the ARS Office of Technology Transfer (OTT) for the 2011 fiscal year (starting October 2011) through February 2014.

Fiscal Year	*2010*	*2011*	*2012*	*2013*	*2014#*
Inventions					
New	16	17	18	15	5
New Patents	9	4	10	4	4

Through April 2014

Examples of Accomplishments and their Impact by Problem Statement

1.A Population System

Goal

The goal under this Problem Statement was to conduct studies in various populations (microbial, animal, plant, or possibly human) that would help bridge the gap between agriculture and public health. The emphasis on understanding and characterizing population systems must include epidemiology, ecology, and host-pathogen relationships. The aims were that epidemiologic studies would provide a scientific approach for population-based studies on new detection methods and interventions, to design and evaluate risk factors for potential control or intervention strategies, and a framework to integrate genomic data with disease in populations. Ecologic studies would determine the attributes and changes in the various communities in order to understand the transmission and dissemination of pathogens and toxins in and among food producing animals and crops, and the interactions and relationships within the population community. Host-pathogen relationship studies would provide an understanding of the acquisition of genetic traits, such as the development and movement of resistance genes; traits connected with colonization and evolution of virulence; the role of protozoa in harboring or transmitting bacterial foodborne pathogens and the role of commensals. It was anticipated that where possible there may be opportunities to establish a metagenomics approach to selected research areas.

Since the populations themselves may be multi-layered, the approaches taken here would hopefully unify pre-and post-harvest food safety into a single entity, as they identify and characterize the movement, structure, and dynamics of populations through the entire food production and processing continuum. At a microbial level, the diversity and complexity within environments and food matrices may change with spatial and temporal influences, or with the competitive or synergistic relationships among pathogens and commensals. In terms of impact, it was anticipated that the knowledge gained would help understand the transmission and dissemination of pathogens and toxins in and among food producing animals and crops; develop effective production practices and intervention strategies; develop and validate predictive microbial models; and provide data for risk assessments.

Introduction

Beef:Dairy Cattle

Animal agriculture has consistently been considered a primary source of the zoonotic food-borne pathogens, Escherichia coli, Salmonella enterica, and Listeria monocytogenes that can contaminate the food chain and lead to illness in humans. Pathogens associated with dairy cows can enter the food chain through milk and meat. Although most milk is pasteurized, eliminating the risk from these pathogens, there are growing numbers of consumers demanding access to unpasteurized milk. Culled dairy animals also contribute significantly to the beef supply mainly as ground beef. Although these pathogens sometimes cause disease in dairy cows it has become evident that some strains infect cows without causing visible disease symptoms. The focus of this research was to elucidate the mechanisms by which bacterial pathogens enter and persist on dairy farms. Specifically understanding the identity, prevalence, and persistence of pathogens in dairy cows and the dairy environment, as well as the distribution of antibiotic resistance within the pathogen population will improve both meat and milk safety and lead to the development of strategies to reduce or eliminate these zoonotic bacteria in products before they leave the farm.

Examples of Accomplishments

- **Shiga-toxigenic E. coli in dairy cows.** Shiga-toxin genes were identified in E. coli populations in the gastrointestinal tracts of cows on three intensely profiled dairy farms over time. Isolation of highly pathogenic serotype O157:H7 was rare, being found on only one farm. The pathogen was isolated intermittently, usually in samples associated with young animals on the farm. One outbreak was detected within the lactating herd on this farm but the duration was very short with no O157:H7 found within 2 weeks of the original outbreak. This study suggests that the risk of O157:H7 contamination from cull dairy beef may be less than in beef cattle, but other Shiga-toxin producing serotypes require further investigation.

- **Persistence of Salmonella on dairy farms in the Northeast U. S.** This was a 5-year study of the ecology and epidemiology of Salmonella on dairy farms the Northeastern United States. Salmonella enterica serotypes Cerro and Kentucky were found to cause extremely long-term infections that may be regional in nature. These infections were difficult to detect as they had no noticeable effect on cow health or milk production demonstrating a risk of contamination of products leaving the dairy farm due to undetectable infections.

- **Genomic differences in transient and persistent strains of Salmonella Kentucky**. Salmonella serotypes differ in their abilities to colonize various hosts and in the severity of disease they can cause. Strains of Salmonella Kentucky were isolated from a dairy herd 6 years apart; one strain was only transiently detected in the herd and the second was isolated during a long-term, asymptomatic infection. Whole genome sequencing of the two strains revealed several differences in regions that have been identified as involved in S. enterica colonization of the bovine intestine. In addition, a virulence repressor was identified that appears to not induce an immune response in the host.

Outcome and Impact

The finding that O157:H7 infections are rare and transient in adult dairy cows, but that other Shiga-toxin producing strains are common, will guide future efforts aimed at controlling these pathogens. Comparative analysis of the genomes of these cow-adapted pathogens with those of serotypes known to infect cows transiently is expected to yield insight into the properties that affect colonization and persistence within the cow gut. These studies also have a direct impact with the FDA-Food Safety and Modernization Act of 2011 since it tasks FDA with developing improved epidemiological tools for obtaining quality exposure data and microbiological methods for classifying cases of foodborne illnesses and expanding capacity tracking systems. FDA has implemented a major program to replace traditional pathogen “tracking” techniques with genomic sequencing. Sequencing of Salmonella enterica serotype Cerro genomes from a long term epidemic has helped define the mutation rate in a host-adapted strain of Salmonella, an important parameter in the development of sequenced-based tracking methods.

Examples of Relevant Publications

- Van Kessel, J.S., Karns, J.S., Lombard, J.E., Kopral, C.A. 2011. Prevalence of Salmonella enterica, Listeria monocytogenes and E. coli virulence factors in bulk tank milk and in-line filters from US dairies. Journal of Food Protection. 75(5): 759-768.
- Van Kessel, J.S., Karns, J.S., Wolfgang, D., Hovingh, E., Schukken, Y. 2012. Dynamics of Salmonella serotype shifts in an endemically infected dairy herd. Foodborne Pathogens and Disease. 9(4): 319-324.
- Van Kessel, J.S., Karns, J.S., Wolfgang, D.R., Hovingh, E. 2013. Regional distribution of two dairy-associated Salmonella enterica serotypes. Foodborne Pathogens and Disease. 10(5): 448-452.
- Van Kessel, J.S., Sonnier, J.L., Zhao, S., Karns, J.S. 2013. Antimicrobial resistance of Salmonella enterica isolates from bulk tank milk and milk filters in the United States. Journal of Food Protection. 76(1): 18-25.

Introduction

Swine

Controlling foodborne pathogens in the food chain is complicated since they can colonize production animals without overtly inducing clinical signs of disease. Control also requires both an understanding of the pathogen as well as it's interaction with the host. To further complicate matters, antibiotic resistance has become a common characteristic of both pathogenic and commensal (foodborne pathogen) bacteria in the intestinal tract of food animals. Currently, there appears to be an increasing prevalence and enhanced virulence of multiple antibiotic resistant strains of Salmonella. To maintain the effectiveness of antibiotics in treating diseases of both humans and animals, alternatives to (traditional) antibiotic use are needed for animal management. Research is ongoing to identify targets for on-farm interventions through identification of the molecular mechanisms that influence resistance and host colonization as well as identifying porcine genetics associated with reduced shedding.

Examples of Accomplishments

- **Effects of antibiotics on the intestinal microbiome** Using culture-based methods, molecular phylotype identifications, and metagenomics analyses (sequence analysis of all genes in an intestinal sample), the impacts of antibiotics on swine intestinal microbiomes and antibiotic resistance was determined. Swine were fed either a commercial diet containing aureomycin, sulfamethazine, and penicillin or the same diet without antibiotics (control group). After 2 weeks on antibiotics, swine shed increased E. coli populations in their feces. Bacterial genes important for energy production and metabolism in the swine digestive tract increased in the antibiotic treated group, a finding consistent with animal performance. The antibiotic diet was found to impact the fecal levels of genes encoding resistance for the antibiotics and as well as increases of certain resistance genes not linked to the antibiotics in the feed.

- **Impact of antibiotic administration on Salmonella virulence.** Research into the effect that various antibiotics have on Salmonella virulence found that the impact of antibiotic use varies with drug type. Florfenicol did not enhance virulence, while tetracycline

promoted virulence attributes in resistant bacteria. This information provided important guidance for the judicious use of antibiotics in livestock.

- **Porcine gastrointestinal microbiota influences Salmonella shedding.** Salmonella typically colonizes the gastrointestinal tract of pigs. Studies determined that the bacterial composition of the intestine prior to Salmonella exposure can impact Salmonella shedding in pigs. Furthermore, the research revealed that sub-clinical Salmonella infections alter the maturation of the microbiota, which is important to animal health and growth performance. Lastly, analysis of the interaction of Salmonella with the porcine intestinal microbiota revealed prospective Salmonella antagonists that are currently under investigation as promising outcomes for intervention approaches.

- **Development of a live Salmonella vaccine.** A live attenuated Salmonella vaccine that provided protection against multiple pathogenic strains of the bacteria was developed. This vaccine also serves to differentiate between infected and vaccinated (DIVA) animals. The vaccine is currently under U.S. Patent review.

Outcome and Impact

The research had impact through the development of new technologies in assessing the microbiome of swine. As a result, the interactions of intestinal bacteria with the host and the impact of external products such as antibiotics were assessed. Studies determined that sub-inhibitory use of some antibiotics increases the invasive properties of Salmonella, while the use of other types of antibiotics at therapeutic doses does not. This information will assist industry in designing intervention strategies to control Salmonella infections by reducing the bacterial load and contamination in our food supply and transmission into the environment.

Examples of Relevant Publications

- Allen, H.K., Levine, U.Y., Looft, T.P., Bandrick, M.M., Casey, T. 2013. Treatment, promotion, commotion: Antibiotic alternatives in food-producing animals. Trends in Microbiology. 21(3): 114-119.
- Bearson, B.L., Bearson, S.M. 2011. Host specific differences alter the requirement for certain Salmonella genes during swine colonization. Veterinary Microbiology. 150(3-4): 215-219.
- Bearson, S.M., Allen, H.K., Bearson, B.L., Looft, T.P., Brunelle, B.W., Kich, J.D., Tuggle, C.K., Bayles, D.O., Alt, D.P., Levine, U.Y., Stanton, T.B. 2013. Profiling the gastrointestinal microbiota in response to Salmonella: low versus high Salmonella shedding in the natural porcine host. Infection, Genetics and Evolution. 16: 330-340.
- Looft, T.P., Allen, H.K. 2012. Collateral effects of antibiotics on mammalian gut microbiomes. Gut Microbes. 3(5): 463-467.
- Looft, T.P., Johnson, T., Allen, H.K., Bayles, D.O., Alt, D.P., Cole, J., Hashsham, S., Stedtfeld, R., Stedtfeld, T., Chai, B., Tiedje, J., Stanton, T.B. 2012. In-feed antibiotic effects on the swine intestinal microbiome. Proceedings of the National Academy of Sciences. 109(5): 1691-1696.

Introduction

National Antibiotic Resistance Monitoring System (NARMS)

Antimicrobial resistance (AMR) and multi-drug resistance in bacteria is a global concern for human and animal health. The prevalence of antimicrobial resistance in bacteria isolated from U.S. food animals has increased over the last several decades as have concerns of antimicrobial resistant foodborne zoonotic human infections. Science based information on the impact of AMR in livestock and the relation to feeding antibiotics is lacking. The goals of this research were to investigate and identify genetic mechanisms of resistance and its spread among bacteria in U.S. food animals using in-depth molecular and phenotypic characterization tools. Additional goals and objectives were to elucidate and provide data, such as prevalence and/or trends, including antimicrobial susceptibilities and molecular subtyping for foodborne pathogens in food animals through the National Antibiotic Resistance Monitoring System (NARMS). The use of genomic technology to study the overall presence of resistance genes and the genetic elements responsible for dissemination of those genes has also been implemented.

Example of Accomplishments

- **Multidrug resistance.** Antimicrobial resistance patterns among foodborne or commensal bacteria are important for defining the genetics behind multi-drug resistance in bacteria. Similar resistance genes were detected regardless of serovar, source, or location in multi-drug resistant S. enterica strains found in animals, retail meat, and human infections in the U.S. and Canada. The presence of similar resistance genes suggests that multi-drug resistant Salmonella collect specific resistance genes from multiple origins and different sources. Multiple origins of resistance are also present in enterococci from poultry and environmental sources as shared multi-drug resistance patterns. The presence of a major clone was not responsible for the multi-drug resistance in enterococci from poultry and environmental sources.

- **Association of multidrug resistance and genetic elements.** Mobile genetic elements appear to be a primary mode of distribution of resistance in foodborne pathogens. Multi-drug resistance was evaluated in Salmonella and generic Escherichia coli from animals using a DNA microarray. The microarray includes a total of 1267 probes; 775 probes detect resistance genes and 487 probes detect two different plasmid replicon types. Significant linkage associations between plasmids, phage type, and animal source found that plasmids and phage are responsible for multi-drug resistance in S. Typhimurium isolated from healthy food animals. Antimicrobial resistance genes detected were consistent with the multi-drug resistance phenotypes of all isolates, and a large number of IncA/C plasmid genes were detected in some of the isolates, indicating the likely presence of this plasmid known to carry multi-drug resistance genes.

Outcome and Impact

The generation of antimicrobial resistance and molecular data impact the development of future mitigation and control strategies to reduce antimicrobial resistance. Generation of genomic and plasmid sequence data benefits several areas of research and identifies potential genes specific for Salmonella and Campylobacter from poultry. By comparing the genomic data for these bacteria from different sources, potential genes specific for Salmonella and Campylobacter from

additional sources (swine, cattle, and humans) may also be identified. These genes will ultimately be used to develop technology to identify the source in foodborne illness outbreak investigations. This data will also identify genes encoding antimicrobial and biocide resistance and the mobile genetic elements responsible for spread of resistance among foodborne pathogens and commensals. Understanding the genesis of antimicrobial resistance is critical to reducing the prevalence of resistance in foodborne pathogens and for eventually finding avenues to control and eradicate resistance.

Examples of Relevant Publications

- McDermott, P., Whichard, J., Cray, P.J., Tate, H., Karp, B., Haro, J.H., Plumblee, J. 2011. Highlights of the NARMS 2009 Executive Report. http://www.fda.gov/downloads/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/UCM275775.pdf
- Molla, B., Byrne, M., Abley, M.J., Matthews, J., Jackson, C.R., Cray, P.J., Sreevatsan, S., Wang, P., Wondwossen, G. 2012. Epidemiology and genotypic characteristics of methicillin-resistant Staphylococcus aureus strains of porcine origin. Journal of Clinical Microbiology. 50(11): 3687-3693.

Introduction

Produce

Concerns about the presence of enteric pathogens in produce have increased due to the numerous recalls of leafy green vegetables due to contamination with Shiga-toxigenic E. coli, Salmonella and Listeria that have occurred in particular, in California at great cost to the produce industry. The focus of this work was to conduct surveillance with the produce growing area. The survey was part of a larger project to determine pathogen incidence with improved spatial and temporal resolution to parameterize and validate a Predictive Geospatial Risk Assessment Model (PGRAM) developed by the Department of Defense Global Emerging Infections, Surveillance and Response System and the National Aeronautics and Space Administration (NASA).

Examples of Accomplishments

- **Pathogens in water sources and wildlife.** Thirty locations on the Central California Coast were sampled for over 2 years to determine the incidence of Shiga-toxin producing Escherichia coli, Salmonella enterica and Listeria monocytogenes. E. coli O157:H7 was found in 8% of samples with the greatest prevalence occurring close to cattle operations or during periods of high precipitation. Non-O157 STEC prevalence was 11% and found at locations similar to O157. However, unlike O157, non-O157 STEC's were also found at high prevalence in the Salinas River during periods of low precipitation. Identical strains were found in bird feces and from samples at several other watershed locations indicating transport. Salmonella prevalence was 65%. PFGE analysis of these and isolates from previous surveys indicated that certain pulsotypes were transported in the region and were persistent in water and wildlife for-up-to 4 years. L. monocytogenes prevalence in the region was 43%, with over 85% of the isolates coding serotype 4b.

Amphibians, reptiles, and associated waters, were also sampled for Salmonella. Studies observed an incidence of 59% in snakes, 15% in lizards, 5% in toads, 1% in frogs, and 18% in water samples. Over 62% of the animal isolates were resistant to one or more antibiotic.

Outcome and Impact

Temporal contamination incidence data will be combined with irrigation management weather data (e.g. CIMIS), remote sensing data, and other high resolution data related to the movement of runoff and water in this region, to further parameterize PGRAM. Predictions provided by PGRAM will be used for validation of the risk assessment model during a third year of sampling. Predictive risk assessment related to hazardous conditions in this region will provide the produce industry with tools to minimize the probability of pre-harvest contamination and occurrence of outbreaks linked to their crops. Wild reptiles and amphibians with access to production environments could be an environmental reservoir for antibiotic resistant Salmonella.

Examples of Relevant Publications

- Benjamin, L., Atwill, E.R., Jay-Russell, M., Cooley, M.B., Carychao, D.K., Gorski, L.A., Mandrell, R.E. 2013. Occurrence of generic E. coli, E. coli O157:H7 and Salmonella spp. in water and sediment from leafy green produce farms and streams on the Central California coast. International Journal of Food Microbiology. 165(1): 65-76.
- Cooley, M.B., Jay-Russell, M., Atwill, E.R., Carychao, D., Quinones, B., Patel, R., Walker, S., Swimley, M., Pierre-Jerome, E., Gordus, A., Mandrell, R.E. 2013. Development of a robust method for isolation of Shiga-toxin positive Escherichia coli (STEC) from fecal, plant, soil and water samples from leafy greens production region in California. PLoS One. 8(6): e65716.
- Gorski, L.A., Parker, C., Liang, A.S., Cooley, M.B., Jay-Russell, M.T., Gordus, A.G., Atwill, R.E., Mandrell, R.E. 2011. Prevalence, distribution and diversity of Salmonella enterica in a major produce region of California. Applied and Environmental Microbiology. 77(8): 2734-2748.
- Gorski, L.A., Jay-Russell, M., Liang, A.S., Walker, S., Bengson, Y., Govoni, J.A., Mandrell, R.E. 2013. Diversity of pulsed field gel electrophoresis pulsotypes, serovars and antibiotic resistance among Salmonella isolates from wild amphibians and reptiles in the California central coast. Foodborne Pathogens and Disease. 10(6): 540-548.

1.B Systems Biology

Goal

The concept of "systems biology" was to use an integrated, systematic approach combining genomics, proteomics, transcriptomics, and metabolomics, as well as bioinformatics to address a research focus. This "omics" approach per se, provided a unique opportunity to understand the basic genetic components of pathogens, their expression, and directly relate this information to the microorganism's biology. Included within this Problem Statement was the study of pathogenicity and virulence. In terms of impact, implementing a "systems biology" approach would for example: generate data for the specific development of molecular phylogenetics, identify genes which code for resistance to antimicrobials and disinfectants, for toxin production, or for the ability to grow in specific ecological niches. Regions of the genome that may have variations in the rate of nucleotide substitution or in the rate of intergenic recombination will be identified. These data can be utilized for the design and optimization of detection technologies and to facilitate comparative genomic analyses for determining critical target strategies for control and to improve molecular tracking. Finally, this approach would provide an understanding and comprehension of organisms and how they may induce disease and provide scientific data that will allow for more complete risk-based decisions.

Introduction

Animals/Beef:Genomics

The prevention and control of Salmonella and Shiga-toxin-producing Escherichia coli entering the food-chain remains an elusive goal, despite intensive research efforts. While a majority of the research has focused on intervention and prevalence, little is known regarding the genetic variation among these pathogens in terms of the virulence and metabolic genes present, nucleotide polymorphisms, and differences in the transcriptional response and control mechanisms employed when they are exposed to adverse environmental stimuli. The specific areas of focus were to: characterize the genomic and transcriptomic differences present in foodborne pathogens (particularly Shiga-toxigenic Escherichia coli (STEC) and Salmonella enterica) and provide information on genetic variation and its association with the ability to cause disease in humans; to survey ecological niches and reservoirs using a systems approach to identify sites for potential interventions to reduce foodborne pathogens; and to identify how foodborne pathogens acquire, maintain and transmit genes for antimicrobial resistance and virulence within cattle from production to processing.

Examples of Accomplishments

- **Salmonella enterica genomic and phenotypic data resource and analysis.** Surveys of ground beef indicate that Salmonella prevalence is generally low, and the most commonly observed serotypes are Montevideo and Anatum. However, disease outbreaks are typically the result of contamination with S. Newport or Typhimurium. Given the potential for future regulation on the presence of certain Salmonella serotypes in ground beef, stakeholders will need tools for rapidly differentiating between serotypes when present as contaminants. Genomic DNA was sequenced from 48 Salmonella strains from the four serotypes described above. The strains were uniformly assembled and annotated to form a reference genome database for S. enterica strains important to the beef industry.

The metabolic and chemical sensitivity profiles of the strains were characterized using the Biolog Omnilog Phenotype Microarray System. Molecular targets that differentiate these serotypes were determined.

- **Genomic targets for detection of non-O157 Shiga toxigenic E. coli.** STEC O157:H7 strains cause the majority of severe disease in the U.S.; however, there is growing concern for the amount and severity of illness attributable to non-O157 STEC. Recently FSIS began regulating the presence of STEC's belonging to serogroups O26, O45, O103, O111, O121, and O145 in certain beef products. Little is known about the genomic content of non-O157 STEC's. In order to design molecular markers for non-O157 STEC serotypes, the genomes from multiple strains of the top six non-O157 STEC, DNA markers were identified that differentiated the STEC serogroups O26, O45, O103, O111, O121, and O145. The results from this research were licensed to a company and used in the development of a commercially available detection assay for non-O157 STEC serogroups O26, O45, O103, O111, O121, and O145.

- **Evolutionary history of Shiga-toxin-producing Escherichia coli serotype O26:H11.** Shiga-toxin-producing Escherichia coli O26:H11 (STEC O26) strains are foodborne pathogens that were recently classified in the U.S. as adulterants in certain beef products. A diverse sampling of human, animal and environmental STEC O26 strains were sequenced to identify nucleotide polymorphisms associated with the ability to cause human disease. From the genomic sequencing, STEC O26 strains were divided by the presence of either the Shiga-toxin 1 or 2 genes, with strains that contain the Stx2 gene associated with the most severe disease.

Outcome and Impact

The research described here focused on addressing the knowledge gaps by generating comprehensive genomic data sets. Sets were generated that identified better DNA targets for detecting foodborne pathogens and identified variability in foodborne pathogens ability to cause disease in humans. For example, the differences in genetic determinants between pathogenic Salmonella compared to commensal organisms were identified. Strains that posed an increased risk to human health could be identified, and thus enabled identification of molecular targets when present as contaminants of ground beef or trim. Gene expression response of Salmonella strains subjected to in-plant interventions, and identifying potential novel targets for intervention was characterized. These data provide information on Salmonella genes and pathways that may be used to survive carcass interventions and could be used to develop intervention strategies.

Genomic targets for non-O157 STEC were found and transferred through a CRADA to develop better detections technologies (see Problem Statement 3). What was unexpected was the observation regarding O26 STEC's evolutionary history, where regardless of their O serogroup, strains were more closely related if they had the same H serogroup indicating that the O serogroup may not be the best method for describing STEC's. This observation may require reconsideration for the development of detection and discrimination technologies for STEC's.

Examples of Relevant Publications

- Arthur, T.M., Bono, J.L., Kalchayanand, N. (2014) Characterization of Escherichia coli O157: H7 Strains from Contaminated Raw Beef Trim during "High Event Periods" Applied and Environmental Microbiology. 80(2): 506-514.
- Bono, J.L., Smith, T.P., Keen, J.E., Harhay, G.P., McDaneld, T.G., Mandrell, R.E., Jung, W., Besser, T.E., Gerner-Smidt, P., Bielaszewska, M., Karch, H., Clawson, M.L. 2012. Phylogeny of Shiga-toxin-producing Escherichia coli O157 isolated from cattle and clinically ill humans. Molecular Biology and Evolution. 28(8): 2047-2062.
- Durso, L.M., Harhay, G.P., Smith, T.P., Bono, J.L., Desantis, T.Z., Clawson, M.L. 2011. Bacterial community analysis of beef cattle feedlots reveals that pen surface is distinct from feces. Foodborne Pathogens and Disease. 8(5): 647-649.
- Harhay, D.M., Arthur, T.M., Bosilevac, J.M., Kalchayanand, N., Schmidt, J.W., Wang, R., Shackelford, S.D., Loneragan, G.H., Wheeler, T.L. 2012. Microbiological analysis of bovine lymph nodes for the detection of Salmonella enterica. Journal of Food Protection. 75(5): 854-858.
- Jung, W.K., Bono, J.L., Clawson, M.L., Leopold, S.R., Shringi, S., Besser, T.E. 2013. Lineage and genogroup-defining single nucleotide polymorphisms of Escherichia coli O157:H7. Applied and Environmental Microbiology. 79(22): 7036-7041.

Introduction

Colonization and Ecology

In the search for new interventions, it is critical to understand the particular ecological niches or reservoirs where pathogens may exist; and to understand the complex factors and interactions that may impact their survival and competitiveness within the gut ecosystem and the production environment. The focus of the research was to identify ecological niches or reservoirs for pathogenic and antimicrobial resistant bacteria, and the nutritional, biological, and environmental factors affecting their ability to colonize, survive, and persist within the gut of food-producing animals and their production environment.

Identification of bacterial factors and elucidation of mechanisms promoting intestinal colonization and adherence of Escherichia coli O157:H7 (O157) and other Shiga-toxin-producing E. coli (STEC's) in cattle are important prerequisites for reducing fecal shedding of these bacteria in cattle. The long-term goal was to develop intervention strategies to reduce or eliminate O157 and STEC's of public health significance from the bovine intestine using a coordinated and multipronged approach. Additionally, the ability of some bacteria to form biofilms in order to colonize surfaces, a risk for cross contamination, was studied.

Research that identifies interventions that prevent or mitigate colonization of the gut of food-producing animals (particularly the lower GI tract before slaughter) or that reduce pathogenic or antimicrobial resistant bacteria in the production environment were performed. This provided an improved understanding of microbial adaption to intrinsic and extrinsic stressors on the acquisition, exchange, and expression of incompatibility plasmids and antimicrobial resistance elements in foodborne pathogens in the production and processing environments.

Examples of Accomplishments

- **Secretion system, factors, and motility in colonization by Escherichia coli O157 in cattle.** By infecting cattle with specific mutants of O157, studies showed that a functional type III secretion system (TTSS) that delivers specific adherence factors (intimin and Tir) is essential for colonization and fecal shedding of O157 in cattle. A TTSS mutant failed to colonize cattle intestine indicated by a rapid decline in the fecal shedding compared to O157 bacteria possessing a functional TTSS. Studies also demonstrated that besides TTSS, bacterial flagella responsible for bacterial motility are essential for colonization and fecal shedding of O157. This was determined by using another O157 mutant with the ability to secrete large amounts of adherence proteins that failed to show increased colonization and fecal shedding in cattle compared to O157 bacteria producing and secreting lower levels of intimin and Tir.

- **Other factors required for O157 adherence to Recto-Anal Junction (RAJ).** Studies demonstrated that squamous epithelial cells, present in the RAJ are used by O157 to adhere and colonize the host. Cultured squamous epithelial cells were used to determine that O157 and six other non-O157 STEC serotypes adhere by expressing novel but as-yet uncharacterized factors. Based on genetic analysis, these factors appeared to be different from intimin and Tir used by O157 to colonize follicular epithelial cells of RAJ. These findings suggest that O157 and STEC adherence to squamous epithelial cells might also be important for colonization and increased fecal shedding of STEC's in cattle.

- **Heat-killed whole cell vaccines for reducing O157 fecal shedding.** (*hha)* mutants produce large quantities of protein factors that are essential for colonization and fecal shedding of O157 in cattle, and *hha sepB* mutants accumulate these factors within bacterial cells. Studies using heat-killed bacterial cells of these two mutants as vaccines demonstrated that vaccinated cattle shed significantly lower quantities of O157 than unvaccinated animals. The *hha* mutant vaccines have been modified to increase their effectiveness in reducing colonization and fecal shedding of O157.

- **High Event STEC isolates**. Individual processing plants experience sporadic peaks in contamination rates with multiple E. coli O157:H7-positive lots clustered in a short time frame, referred to as "High Event Periods" (HEP) of contamination. Compared to control strains, the HEP strains had significantly higher potency of mature biofilm formation after incubation for 3 to 6 days and exhibited significantly stronger resistance to sanitizer treatments as well as higher recovery capability after sanitization. These data suggest that biofilm formation and sanitization resistance may play critical roles in HEP beef contamination by E. coli O157:H7, which highlights the importance of proper sanitization in commercial meat plants.

- **Impact of distillers' grains vs citrus pulp.** Comprehensive metagenomic studies in addition to traditional cultural methodologies provided new information on the effects of distillers' grains, citrus pulps and commonly used ionophore antibiotics on microbial population dynamics within the gut ecosystem. Results revealed that feeding cattle distillers' grains, concentrated in nonstarch components such as lipid, bypass protein, and lignocellulose, resulted in an enrichment of bacteria belonging to phylum Bacteriodetes at

the expense of bacteria belonging to phylum Firmicutes. Conversely, feeding cattle increasing amounts of citrus pulp products consisting of concentrated essential oils and organic acids, tended to enrich for bacteria belonging to the phylum Firmicutes at the expense of those belonging to Bacteriodetes.

Outcomes and Impact

The identification of bacterial factors and understanding the mechanisms by which these factors promote STEC colonization of RAJ are critical for developing interventions, such as vaccines, chemical inhibitors, or dietary alternatives, for reducing fecal shedding of these bacteria. These studies showed that a global regulator [Hha] controlled colonization and fecal shedding and a study of *hha* mutants expressing or unable to express one or more of these pathways correlated with changes in fecal shedding of STEC in cattle as well as production of biofilms on solid surfaces. Additionally, the development of *hha* mutant-based vaccines and/or the use of a non-antibiotic metabolic inhibitor for reducing fecal shedding of O157 in cattle show promise for control. Studies showed that foodborne pathogens exhibited variability in biofilm forming potency and resistance to sanitization, especially in multi-species biofilms. A combination of sanitizers and hot water was effective at removing mixed pathogen biofilms from surfaces. The impact of these studies indicated that foodborne bacterial pathogens are not a homogenous group, but are a collection of organisms with different abilities to survive their environment and adapt to changes. New tools and information from this research will help develop successful strategies to reduce foodborne pathogens in the food continuum, capitalizing on the variation in these populations, and targeting interventions to address specific niches.

The research revealed differential gut microbial population shifts at the phylum level in cattle being fed distillers' grain products or citrus pulps. Recognizing, however, that the gut microbiome is extremely complex we are cautious to avoid generalized conclusions about population changes at the phylum level. Nevertheless, ongoing research utilizing next generation sequencing with deeper coverage and greater resolution will yield important new knowledge applicable to the ultimate development of designer diets composed of mixtures of feedstuffs and bioactive ingredients to select for populations able to exclude pathogens yet optimize nutrient utilization.

Examples of Relevant Publications

- Kudva, I.T., Griffin, R.W., Krastins, B., Sarracino, D.A., Calderwood, S.B., Manohar, J. 2012. Proteins other than the Locus of Enterocyte Effacement-encoded proteins may contribute to Escherichia coli O157:H7 adherence to bovine rectoanal junction stratified squamous epithelial cells. BMC Microbiology. 12: Article 103.
- Kudva, I.T., Hovde, C.J., John, M. 2013. Adherence of non-O157 Shiga-toxin Escherichia coli to bovine recto-anal junction squamous epithelial cells appears to be mediated by mechanisms distinct from those used by O157. Foodborne Pathogens and Disease. 10(4): 375-381.
- Sharma, V.K., Nystrom, E.A., Casey, T. 2011. Evaluation of hha and hha sepB mutant strains of E. coli O157:H7 as bacterins for reducing E. coli O157:H7 shedding in cattle. Vaccine. 29(31): 5078-5086.

- Sharma, V.K., Sacco, R.E., Kunkle, R.A., Bearson, S.M., Palmquist, D.E. 2012. Correlating levels of type III secretion and secreted proteins with fecal shedding of Escherichia coli O157:H7 in cattle. Infection and Immunity. 80(4): 1333-1342.

Introduction

Animals/Poultry

Host interactions are complex competitions during which both the host and the pathogen have adapted to each other in order for one or the other, or both, to survive. Understanding how the host micro environmental signals direct the microbe's virulence gene expression for efficient colonization and/or persistence, contributes to defining the mechanisms of pathogenicity. Additionally, understanding host-pathogen interactions should provide insights into host defenses and the tactics used by pathogens to overcome them. For example, non-typhoid serotypes of S. enterica are zoonotic bacteria that cause diarrheal disease in humans. This research focuses on the molecular mechanisms associated with pathogen persistence in animals (livestock). Research was performed on the differential host-pathogen interactions of pathogens in human, chicken, and swine intestines using emerging genomic technologies including quantitative proteomics, identifying virulence-associated microbial genes and host defense strategies, and developing strategies targeting the host innate immune system

While a variety of intervention strategies have been developed for the reduction of pathogens in broiler chicken production and processing, nevertheless the organism continues to contaminate both production flocks as well as the final consumer product. Interventions applied at the processing level may appear to have an immediate impact on pathogen reduction for the final consumer product; however, there are increasing concerns (emerging bacterial resistance as well as the presence of chemical residues) regarding the use of many of these interventions and contamination of poultry products continues to be a problem. Therefore, it was clear that the reduction of pathogens during the production phase was necessary for the delivery of less contaminated birds to the processing facility, and ultimately for the supply of safer poultry products. The research conducted employed a systems biology approach (incorporating pathogen genomics, transcriptomics, proteomics, metabolomics, and metagenomics) to facilitate/ focus on the development of scientifically sound intervention strategies for the mitigation of pathogens during poultry production.

Examples of Accomplishments

Omics/Colonization (preharvest)

- **Gut health and microbiome**. Using multiple "omics" approaches differences were observed in the microbiome and intestinal immunity associated with the dietary changes. Specifically, at 10 days post-hatch, Gallibacterium and Lactobacillus were over-represented in fecal samples, while Bacteroides was significantly more abundant in the cecum. By 31 days post-hatch, Clostridium and Caloramator had increased in the cecum and Lactobacillus remained over-represented in fecal samples. Cytokine gene expression changed over time. At 10 days post-hatch, the response was skewed towards a pro-inflammatory response. Following change in the diet from starter to grower and grower to

finisher, the cytokine profile changed to predominately anti-inflammatory/ regulatory while the pro-inflammatory cytokines were significantly down-regulated.

- **Immunoinfectomics of bacterial/viral infections.** Specialized receptors [Toll-like] (TLR) bind to components of microbes, activate cellular signal transduction pathways and stimulate innate immune responses. Chicken monocytes in combination with various inducers and receptors results in a synergistic immune response. This showed that the synergistic signaling is not a simple addition of TLR pathways, but rather a set of secondary pathways that appear to be stimulated leading to activation of various cytokines, nitric oxide synthase and the induction of a protective Th1 biased immune response. Finding a protective, non-pathogenic signaling pathway may be important for the induction of pathogen specific immune responses without the use of live vaccines.

- **Intestinal interactome.** Similar to humans, non-typhoidal Salmonella enterica infection induces an early pro-inflammatory response in chickens. However, unlike in humans, the response is short-lived and asymptomatic. It results in a persistent colonization of the gastrointestinal (GI) tract that can transmit infections to naïve hosts via bacterial fecal shedding. Persistent colonization of the ceca of chickens by Salmonella is accompanied by increased expression of several cytokines. This suggests the induction of a state of immunological tolerance mediated by an alteration of host signaling pathways that result in the influx and functional activation of T regulatory (Treg) cells and induction of anti-inflammatory immune responses. The results demonstrate that following an early pro-inflammatory reaction to initial colonization of the ceca, a state of immunological tolerance is developed within 3 days post-infection and maintained during persistent Salmonella colonization in the gut of chickens.

- **Genomics.** Research identified two distinct populations of sires within a line of chickens characterized by either inherently high or low pro-inflammatory cytokine/chemokine profiles. The progeny from high sires had significantly higher cytokine and chemokine mRNA expression levels compared to progeny from low sires. These were used to establish two new experimental lines of broilers (a high and low line). Evaluation of the high and low lines clearly showed the high line was highly resistant to foodborne and poultry pathogens including Salmonella, Campylobacter, and coccidiosis.

- **Immunoinfectomics for immunity to antibiotic resistance.** Research identified an adjuvant immunotherapy that selectively stimulates protective immune responses for Salmonella and Campylobacter infections. This established that a group of small peptides possess antimicrobial activities. These peptides, when provided to neonatal chicks as a feed additive for four days after hatch, significantly decreased intestinal and extra-intestinal colonization by Salmonella enteritidis. Feeding the peptide-supplemented diet primed cecal tissue for immune-mediated antibacterial activity without being directly antimicrobial. The significance is that the orally delivered cationic peptides stimulate the innate response during the first week after hatch, normally a time of immunologic inefficiency and increased susceptibility to bacterial infections.

Outcome and Impact
The research improved technology with respect to refinement of molecular methodology, and cultural recovery of Campylobacter spp. from poultry. The data generated also provided important information as to why previously applied laboratory identification techniques were not always accurate and efficacious.

Using genomic and proteomic technologies, new insights into individual regulatory control mechanisms of Salmonella and Campylobacter persistence in poultry and its ability to evade the innate host defenses was determined. Investigations of leukocyte-mediated innate immunity and genetic control of the functional innate response provided convincing evidence for the potential of their use as alternatives to antibiotics by boosting innate responses. Recognition of Salmonella by a variety of host immune receptors induces various extracellular activation cascades and intracellular signaling pathways leading to an inflammatory response, recruitment of immune cells for clearance of the pathogens, and mobilization of professional antigen-presenting cells. The signal transduction events that lead to the activation of transcription factors and the induction of specific, pathogen-directed immune responses are controlled, not at the transcriptional or translational level, but post-translationally via a reversible series of phosphorylation of proteins which act as a molecular switch controlling the activities of signaling molecules and downstream target proteins. Lastly, the role of some bacterial virulence factors in intestinal colonization and persistence were evaluated.

Although much of this work is fundamental, some major impacts were identified which have attracted industry partners, especially in the poultry production and pharmaceutical industries. For example, the development of "green" commercial lines of birds; identification of cationic peptides, useful as alternatives to antibiotics; and the finding of a unique protective, non-pathogenic signaling pathway may be an important consideration for the induction of pathogen specific immune responses without the use of live vaccines.

Examples of Relevant Publications

- Genovese, K.J., He, L.H., Swaggerty, C.L., Kogut, M.H. 2013. The avian heterophil. Developmental and Comparative Immunology. 41(3): 334-340.
- Conlan, A.J., Line, J.E., Hiett, K.L., Coward, C., Van Diemen, P.M., Stevens, M.P., Jones, M.A., Maskell, D.J. 2011. Transmission and dose–response experiments for social animals: a reappraisal of the colonization biology of Campylobacter jejuni in chickens. Journal of the Royal Society Interface. 8(65): 1720-1735.
- Kogut, M.H., Genovese, K.J., Nerren, J., He, L.H. 2012. Effects of avian triggering receptor expressed on myeloid cells (TREM-A1) activation on heterophil functional activities. Developmental and Comparative Immunology. 36(1): 157-165.
- Line, J.E., Hiett, K.L., Guard, J.Y., Seal, B.S. 2010. Differential carbon source utilization by Campylobacter jejuni strain 11168 in response to growth temperature variation. Journal of Microbiological Methods. 80(2): 198-202.
- Oakley, B., Morales, C., Line, J.E., Berrang, M.E., Meinersmann, R.J., Tillman, G.E., Wise, M.G., Siragusa, G.R., Hiett, K.L., Seal, B.S. 2013. The poultry-associated microbiome: network analysis and characterization along the farm-to-fork continuum. PLoS One. 8(2): e57190.

- Swaggerty, C.L., He, L.H., Genovese, K.J., Duke, S.E., Kogut, M.H. 2012. Loxoribine pretreatment reduces Salmonella enteritidis organ invasion in 1-day-old chickens. Poultry Science. 91(4): 1038-1042.

Introduction

Interventions (preharvest)

Molecular technology has brought dramatic advancements in understanding foodborne pathogens, and yet this technology has not provided large scale cost-efficient solutions. By working with the poultry/animal industries our goal was to investigate cost-effective new protocols that could mitigate pathogens in food producing animals. Considering alternative management protocols was critical. Specifically the focuses were: to identify/characterize management practices and environmental factors that would reduce the fitness characteristics of foodborne pathogens in poultry associated with persistent colonization, survival growth, virulence, and antimicrobial resistance; to develop/evaluate new intervention strategies that prevent or reduce enteric colonization that could be integrated into existing management practices and decrease bacterial shedding; to understand the effect of waste management conditions in poultry, both extrinsic and intrinsic, and determine the complex interactions among waste management practices on survival and dispersion of pathogens; and finally to determine the complex interactions among fungi/protozoa/microbial population within the gastrointestinal tract of poultry and how it affects food safety.

Examples of Accomplishments

- **Vaccine management programs**. Demonstrated that vaccinating against Marek's disease can increase the Salmonella load. Vaccinating the chick embryos on Day 18 of incubation instead of day-of-hatch can reduce Salmonella levels10-fold in the chick. Additional studies have also been conducted with the infectious bronchitis vaccine.

- **Darkling beetles retain Salmonella through metamorphosis**. The lesser mealworm beetle is a serious pest in poultry facilities and is known to carry pathogens affecting both human and animal health. Lesser mealworm beetles were exposed to Salmonella and then evaluated for Salmonella shedding. Exposed larvae were also exposed to Salmonella and followed through pupation, and the newly emerged adults examined for Salmonella. Exposed adults and larvae produced Salmonella-positive feces for up to 12 days. Current management programs reuse poultry litter and since these beetles survive between flock rotations, the reutilized litter may be an ongoing source of Salmonella.

- **Salmonella in cecal biofilms.** Established that successful gastrointestinal pathogen colonization was not just dependent on the microbial community complexity, but on the presence of Salmonella within a biofilm component that facilitates long-term infection. Using a continuous-flow chemostat culture derived from ceca of chicks to model gastrointestinal microbial communities, the work determined the bacterial communities in planktonic and biofilms components. The capacity for Salmonella to invade and colonize the gastrointestinal system of poultry was shown to be due to the complexity of the bacterial community and sequestering of Salmonella in the biofilm.

- **Microbial ecology of poultry litter composting.** Due to economic conditions, poultry litter is reused one or more years with the potential to be contaminated with pathogens and antibiotic resistant bacteria. Current interventions to control these bacteria include in-house composting or windrowing. The studies concluded that current in-house composting or windrowing is not effective in reducing pathogens or antibiotic resistant bacteria. It was found that by including certain composting bacteria during commercial processing (allowing for time restraints) allows composting the potential to be an effective way to solve the growing concern of solid waste management.

Outcome and Impact

The research suggests changes to existing management programs that do not dramatically increase costs to the producer. These changes include regulating the timing of vaccination and litter management programs without adding cost and without the use of antibiotics. The studies describe the importance of beneficial bacterial and fungal populations and how these populations can be manipulated to reduce both animal health issues and food safety issues. In addition, research has focused on arthropods, such as the lesser mealworm, as reservoirs of Salmonella. With the information gained this problem can be effectively addressed through the development of attractants/repellants and natural antimicrobial pesticides. Finally, research provided effective antibiotic alternative strategies that incorporate common compounds into feed to significantly reduce Salmonella cecal contamination in young broilers.

Examples of Relevant Publications

- Crippen, T.L., Zheng, L., Sheffield, C.L., Tomberlin, J.K., Beier, R.C., Yu, Z. 2012. Transient gut retention and persistence of Salmonella through metamorphosis in the lesser mealworm, Alphitobius diaperinus. Journal of Applied Microbiology. 112(5): 920-926.
- Hume, M.E., Barbosa, N.A., Dowd, S.E., Sakamura, N.K., Nalian, A.G., Kley, A.M., Oviedo-Rondon, E.O. 2011. Use of pyrosequencing and denaturing gradient gel electrophoresis to examine the effects of probiotics and essential oil blends on digestive microflora in broilers under mixed Eimeria infection. Foodborne Pathogens and Disease. 8(11): 1159-1167.
- Stringfellow, K., Caldwell, D.J., Lee, J., Byrd II, J.A., Carey, J., Kessler, K., McReynolds, J.L., Bell, A.A., Stipanovic, R.D., Farnell, M. 2010. Pasteurization of chicken litter with steam and quicklime to reduce Salmonella Typhimurium. Journal of Applied Poultry Research. 19(4):380-386.
- Volkova, V.V., Wills, R.W., Hubbard, S.A., Magee, D.L., Byrd II, J.A., Bailey, R.H. 2011. Risk factors associated with detection of Salmonella in broiler litter at the time of new flock placement. Zoonoses and Public Health. 58(3):158-168.

Introduction

Omics (postharvest)

The human pathogens Campylobacter, Salmonella and Listeria are commonly associated with poultry products. The processing plant is a post-harvest site in which numerous manipulations are made to poultry carcasses and meat, many of which impact the microbial safety of the end products. This research was designed to study the distribution and dispersion of bacterial pathogens in and around poultry processing plants and poultry products. The research undertook a molecular systems approach to foodborne pathogens and their movement in the poultry processing environment. Through the use of population genetics, bacterial migration and adaptation of foodborne pathogens were tracked through poultry processing and the associated environment evaluated for variations and influence of genetic and strain diversity from the animal through the processing plant. Additional research on the host-microbial interaction of protozoa and foodborne pathogens, and other biological populations that may provide important interventions at the animal production or processing level was investigated. Intervention techniques and processing modifications to lessen microbiological contamination of poultry meat were developed, tested and validated (see under Program Statement 4). Finally, the role or influence of other microbiological entities, specifically protozoa, in the dynamics of pathogen survival in the processing environment was also examined.

Examples of Accomplishments

- **Population studies of Campylobacter.** In collaboration with Cornell University, the evolutionary history of Campylobacter using microarray comparative genomic analysis and an expanded multi-locus sequence typing (MLST) scheme were evaluated. Even though populations of Campylobacter share DNA by recombination, population structures could be identified that correlated with the animal source of the bacterial isolates as well as the geographic source of the isolates. Human isolates showed the most overlap with the population from chickens.

- **Selection of antimicrobial resistance by carcass processing.** The effect of broiler processing chemicals on the antimicrobial resistance and subtype of Campylobacter is not entirely clear. A culture collection of Campylobacter from a previous ARS study includes individual isolates exposed to pH extremes during broiler processing. The antimicrobial resistance profile of all isolates was determined in order to elucidate if the pH treatments selected for organisms more resistant to traditional antimicrobial treatments. No differences were found.

- **Validation of sequence-based typing for Listeria monocytogenes.** Total genome sequences for 244 isolates of L. monocytogenes were analyzed to determine which genes jointly evolve. Analysis indicated that the genes were not linked as expected, and there appeared to be extensive exchange of genetic material between different lineages. It was observed that there were five groups of genes based on their evolutionary profiles and many genes had evolved independently. This was indicative of genes being cross-combined from different lineages.

- **Evolution of IncA/C plasmids.** Plasmids are important pieces of DNA that are often exchanged between bacteria and they often encode genes that give the bacteria a survival or growth advantage, such as antimicrobial resistance genes. To develop a model of how the class of IncA/C plasmids evolved, studies determined the DNA sequence for 39 recently isolated plasmids and compared these with each other, and 26 other sequences that were publicly available. The pattern of changes indicated that the transfer of any plasmid from one strain to another led to alterations in the DNA of the plasmid. This study served as a role model for whole-genome MLST analyses of population structures.

Outcome and Impact

The population structure studies of Campylobacter and Listeria have provided interpretive criteria for analyzing whole genome sequences for both epidemiological studies and selective evolution of the pathogens correlating with presence in poultry and processing environments. Some critical findings were: change in ribosomal genes appeared so rarely that they could be used for diagnostic procedures for accurately identifying the Intergenic Sequence Region (ISR). Although contaminated water was thought to be a mechanism for the passage of Campylobacter, the studies suggests that water plays only a minor role if at all in the transmission of human associated Campylobacter. Agricultural practices do not substantially impact the Campylobacter population. Within Listeria populations there appears to be a great deal of gene transfer. This was unexpected and such trading of genes means that the organism is more adaptive than expected and more genomic information is needed to fully trace lineages of the organism.

Examples of Relevant Publications

- Berrang, M.E., Smith, D., Meinersmann, R.J. 2011. Variations on standard broiler processing in an effort to reduce Campylobacter numbers on post-pick carcasses. Journal of Applied Poultry Research. 20(2): 197-202.
- Berrang, M.E., Frank, J.F., Meinersmann, R.J. 2013. Contamination of raw poultry meat by airborne Listeria originating from a floor drain. Journal of Applied Poultry Research. 22(1): 132-136.
- Lindsey, R.L., Frye, J.G., Cray, P.J., Meinersmann, R.J. 2011. Microarray-based analysis of IncA/C Plasmid-Associated genes from multidrug-resistant Salmonella enterica. Applied and Environmental Microbiology. 77(19): 6991-6999.
- Lomonaco, S., Verghese, B., Gerner-Smidt, P., Tarr, C., Gladney, L., Joseph, L., Katz, L., Turnsek, M., Frace, M., Chen, Y., Brown, E., Meinersmann, R.J., Berrang, M.E., Knabel, S. 2013. Novel epidemic clones of Listeria monocytogenes, United States, 2011. Emerging Infectious Diseases. 19(1): 147-150.
- Meinersmann, R.J., Ladely, S.R., Lindsey, R.L. 2010. Ribosomal operon intergenic sequence region (ISR) heterogeneity in Campylobacter coli and Campylobacter jejuni. Letters in Applied Microbiology. 51(5): 539-545.
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Introduction

Egg Safety and Quality: Production Systems/Omics

Egg safety and quality issues often occur in production and storage before entering the processing facility. There was a need for a farm-to-fork approach to product safety and quality, especially in light of Federal and State regulations and increased interest in alternative housing systems to replace housing hens in conventional cages. There was a deficiency of information regarding the effectiveness of Federal and State regulations and guidelines concerning egg storage, transportation, processing and marketing for alternatively produced eggs. Approximately 20 serotypes from the 1400 Salmonella enterica subspecies I are frequently associated with human disease. Salmonella enterica serovar Enteritidis (S. Enteritidis) is the only serotype known that can efficiently contaminate the internal contents of eggs produced by otherwise healthy hens. In contrast, S. Heidelberg has been associated with colonization of the cloaca of hens and contamination of the external surface of the eggshell. Risk of an outbreak is in part associated with the serotype involved. Research of the pathobiology of more problematic Salmonella serotypes within the laying hen and developing a better understanding of how serotypes differ in their ability to colonize associated environments is highly relevant for protecting the safety of the food supply. S. Enteritidis emerged as a persistent leading cause of human salmonellosis in modern times and substantial regulatory practices are aimed at reducing it on-farm; however, a comparative approach for understanding the pathobiology and epidemiology of other poultry-associated serotypes is also needed.

Examples of Accomplishments

- **Effect of production systems on naturally occurring flora and pathogens.** The impact of commercial conventional cage, enriched colony cage, and cage-free aviary production systems on egg and environmental microbiology was compared over the life of a production flock. The prevalence and diversity of Salmonella spp. and Campylobacter spp. was determined, as well as the levels of aerobic organisms and coliforms. Aviary floor eggs had the highest levels of total aerobes and coliforms, while conventional cage and enriched colony cage nest box eggs had the lowest. Salmonella was detected in 69% of aviary drag swab samples throughout the study. Campylobacter was detected in all housing systems with the greatest prevalence in aviary drag swabs (100%) and the lowest in aviary nest box swabs (10%). All housing systems resulted in fecal shedding of Salmonella, but conventional cage hens did not shed Campylobacter in any fecal samples. The impact of organic egg production was also investigated. Hens were rotated on dedicated paddocks or behind an organic dairy herd. There were no significant differences in the levels of total aerobes and Enterobacteriaceae detected in the production environment or eggs from the grazing treatments. Salmonella, Campylobacter, and Listeria prevalence was not different between the grazing regimens. The highest levels of microbes were found on the nest box straw and paddock grass which serve as microbial reservoirs.

- **Effect of production environments on Salmonella transmission, persistence, organ invasion, and egg contamination in laying hens.** Studies compared two different types of housing (conventional cages and enriched colony cages with perches and enclosed nesting areas) for their effects on internal organ colonization in laying hens infected orally with S. Enteritidis. There was no difference between housing systems in the

frequency of colonization of the intestinal tract (95.3% of all birds) after experimental oral inoculation with a dose of 10^7 cells of S. Enteritidis, but the pathogen was found at a significantly higher frequency in liver (96.9% vs. 75.0%), spleen (93.8% vs. 53.1%), ovary (25.0% vs. 10.4%), and oviduct (19.8% vs. 2.1%) samples taken 5-6 days post-inoculation from hens in conventional cages compared to hens in enriched cages. A second study assessed the effects of two different housing systems (conventional cages and colony cages enriched with perching and nesting areas) on the frequency of Salmonella Enteritidis contamination inside eggs laid by experimentally infected laying hens. No significant differences ($P > 0.05$) in the frequency of egg contamination were observed between the two housing systems.

- **Effects of level of Salmonella exposure to detection in laying hens and eggs.** Government and industry continue to devote substantial resources to controlling S. Enteritidis contamination of eggs by testing and developing risk reduction programs for commercial laying flocks. However, a comprehensive understanding of the process by which contaminated eggs are produced remains undefined. Eggs from specific-pathogen-free laying hens experimentally inoculated with oral doses of 10^4, 10^6, or 10^8 cells of S. Enteritidis were collected and the yolk and albumen of each egg were cultured separately to detect contamination. The studies showed that higher oral doses of S. Enteritidis were associated with significant increases in the detection of contamination of both yolk and albumen. S. Enteritidis was found at the highest level in the albumen of eggs from hens given the largest dose. In addition, the frequency at which the pathogen was shed in voided feces was determined. At 1 week post-inoculation, the frequency of fecal shedding of the pathogen ranged from 23.8% for the 10^4 dose to 87.5% for the 10^8 dose. No fecal shedding was detected after 3 weeks post-inoculation from hens inoculated with 10^4 cells, but a small proportion (2.5% to 5.0%) of hens that received doses of 10^6 or more were still shedding at 8 weeks post-inoculation. The results indicate that the oral exposure dose can significantly influence both the frequency and location of deposition of this pathogen inside eggs and the frequency and duration fecal shedding into the environment by infected laying hens. Accordingly, the probability of detecting infection by environmental testing protocols which depend on fecal shedding may be relatively low when hens in a flock are exposed to low doses of S. Enteritidis.

- **Cost effective serotyping of Salmonella enterica.** The cost, availability and delivery of timely results on serotype are problems that interfere with epidemiological studies of Salmonella in complex environments where multiple serotypes can be present. The cost of serotyping can range from $40 to $300 per isolate. Antibody reagents for determining serotype are becoming unavailable; while the molecular techniques developed, require expensive equipment, reagents and training to use. A discrete region of the Salmonella enterica subspecies I genome of approximately 500bp was discovered that was ideal as a target for serotyping because of the amount of heterogeneity present. The method is called dkgB-linked Intergenic Sequence Ribotyping (ISR). Sequences from ISR regions from over 100 serotypes of Salmonella were compared using DNA microarray as well as to a antibody-based method, Kauffman-White-LeMinor (KWL). Triangulation of techniques indicated that ISR was as accurate as the DNA microarray and KWL, and in some cases was more discriminatory than either. The ISR method can detect mixed

serotypes in culture if forward and reverse reactions are performed. Hundreds of isolates can be processed for about 1/10 of the cost associated with KWL or DNA microarray. A Material Transfer Agreement (MTA) was generated to transfer the USDA database on dkgB-linked Intergenic Sequence Ribotype (ISR) between ARS, the University of Georgia, Mississippi State University, collaborators in South America organized under AMEVEA, and companies involved in the poultry industry.

Outcomes and Impact

Salmonella contamination of eggs continues to be a major source of public health concern. The advent of State (and potentially Federal) legislation in the U.S. mandating laying hen housing requirements, along with consumer demand for diverse egg production options, has led to a need for greater understanding of how housing systems impact egg safety, hen health, and egg quality. The impact of the changes occurring at the level of the single nucleotide is an important type of evolution of Salmonella that impacts human health and well being. Efficiencies in serotyping of Salmonella in general will aid epidemiological investigations of this important food safety pathogen [as it occurs] on-farm, in the market place, in animal populations and in humans. Knowledge of single nucleotide polymorphisms that contribute to niche specialization will help in the selection of strains most appropriate for the testing and production of vaccines. Future issues include streamlining genomics information of S. enterica to make it user friendly to those without specialized backgrounds.

Examples of Relevant Publications

- Gast, R.K., Guraya, R., Guard, J.Y. 2013. Salmonella Enteritidis deposition in eggs after experimental infection of laying hens with different oral doses. Journal of Food Protection. 76(1): 108-113.
- Gast, R.K., Guraya, R., Jones, D.R., Anderson, K.E. 2013. Colonization of internal organs by Salmonella Enteritidis in experimentally infected laying hens housed in conventional or enriched cages. Poultry Science. 92(2): 468-473.
- Guard, J.Y., Gast, R.K., Guraya, R. 2010. Colonization of avian reproductive tract tissues by variant subpopulations of Salmonella Enteritidis. Avian Diseases 54(2): 857-861.
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- Jones, D.R., Anderson, K.E., Guard, J.Y. 2012. Prevalence of coliforms, Salmonella, Listeria, and Campylobacter associated with eggs and the environment of conventional cage and free range egg production. Poultry Science. 91(5): 1195-1202.
- Jones, D.R., Anderson, K.E. 2013. Housing system and laying hen strain impacts on egg microbiology. Poultry Science. 92(8): 2221-2225.
- Pulido-Landinez, M., Sanchez-Ingunza, R., Guard, J.Y., Pinheiro Do Nascimen, T. 2013. Assignment of serotype to Salmonella enterica isolates obtained from poultry and their environment in Southern Brazil. Letters in Applied Microbiology. 57(4): 288-294.

Introduction

Produce: Attachment/Survival/Growth/Omics

The rationale for the research focus described here was that enteric pathogens such as enterohemorrhagic Escherichia coli, Salmonella and Listeria are some of the most important foodborne pathogens associated with fresh produce consumption. They are capable of survival and proliferation in various adverse environments outside of animal hosts, and it has been postulated that these bacterial pathogens have developed fitness for association with plant growth environments. The mechanisms for these pathogens to attach and adhere to food and environmental matrices, and to survive and persist in such environments are poorly understood and needs increased research.

Examples of Accomplishments

- **Biofilms and produce**. Increased E. coli O157:H7 biofilm formation on stainless steel surfaces was observed in the presence of spinach lysates. Using physiological and metagenomic approaches, the success of E. coli O157:H7 at the early stage of mixed biofilm formation was attributed to its efficient utilization of spinach nutrients, whereas the rapid declining of E. coli O157:H7 population in later-stage biofilms was due to its poor competition for macronutrients with the natural plant microflora.

- **STEC's in California**. Shiga-toxin-producing Escherichia coli (STEC's) are frequently isolated from produce but their virulence potential and threat to human health are unknown. The virulence gene content of 85 E. coli O145 strains isolated from various environmental samples collected in the Salinas Valley region was determined. Results showed that the core virulence determinants of enterohemorrhagic E. coli are conserved in the majority of O145 environmental strains, including genes encoding Shiga-toxin, enterohemolysin, and the locus of enterocyte effacement. Using sequenced genomes as references, the genetic diversity of O145 was characterized. The results indicated that environmental E. coli O145 have the arsenal required to cause human illness and that their contamination of produce must be minimized. The genetic information can be used to identify molecular markers targeting hyper-virulent O145 strains.

- **E. coli O157 and lettuce.** Studies characterized the physiology of E. coli O157:H7 on cut lettuce using transcriptomics and demonstrated that there is a stress response with exposure to lettuce wounds, including enhanced tolerance to oxidative stress. This enables E. coli O157:H7 to survive subsequent treatment with sodium hypochlorite, the most broadly used sanitizer in the produce processing industry. These findings help explain the often-failure of current decontamination approaches in the fresh-cut industry and provide critical information about E. coli O157:H7 physiology for the design of new more efficacious technologies to reduce microbial contamination of produce.

- **Salmonella and retail produce.** The incidence of Salmonella contamination of retail produce has been positively correlated with the presence of soft-rot, a common post-harvest disease that involves maceration of plant tissue and affects all fruit and vegetables. Studies using transcriptomics determined that Salmonella experiences in macerated lettuce and cilantro leaves, nutritional and physical conditions that overlap with those in the animal host intestine. This high adaptation of Salmonella to efficient

colonization of degraded plant tissue results in extensive pathogen proliferation needed to cause human illness. These observations have serious implications for safe shelf-life standards set by the produce and food industry, and indicate that the presence of soft-rot should be included in microbial risk assessment models for produce.

- **Salmonella and protozoa.** The infectious dose of Salmonella from contaminated produce in humans is unclear. Previous studies showed that certain protozoa commonly inhabiting lettuce and spinach leaves fail to digest Salmonella in their food vacuoles and release it as viable cells in their fecal pellets, where it has increased persistence in the environment. Our transcriptomic studies revealed that Salmonella induces genes for survival and replication in the vacuoles of the common protozoan, Tetrahymena, similar to those induced in the vacuole of macrophages and human epithelial cells. This adaptation resulted in Salmonella sequestration in the protozoan fecal pellets and its increased resistance to low pH, such as that found in the human stomach.

- **Salmonella and almonds.** The biology of Salmonella in almond orchards and its contribution to kernel contamination that leads to outbreaks of salmonellosis, and costs the industry millions of dollars are unknown. Studies investigated the interaction of Salmonella with other microbes present in almond orchards and discovered that Salmonella forms large complex biofilms with a common epiphytic fungus, Aspergillus niger. Molecular studies identified cellulose-chitin interactions as a main factor in the production of these biofilms, which may facilitate the high persistence of Salmonella in almond orchards. California is the largest almond producer in the world therefore any contamination issues are a major concern.

- **Protozoan control**. Studies demonstrated that controlling certain protozoa with monensin, a dietary feed supplement for improved milk production and meat quality extends the survival of E. coli O157:H7 in dairy wastewater. Monensin also alters the community composition of both protozoa and bacteria in wastewater. These data suggest prudent use of antibiotic dietary supplements is warranted as such treatment enhances the persistence of E. coli O157:H7 in the agricultural environment.

- **Listeria monocytogenes and cantaloupe-borne outbreak.** Using molecular subtyping methods studies characterized and compared L. monocytogenes isolates from the 2011 Colorado cantaloupe outbreak, with isolates from other food-related outbreaks, clinical collections, defined epidemic clones, and isolates from chicken processing plants It was determined that the cantaloupe outbreak isolates matched isolates from food-related outbreaks in Canada, other worldwide clinical collections and chicken processing plants in the U.S. None of the cantaloupe isolates fit into previously described epidemic clones but two novel epidemic clones were created to include these strains. Thus, a new type of highly virulent Listeria has been recognized and needs to be monitored.

Outcome and Impact
There was significant progress and impact in our understanding of plant pathogen interactions, and of pathogen survival in stressful environments. The function of osmoregulated periplasmic glucans (OPGs) for bacterial survival in low osmolarity low nutrient environment was demonstrated, and multiple cellular proteins for the optimal growth of bacterial in such environment were identified.

Several studies showed the importance of native microflora; especially in promoting biofilm formation by bacterial pathogens. This suggests that native microflora-based biofilms may play an important role in the survival of bacterial pathogens in produce processing environments. The presence of the natural microflora on produce also has great value as an additional hurdle among other strategies to minimize enteric pathogen levels, and in the produce processing environment in order to reduce human illness.

The sequenced STEC genomes provided new genomic information about important serotypes and will facilitate the identification of STEC signature genes in order to improve their detection and characterization. Studies provided insight into the evolution of E. coli O157:H7 and which genes are critical to understanding survival strategies during cycling between host and non-host environments, and its epidemiology. The studies suggest the important role of EHEC accessory genes in its environmental fitness and provide science-based knowledge to identify molecular targets for the development of improved sanitation methods.

Other studies identified that proximity to animal/dairy operations is a risk factor in the contamination of produce. This could be a critical finding since bacterial profiles specific to each animal operation may be used as tracers for detecting the source of pathogens that are transported to produce or fruit grown nearby. Further, passage through protozoa confers a physiological advantage to Salmonella that may lower the number of cells required to make a human sick by enhancing its resistance to acidic conditions in the stomach. Salmonella in almond orchards and other food- or medically relevant systems may form biofilms with fungi. Studies revealed L. monocytogenes fitness factors in soil, which is considered as an important reservoir of the pathogen, and that the biology of human pathogens on plants can be used to develop more effective interventions to mitigate contamination of produce. Industry shows interest in the development of sanitizers based on the physiology of enteric pathogens. Continued studies on plant and plant pathogen/commensal interactions should be a future research focus.

Examples of Relevant Publications

- Brandl, M., Carter, M.Q., Parker, C., Chapman, M.R., Huynh, S., Zhou, Y. 2011. Salmonella biofilm formation on Aspergillus niger involves cellulose -chitin interactions. PloS ONE 6(10): e25553.
- Brandl, M., Cox, C.E., Teplitski, M. 2013. Salmonella interactions with plants and their associated microbiota. Phytopathology. 103(4): 316-325.
- Carter, M.Q., Xue, K., Brandl, M., Liu, F., Wu, L., Louie, J.W., Mandrell, R.E., Zhou, J. 2012. Functional metagenomics of Escherichia coli O157:H7 interactions with spinach indigenous microorganisms during biofilm formation. PLoS One. 7(9): e44186.

- Gorski, L.A., Parker, C., Liang, A.S., Cooley, M.B., Jay-Russell, M., Gordus, A., Atwill, R.E., Mandrell, R.E. 2011. Prevalence, distribution and diversity of salmonella enterica in a major produce region of California. Applied and Environmental Microbiology. 7798): 2734-2748.
- Goudeau, D.M., Parker, C., Zhou, Y., Sela, S., Kroupitski, Y., Brandl, M. 2013. The Salmonella transcriptome in lettuce and cilantro soft rot reveals a niche overlap with the animal host intestine. Applied and Environmental Microbiology. 79(1): 250-262.
- Patel, J.R., Millner, P.D., Nou, X., Sharma, M. 2010. Persistence of enterohemorrhagic and non-pathogenic E. coli on spinach leaves and in rhizosphere soil. Journal of Applied Microbiology. 108(5): 1789-1796.
- Patel, J.R., Sharma, M., Ravishankar, S. 2011. Effect of curli expression and hydrophobicity of E. coli O157:H7 on attachment to fresh produce surfaces. Journal of Applied Microbiology. 110(3): 737-745.
- Smith, C.D., Berk, S.G., Brandl, M., Riley, L.W. 2012. Survival of diarrheagenic E. coli pathotypes in the free-living ciliate tetrahymena sp. FEMS Microbiology Ecology. 83(2) 574-583.

Introduction

General/Postharvest: Biofilms/Environmental Stress

Most microbes in the environment, including foods and food processing environments, grow on surfaces, in a complex community of microorganisms. Microorganisms in these communities often engage in a wide range of intercellular behaviors that may affect the presence and persistence of pathogens in foods. The primary focus of this research was to gain a better understanding of some of the complex [social] behaviors of foodborne pathogens and to develop methods to catalog bacterial communities associated with selected foods and food processing environments. This focus was accomplished primarily by studying the genetic and environmental factors contributing to biofilm formation in Shiga-toxin-producing E. coli (STEC); studying the potential for mixed biofilm formation between STEC and non-pathogenic environmental flora; and applying sampling and sequencing methods to qualitatively and quantitatively determine the members of microbial communities in food and food processing facilities.

Examples of Accomplishments

- **Biofilm Formation by STEC.** Shiga-toxin-producing E. coli (STEC) are an important cause of foodborne illness and the USDA-FSIS has adopted a zero tolerance policy for isolates of STEC O157:H7 and 6 serotypes of non-O157 STEC. A large collection of STEC O157:H7 and non-O157 STEC strains were characterized for their biofilm-forming capabilities and it was discovered that STEC do not generally form robust biofilms. While less than 5 percent of strains of serotype O157:H7 are able to form robust biofilm, 20-30% of non-O157 strains were able to form biofilms. Many strains of STEC O157:H7 contained two different mutations that disrupt the ability to form biofilms.

- **Regulation of oxidative stress tolerance in E. coli biofilms.** Pathogenic E. coli are able to form biofilms that increase the bacteria's resistance to environmental assaults and their persistence in food processing facilities. Research was undertaken to gain a better

understanding of oxidative stress tolerance in E. coli biofilms. Some important findings in this study include: peroxide resistance was greater in biofilm cells than in swimming cells; the major protective peroxidases for biofilm cells were identified; regulatory genes required for full oxidative stress resistance were identified; and complex regulatory systems for peroxidase gene regulation were determined. Furthermore, studies using mixed strain biofilms demonstrated that non-biofilm forming STEC O157:H7 strains were retained on solid surfaces associated with biofilms generated by companion strains and this biofilm association conferred increase resistance to oxidative stress.

- **Construction and characterization of STEC control strains for FSIS.** Strains of E. coli O157:H7, [6] non-O157 STEC's and Salmonella containing a unique DNA sequence were constructed to provide positive controls for FSIS. This DNA sequence will allow FSIS to distinguish isolates and minimize the possibility of false-positives. End-point and real-time PCR assays for specific detection of these control strains were developed and validated. The control strains were useful in studies on pathogen growth modeling.

Outcome and Impact

The basic knowledge of the various genetic mutations that effect biofilm formation in STEC, the fact that most STEC, particularly strains of STEC O157:H7, are not able to form robust biofilm in pure culture, suggests that this organism may not form robust biofilm on foods or in food processing environments. It may be that STEC need not persist in the food processing environment but incidentally contaminate beef products during slaughter and merely need to survive processing. If this is the case, every effort must be made to reduce the pre-harvest incidence of STEC as well as prevent incidental contamination at slaughter. The discovery of the unique growth morphology of the common spoilage bacteria Brochothrix thermosphacta by others poses several interesting basic research questions. Most importantly may be how it is involved in allowing the association of pathogens with this complex macroscopic structure?

Examples of Relevant Publications

- Chen, C., Hofmann, C.S., Cottrell, B.J., Strobaugh Jr, T.P., Paoli, G., Nguyen, L.T., Yan, X., Uhlich, G.A. 2013. Phenotypic and genotypic characterization of biofilm forming capability in non-O157 Shiga-toxin-producing Escherichia coli strains. PLoS One. 8(12): e84863.
- Uhlich, G.A., Rogers, D., Mosier, D.A. 2010. Escherichia coli serotype O157:H7 persistence on solid surfaces and peroxide resistance is enhanced by dual-strain biofilm formation. Foodborne Pathogens and Disease. 7(8): 935-943.
- Uhlich, G.A., Chen, C., Cottrell, B.J., Irwin, P.L., Phillips, J.G. 2012. Peroxide resistance in Escherichia coli serotype O157:H7 biofilms is regulated by both RpoS dependent and independent mechanisms. Microbiology. 158(9): 2225-2234

Introduction

Foodborne Parasites

There is no required reporting for illnesses attributed to parasites, thus the full extent of the burden and cost is currently unknown. Many of these parasites are zoonotic, spread by animals through direct contact, or by contaminated water and food. There were several research focus' within this Program Statement: to improve recovery of the parasites from foods using new reagents to dissociate the parasites from food material, discovery of new species, genotypes, and subtypes of zoonotic parasites, test specialized drugs against a virus within parasites, determine proteins for vaccine development and drug discovery, test probiotics to protect against parasite infection, determine what genetic and genomic features distinguish Trichinella species, determine if microsatellite loci can be used to trace zoonotic outbreaks; and determine the genetic features that account for the epidemic spread of certain strains of Toxoplasma.

Examples of Accomplishments

- **New pathogen species infectious for humans and animals.** A new species of Cryptosporidium, infectious for a wide range of animals and humans was discovered and named Cryptosporidium ubiquitum. The species infects cattle, goats, sheep, wildlife, and rodents as well as humans. Recognition of this organism is important because of it may be a human pathogen and potential source of infection.

- **Protective immunity using unique Giardia proteins (giardins).** There are no vaccines against giardiasis and drug treatment can be lengthy and unpleasant; therefore to stimulate immunity and avoid illness is preferable. Giardia has unique proteins called giardins. Antibodies produced against beta- and delta-giardins were identified and provides a basis for developing methods of prevention/treatment of Giardia infections. Giardin antibodies, immunizations, or drugs to block attachment to cell surfaces are being assessed.

- **Toxoplasma.** Pregnant women, their fetuses, and persons with AIDS are vulnerable to infections with Toxoplasma gondii, which may be acquired from infected meat or from contact with the feces of cats. Working with an international team, studies developed a broader view of parasite diversity by defining the amount of variation in parasite genes, and by evaluating the importance of recombination in generating new parasite strains. These results suggest that the parasite endures prolonged intervals of genetic stasis, punctuated by occasional (but consequential) episodes of genetic re-assortment. Waterborne outbreaks have been subsequently established to represent bursts of replication from highly inbred parasites.

- **Trichinella and swine.** Increasing concern that trichinellosis (once nearly completely eliminated from the U.S. pork supply) may resurge as a foodborne parasitic threat, due to raising swine outdoors. Studies compared a parasite capable of thriving and persisting in swine with a closely related parasite adapted to wildlife hosts. In spite of the fact that the parasites are more similar than different, proteins were discovered that specifically differentiate between these parasites.

- **Trichinella and risk.** Most cases of trichinellosis are attributed to consuming poorly cooked wild game, with conventionally raised pork no longer a risk. Studies established that pigs raised in familial consumption are twice as likely to be infected as wild boar. By contrast, swine raised indoors were entirely free of infection. Thus, risk varies not geographically but rather according to animal husbandry practices. Risk is greatest where swine are raised near people, with required attention to rodent control and quality feed.

Outcome and Impact

Diagnosis of the enigmatic parasite potentially linked with irritable bowel syndrome (IBS) in humans and diarrhea in animals, Blastocystis, is difficult. However, it can now be quickly and clearly identified using a commercially marketed fluorescent antibody stain visualized by microscopy. Undiscovered species and genotypes of parasites in food animals that impact food safety continue to emerge with the application of molecular identification methods, enabling distinction between microscopic forms that previously appeared indistinguishable including three new species of Cryptosporidium. The microsporidian parasite E. bieneusi, first seen in HIV-AIDS patients and now in non-AIDS patients, was found to be composed of over 120 genotypes (25 discovered in this study) some of which are shared by food animals. It is likely that more will be found in companion animals and wildlife. Assays for the newly identified species of Cryptosporidium and its viral symbiont have been developed for assessing the effectiveness of anti-protozoal and anti-viral drugs Proteins useful in the development of a vaccine against giardiasis are identified and further research should result in commercialization.

Studies clarified how infections in wildlife influence the safety of pastured pork by identifying heritable differences between two related species of Trichinella, only one of which (T. spiralis) severely compromises pork safety. The other species, Trichinella murrelli, fails to thrive in swine. Understanding what makes pigs vulnerable to T. spiralis and the means to trace chains of transmission of Trichinella spp. was established. The genetics of T. gondii reproduction was characterized. The differences in protein expression by the two parasites at various stages of their development was determined and immunological assays were designed in order to establish which of these might offer the most potential to diagnose animals exposed to one or the other of these parasites. To improve the ability to trace outbreaks of T. spiralis, additional variable genetic markers were validated. Analyzing these markers demonstrated that in nature, animals can become infected more than once, but seldom do which is relevant for potential vaccines. Finally, the process of strain formation and dissemination of toxoplasmosis was elucidated. We established new criteria for recognizing a fourth major lineage in North American wildlife, and contributed to a comprehensive assessment of global diversity of T. gondii, providing a common set of reference strains which will hastened progress in the field of toxoplasmosis epidemiology.

Examples of Relevant Publications

- Fayer, R., Santin, M., Macarisin, D. 2010. Cryptosporidium ubiquitum n.sp. in animals and humans. Veterinary Parasitology. 172(1-2): 23-32.
- Fayer, R., Santin, M., Macarisin, D. 2012. Detection of concurrent infection of dairy cattle with Blastocystis, Cryptosporidium, Giardia, and Enterocytozoon by molecular and microscopic methods. Parasitology Research. 111(3): 1349-1355.
- Khan, A., Dubey, J.P., Su, C., Ajioka, J.W., Rosenthal, B.M., Sibley, L.D. 2011. Genetic analyses of atypical Toxoplasma gondii strains revealed a fourth clonal lineage in North America. International Journal for Parasitology. 41(6): 645-655.
- Su, C., Khan, A., Zhou, P., Majumdar, D., Ajzenberg, D., Darde, M.L. Zhu, X.-Q., Ajioka, J.W., Rosenthal, B.M., Dubey, J.P., Sibley, L.D. 2012. Globally diverse Toxoplasma gondii isolates comprise six major clades originating from a small number of distinct ancestral lineages. Proceedings of the National Academy of Sciences. 109(15): 5844-5849.
- Turner, M., Lenhart, S., Rosenthal, B.M., Sullivan, A., Zhao, X. 2013. Modeling effective transmission strategies and control of the world's most successful parasite. Theoretical Population Biology. 86: 50-61.

1.C Technologies for the Detection and Characterization of Microbial Contaminants

Goal

The goal of the research under this Problem Statement was that the Program would lead in developing and validating detection, differentiation and characterization technologies that have public health, regulatory, trade, industry and research use; a commonality of interests between government and stakeholders. Effective and efficient detection and characterization technologies that can be readily implemented will allow improved response times to events, and subsequently allow for the development of mechanisms for treating foods taken out of commerce. The technologies would provide data to identify areas where interventions are most critically needed, thus assisting the implementation of HACCP/GAP/GMP programs by USDA-FSIS, AMS; DHHS-FDA, and their regulated industries. The data would also assist to develop and validate predictive microbial models and to help fill identified data gaps.

A challenge was to address the specific needs of the various stakeholders, while balancing the inherent capabilities of the Program. Thus, the need to focus on the most promising technologies (depending on the matrix) or point of use, and whether the technology was to be used for baseline studies, traceability and/or forensics. Decisions had to be made relative to what should/could be detected, and the required level of detection and characterization. Further, technologies that had the highest level of detection/characterization capability might not necessarily be the most practical, useful, economically viable, or be readily implemented. High-throughput analysis was important, but it may also be impractical. Finally, in developing technologies decisions were not made in isolation; there needed to be an integration of biology, epidemiology and the physical sciences systems.

Introduction

Traditional microbial culture methods are able to detect and identify a single specific bacterium, but may require days or weeks, and often do not produce quantitative data. The quest for faster, quantitative results has spurred development of "rapid methods". However, the high levels of non-target microorganisms and other materials in food samples often interfere with detection limits required for food safety. Physical and/or microbiological processes, which separate or isolate the target pathogen from the sample matrix and/or other microorganisms and substantially increase its concentration prior to detection, are essential for effective detection of foodborne pathogens. These processes must preserve the unique genetic and phenotypic features which allow detection and identification of the target organism. Underlying the detection process are sampling, separation, isolation, and concentration. Further, screening applications for the detection of bacteria are not as technically demanding as those that seek to validate the identity of the detected microorganism. Furthermore, the complexity to develop novel identification methods is compounded when rapidity is considered a key aspect. Research efforts were focused on multiple efforts: improvements to detection such as accounting for microbial integrity (genotypical or phenotypical attributes) during isolation and/or enrichment, considerably more efficient nucleic acid extraction strategies, and enhanced techniques for bacterial separation and concentration.

The development of novel biorecognition elements such as generated PCR and/or hybridization probes as well as stable aptamers were also area of need. This research would set the stage for consolidating our findings with existing technologies in order to ultimately generate novel typing platforms whether they-be variations on existing typing methods such as MLST, RFLP or greatly expanded DNA and/or antibody (aptamer) microarrays.

Additionally, ARS has collaborative interactions with Purdue University's Center for Food Safety Engineering, building on each partner's strengths to develop and integrate operational technologies to rapidly and effectively concentrate viable target cells from food matrices in a self-validating system into an automated instrument; and develop, evaluate, and adopt novel technologies for rapid detection, identification, and quantification of viable and non-viable target microorganisms, including light scattering technologies (BARDOT), biochips, biosensors, and bacteriophage. Some technologies included were developed as a consequence of other research, for example, produce ecology or biosecurity related toxins studies.

Current agricultural production and processing practices have also led to an increase in the types and virulence of human pathogens adapted to grow or survive on foods. Escherichia coli O157:H7, Salmonella spp. and Campylobacter spp. remain major causes of outbreak and sporadic illnesses associated with meat, poultry, fresh produce and raw milk. Indeed, some unexpected foods are now associated with outbreaks for the first time (e.g., peanuts, tree nuts, cookie dough, hydrolyzed vegetable protein in many foods). In addition, non-O157 E. coli have emerged and demand increased efforts to identify and evaluate the types and functional activities of associated virulence factors such as Shiga-toxins. Current methods for identifying and typing pathogens remain inadequate for robust studies of the ecology, epidemiology and source tracking of pathogens in the environment. Another aim was to integrate an "omics approach" for enteric foodborne pathogens; to characterize genomic and phenotypic variations that we hypothesize are relevant to persistence and virulence in diverse and complex agricultural environments and to public health; and to develop and validate enhanced detection and typing methods.

There were specific areas of focus: Comparative genomic analyses of Campylobacter spp., Arcobacter spp., E. coli spp. and S. enterica to identify novel genetic elements or polymorphisms that are associated with virulence, niche specialization or other adaptive traits; developing sequence-based typing methods to detect and analyze multiple food-borne pathogens from numerous sources; generation of gene expression profiles for pathogens from varying sources and in response to various environmental stresses to identify factors contributing to virulence and survival in diverse habitats; development of rapid, simple and inexpensive multiplex assays for pathogen detection and virulence characterization using novel technology for use in surveillance and outbreak epidemiology; and establishing proteomic approaches for detecting and typing foodborne pathogens and toxins, and measure pathogen response to environmental stresses by mass spectrometry methods. The accomplishments/technologies below are divided based on the stage of detection.

Examples of Accomplishments

Sampling

- **Microbiological sampling of poultry products.** A study for FSIS to determine the best means to sample broiler skin for the recovery of Salmonella and Campylobacter was completed. Carcasses were inoculated with known numbers of both pathogens. Breast skin was sampled by a non-destructive sponge method and by a skin excision method. Skin excision allowed recovery of 0.1 to 0.2 log10 more inoculated bacteria than did sponge sampling. When excision was used on the same skin previously sampled by sponging, the combination of both methods did not significantly improve recovery compared to sponging alone. Skin excision is slightly more sensitive than sponge sampling; however, for repeated nondestructive sampling of broiler carcasses during processing, sponge sampling may be preferable within 60 s of a contamination event.

Media for growth enhancement

- **Enhanced growth of Listeria monocytogenes**. L. monocytogenes does not grow well in the presence of background microflora and/or major pathogens. Since most rapid methods have detection limits of $\geq 10^3$ CFU/mL, the need to improve the growth of L. monocytogenes is apparent. Synergistic effects of growth on casamino acids and Oxyrase in multiple broths was demonstrated to enhance growth of L. monocytogenes to $>10^5$ CFU/mL in an overnight culture of either pristine broth or ground pork homogenate. A CRADA with media manufacturers to extensively test the formulation on multiple strains of pathogens in various food systems is under development.

Separation, isolation, and concentration

- **Cell concentration technology.** A cell concentration and recovery system called C^3D was developed. It is a self-validating, automated instrument which minimizes risks of contamination and requires low labor intensity. A commercial hollow fiber membrane module is used to microfilter 50 to 400 mL volumes of enzyme-treated food extracts. The microfiltration step recovers 50 to 80% of the cells in a viable form and at 500 to 1000 higher concentrations than originally present in the sample. The C^3D system is designed to be efficiently cleaned and sterilized, and the membrane module can be reused. The key is the use of enzymes to hydrolyze protein or other water soluble macromolecules so that fouling of the microfiltration membranes is minimized. The cost for the whole process has been reduced by using a stepwise hydraulic and chemical cleaning procedure. Combined operational cycles of concentration, recovery, cleaning, and re-equilibration occur in one hour. The software of the C^3D system has been developed for simple operation, following instructions for each step that can be visualized on the monitor during the process. The C^3D system has been tested on inoculated and naturally contaminated food samples, and its application has been validated using real-world samples provided by the food industry. (CSFE at Purdue University were principal investigators)

Culture based

- **Novel agar medium to simultaneously isolate and detect non-O157:H7 STEC.** A single chromogenic agar medium was developed to simultaneously isolate and detect all six non-O157:H7 STEC serogroups [O26, O45, O103, O111, O121, and O145]. The ability to isolate and distinguish among these serotypes is based on the utilization of various carbohydrates, b-galactosidase activity, and resistance to selective agents. This chromogenic agar medium could help significantly in routine screening for the top six non-O157 STEC serogroups from beef cattle and other food. An industrial cooperator has commercialized the improved medium.

Nucleic acid based (and phenotypic expression)

- **PCR-based kits for STEC and Salmonella detection.** In collaboration with FSIS methods for detection, isolation, and identification of non-O157 STEC in ground beef were developed. The methods were included in the FSIS Microbiology Laboratory Guidebook (MLG). In addition, through a collaboration with a commercial company, PCR-based non-O157 STEC screening kits were developed, and these are now included in the MLG.

- **Campylobacter species identification and strain typing.** Multilocus sequence typing methods for emerging Campylobacter's were developed and expanded; an improved atpA typing method to identify Campylobacter species; and a porA-based typing method for C. jejuni and C. coli. The expanded multilocus typing methods were used to type campylobacter's isolated during a multi-year survey in the Salinas Valley, California. The atpA method can also identify other clinically relevant organisms, such as Helicobacter pylori. The porA typing method was used successfully during multiple milk-associated Campylobacter outbreaks to help identify the source.

Serological based

- **Non-O157 Shiga-toxin-producing E, coli (STEC).** To comply with FSIS testing protocols, components required for rapid and accurate testing of O26, O45, O103, O111, O121, and O145 serogroups were developed under a CRADA. The materials commercialized as a latex agglutination tests (LAT) and immunomagnetic separation (IMS) beads were used for efficient isolation, concentration, and identification of the target STEC from food samples.

- **Toxin typing assay for E. coli (STEC) using antibody microarray.** A colorimetric ELISA in microarray format was developed as an inexpensive alternative to traditional fluorescence-based array scanning. This multiplexed, high-throughput technique uses inexpensive, multi-well polystyrene plates as microarray substrates as well as low-cost flatbed image scanners employed in typical office suites. Monoclonal antibodies were used to demonstrate the platform and its ability to toxin type STEC's that produced either Shiga-toxin 1, 2, or 1 and 2. In addition, toxins were generated and detected in situ using enrichment cultured STEC's with the assistance of the antibiotic mitomycin C and a protein extraction reagent. Detection was both quantitative and qualitative.

Optical/Sensing

- **Phage-based technology.** A phage-based technology to produce a visual or optical signal upon infection of E. coli O157:H7 was developed. To accomplish this, the E. coli O157:H7 specific bacteriophage [phiV10] was genetically modified, so upon infection of the pathogen it reprograms to biochemically produce a colorimetric or optical signal which can be visually detected. Primarily the phage-based technology detects the target organism during the selective growth step during testing protocols allowing for faster results. (CSFE at Purdue University were principal investigators)

- **Light scattering method developed for identifying pathogenic bacteria.** The innovative optical light scattering sensor called BARDOT (*BA*cterial *R*apid *D*etection using *O*ptical light scattering *T*echnology), is a noninvasive label-free detection system which allows detection and identification of bacterial colonies in real-time. In this semi-automated device, a Petri-dish containing bacterial colonies is scanned and a colony map of the plate is acquired. In addition to providing an accurate count of the number of colonies on the plate, a laser beam sequentially runs through each preselected colony to generate a scatter signature image. Software developed by the project compares this image to a database of images for detection and identification. Image databases have been generated for serovars of Salmonella and Shiga-toxin-producing E. coli, Bacillus, Staphylococcus, and several other foodborne pathogens. Detection and discrimination of numerous pathogens such as Listeria, Salmonella, Vibrio, Escherichia, and Bacillus at the genus, species, and serovar levels from food have been demonstrated. Additionally, detection and classification of unknown pathogens is also possible using the BARDOT, as demonstrated in a study of non-O157 pathogenic E. coli wherein even for the two most difficult classes of E. coli studied (O103:H11, O103:H12) BARDOT was able to detect the presence of previously unseen classes. The technology has been patented and commercialized. A portable BARDOT instrument was also developed with the identification capabilities of the larger units. The portable unit has travelled extensively overseas for demonstrations without issue. (CSFE at Purdue University were principal investigators)

Alternate technologies/pathogens

- **Nanobiosensors** A nanobiosensor for simultaneous detection of several pathogens using a method for specific detection of pathogen DNA was developed. Unlike traditional DNA amplification which involves numerous temperature changes, the developed nanobiosensor is simplified because it uses a DNA amplification method that occurs at one temperature. The biochip sensors are able to rapidly and simultaneously detect L. monocytogenes, Shiga-toxin-producing E. coli, and Salmonella. A second nanobiosensor based on a fluorescent nanoparticle strategy was developed, where nanoparticle magnetic beads are modified to bind specific pathogenic bacteria, and upon binding of the target pathogen to the beads, a fluorescent signal is generated that is easily detectable by common laboratory equipment. This nanoparticle magnetic bead method detected low numbers of E. coli O157:H7, Salmonella Typhimurium, and L. monocytogenes in less than 2 hours. The biosensor was able to detect each pathogen individually or in a mixture with very little cross-reactivity. The limit of detection for the sensor was $\sim 10^3$ CFU/ml for all three pathogens.

- **Human norovirus**. An optimized detection and capture method for human norovirus (HuNoV) from food samples was developed. The specificity and kinetics of HuNoV attachment to histo-blood group antigens conjugated to magnetic beads (HBGA-MB), which were developed previously as a sensitive detection method for HuNoV, had not been determined. Optimized pH and ionic strength conditions for fast and efficient binding of HuNoV to the beads and showed enhanced subsequent recovery from the beads by temperature treatment. The optimized method provides a significantly higher recovery rate than extraction with the commercial reagents commonly used for HuNoV detection and has broad specificity because it captures most GI and GII strains.

Omics

- **Escherichia coli.** Shiga-toxin producing E. coli (STEC) strains are not equally virulent and this may be related to genomic differences. Sequencing and comparative genomic analysis on the complete genome of an O157 progenitor strain, EPEC O55:H7 was done. Results suggested that the placement and presence of lambda-like bacteriophages (including those containing the Shiga-toxin-encoding gene *stx*) should not be considered stable evolutionary markers or be required in placing O55:H7 and O157:H7 strains within the stepwise evolutionary models. Comparative genomic analysis on two STEC O145 strains associated with two distinct outbreaks (ice cream in Belgium and lettuce in the USA) provided evidence that STEC O145 and STEC O157 evolved from a common lineage that was distinct from STEC O111 and STEC O26. Ultimately, each serotype and strain evolved via a lineage-independent manner by acquisition of the core set of EHEC virulence factors, including the genes encoding Shiga-toxin on bacteriophage.

- **Campylobacter/Arcobacter**. Although C. jejuni is responsible for most cases of campylobacteriosis, recent data suggest that multiple instances of sporadic and possibly outbreak-related, food-borne illnesses are caused by other less-characterized members of the Campylobacteraceae. Little if any genomic data exist for these emerging organisms, especially for host-association, virulence, antibiotic resistance, lateral gene transfer and novel metabolic pathways. The genomes of all Campylobacter taxa not already sequenced (33 taxa in total), were sequenced to completion, assembled and annotated. In addition, the genomes of 16 Arcobacter spp. were likewise characterized. Virulence genes were identified in many organisms, suggesting that these taxa might contain potential pathogens. Also, genes encoding novel metabolic pathways and genes tentatively related to host-association were identified. The genomic data was used to develop new or improve existing Campylobacter/Arcobacter typing and species identification methods.

- **Proteomic approaches**. Top-down proteomic software was further developed for in silico database construction (ISDB Processor v1.2). This new software has allowed development of a database of over 250,000 bacterial protein sequences. The most significant application has been its use in identification of sequence-specific subtypes of bacterial protein toxins. Sequence-specific subtypes of Shiga-toxin 2 (Stx2a-g) have been linked to differences in relative toxicity. Stx2 subtypes were identified from

bacterial cell lysates using tandem mass spectrometry and developed top-down software. As the protocol involves antibiotic-induced toxin production, STEC response to a specific antibiotic has clinical relevance. Top-down proteomic identification of acid stress proteins critical to pathogen survival at low pH, was also demonstrated. Another component of the foodborne pathogen proteomics effort has been characterizing surface-exposed biomolecules critical to attachment or virulence.

Outcome and Impact

These ranged from modest developmental and validation efforts to highly innovative, new, Patentable technologies. Each development, validation or improved/ new technology however, presented its own successes and challenges. Some efforts may appear duplicative, for example various methods were developed for STEC's. However, it was often found that a method developed for animal derived products was not applicable for another system such as produce, or manure. One of the critical issues regarding this Problem Statement was how to measure any accomplishments impact. Method development can be seen as the first leg of a relay, that is, development/validation of the technology, then passing it to our stakeholders for their validation, and use-or non-use. The Program works with stakeholders/partners to resolve technology issues but leave final determination of any methods use to their discretion. It should be also stressed that despite efforts, some studies simply do not work out; for example, the production of stable RNA aptamers as replacement of antibodies for biorecognition.

Significant advances were made to establish a framework for comparative genomic analyses based on complete genome sequencing of several bacterial pathogens, including: Campylobacter, E. coli, and S. enterica and some of their non-pathogenic relatives. This genomic sequencing resulted in the annotation of the complete genome content of the bacteria; and identification of novel genetic elements and metabolic pathways that will assist in understanding and modeling these bacterial pathogens at various stages within the food environments. Our analysis of the genomic data identified new bacterial taxa and revealed clues into evolutionary shifts that may lead to the creation of bacterial pathogens. The genomics also led to the development of molecular and sequence-based methods that detect and type foodborne pathogens.

Transcriptomics and metabolomics of foodborne pathogens from varying sources have revealed differences, in response to various environmental stresses, between variants within bacterial species and identified genes and operons critical for mounting stress responses. DNA based-technologies were developed and validated for the specific identification of bacterial and viral pathogens. Some of these technologies have proven to be sensitive, rapid, and practical methods with commercial potential. Development of top-down proteomics methods and databases has allowed us to examine the induction of toxin expression.

Examples of accomplishments implemented/commercialized/obtained awards

- A multiplex real-time PCR method for E. coli O157, Salmonella spp., and Listeria monocytogenes in meat, cheese and produce samples successfully adopted by the FDA
- A method for confirming presumptive positive non-O157 STEC's. Incorporated into the FSIS Microbiology Laboratory Guidebook (MLG) Chapter 5B.02. Adoption contributed

to the implementation of the USDA 'zero tolerance policy' for the six non-O157 STEC's in June, 2012. The scientists who developed the technology received the USDA Secretary's Award "For excellence in leadership to protect the U.S. food supply through timely development and implementation of a robust science-based program for controlling non-O157 Shiga-toxin-producing E. coli in beef.

- BARDOT technology. Prototype and commercial instruments (bench-top and portable) are available. This technology produced 1 commercial license, 2 Patent applications, 2 provisional Patents, 1 utility Patent, and various material transfer agreements.

Examples of Relevant Publications

- Ahmed, W.M., Bayraktar, B., Bhunia, A.K., Hirleman, E.D., Robinson, J.P., Rajwa, B., 2013. Classification of bacterial contamination using image processing and distrubted computing. IEEE Journal of Biomedical and Health Informatics 17(1): 232-239.
- Bhaduri, S., Phillips, J.G. 2013. Growth of a plasmid-bearing (pYV) Yersinia pestis KIM5 in retail raw ground pork. Foodborne Pathogens and Disease. 10(5): 467-471.
- Carter, M.Q., Xue, K., Brandl, M., Liu, F., Wu, L., Louie, J.W., Mandrell, R.E., Zhou, J. 2012. Functional metagenomics of Escherichia coli O157:H7 interactions with spinach indigenous microorganisms during biofilm formation. PLoS One. 9: e44186.
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- Huff, K., Aroonnual, A., Bae, E., Banada, P., Rajwa, B., Rajwa, B., Hirleman, E.D., Robinson, J.P., Richards, G.P., Bhunia, A. 2012. Light scattering sensor for real-time identification of Vibrio parahaemolyticus, V. vulnificus and V. cholera colonies on solid agar plates. Microbial Biotechnology. 5(5): 607-620.
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- Kalchayanand, N., Arthur, T.M., Bosilevac, J.M., Wells, J., Wheeler, T.L. 2013. Chromogenic agar medium for detection and isolation of Escherichia coli serogroups O26, O45, O103, O111, O121, and O145 from fresh beef and cattle feces. Journal of Food Protection. 76(2): 192-199.

- Li, X., Ximenes, E., Amalaradjou, M.A.R., Vibbert, H.B., Foster, K., Jones, J., Liu, X., Bhunia, A., Ladisch, M., 2013. Rapid sample processing for foodborne pathogen detection via crossflow microfiltration. Applied and Environmental Microbiology 79(22): 7048-7054.
- Miller, W.G., Chapman, M.H., Yee, E., On, S.L., Mcnulty, D.K., Lastovica, A.J., Carroll, A.M., Mcnamara, E.B., Duffy, G., Mandrell, R.E. 2012. Multilocus sequence typing (MLST) methods for the emerging Campylobacter species C. hyointestinalis, C. lanienae, C. sputorum, C. concisus and C. curvus. Frontiers in Cellular and Infection Microbiology. 2:45
- Tian, P., Mandrell, R.E. 2011. A simple method to recover Norovirus from fresh produce with large sample size by using histo-blood group antigens conjugated magnetic beads re-circulating immunomagnetic separation system. International Journal of Food Microbiology. 147(3): 223-227.

1.D Intervention and Control Strategies

Goal

The goal of the research under this Problem Statement was to develop strategies to control and/or eliminate microorganisms in animals, seafood and plants, and their derived products, and production, processing and storage systems. An underlying assumption was that production control interventions reduce downstream contamination which subsequently reduces disease risk. Thus the special challenge was that intervention/control strategies must be developed that reduce the pathogen load during the entire food continuum. Efforts would focus on developing environmentally compatible technologies. Strategies would be developed for operations of all sizes (large to very small). Pathogens may develop resistance to some interventions; thus, efforts would focus on development of combinations of new or innovative intervention technologies for (minimal) processing. Interventions would be developed based on an understanding of their modes of action and effects on the microbial ecology of a food product, since inadequate suppression of spoilage could create an opportunity for human pathogen growth and toxin production. Development of intervention strategies would provide critical input to industry, commodity organizations and regulatory/action agencies that allowed for the development, evaluation and implementation of Good Agricultural Practices (GAP's); Good Manufacturing Practices (GMP's) or regulations based on sound science. Transfer of any developed technologies (through OTT) would be done expeditiously, and technologies that required the purchase of expensive equipment would be carefully reevaluated and if necessary alternate approaches formulated. Research focused on both pre- and post harvest interventions.

Introduction

Beef

The overall goal was to increase the microbiological safety of beef by reducing or eliminating primary foodborne pathogens. Research was weighted heavily towards E. coli O157:H7 as well as new STEC's (non-O157) and Salmonella. These pathogens were a high priority for the beef industry. Research was also directed towards providing information and technologies to assist the industry in controlling these pathogens throughout the farm to fork continuum.

Preharvest research addressed needs in developing interventions that prevent or mitigate colonization of the gut in particular the lower GI tract before slaughter or that reduce pathogenic or antimicrobial resistant bacteria in the production environment; reduction of pathogen shedding, antimicrobial intervention, pathogen detection, and host-pathogen interaction. Studies were conducted to develop and evaluate novel interventions for pathogen mitigation for the live animal in production and lairage environments. In conjunction with intervention development, improvements to sampling and detection methodology and pathogen source identification were investigated for cattle production. Finally, research on host-pathogen interactions (see Problem Statement 1.B) would potentially provide the basis for new, improved and specifically targeted developments in pathogen detection and intervention.

Postharvest research addressed needs in antimicrobial intervention, and pathogen detection. Studies were conducted to develop and evaluate novel interventions for carcasses and products at multiple stages of processing, and for the finished product. In conjunction with intervention

development, improvements to sampling and detection methodology and pathogen source identification were investigated for cattle processing, as well as the finished product.

Examples of Accomplishments

- **Feeding technologies.** Studies with GRAS organic acids, essential oils, or lactoferrin revealed only modest efficacy in reducing colonization by foodborne pathogens. In some cases, research elucidated mechanistic reasons for low activity in vivo and led to modification of these GRAS compounds to be more effective. With CRADA partners studies continued to evaluate the efficacy of the chlorate-[and nitro]based technologies developed for application as an antibiotic alternative to control E. coli and Salmonella infections in growing piglets and calves. This chlorate technology is still undergoing regulatory review by the FDA. The chlorate and nitro-compound technologies are efficacious when administered individually, but when applied as a combined technology they yielded a product more than 100-fold more efficient.

- **E. coli O157:H7 in cattle fed wet distillers grains (WDGS).** The use of WDGS in cattle finishing diets was associated with increased E. coli O157:H7 in feces and on hides of animals when fed at 40% of the dry matter intake, compared to cattle fed a typical corn grain diet. Shifting from high levels of WDGS (40 or 70%) to lower levels of WDGS (0 or 15%) reduced the levels and frequency of E. coli O157:H7 in feces and on hides. However, full benefits of the change in diet were not observed until 56 days after the reduction of WDGS.

- **Control of E. coli O157:H7 in deep-bedded cattle confinement facilities**. Deep-bed confinement barns for feedlot cattle are used in the winter months. E. coli concentrations were lower in manure/bedded packs containing wood shavings, compared to manure/ bedded packs with shredded paper and crop-based materials including corn stover, soybean stover, ground corn cobs, wheat straw, and switch grass. Controlling microbial survival in the manure/bedded packs is important: this waste can be a source of pathogens for contamination of additional cattle, or alternately of water, food, and feed crops when the material is applied to cropland.

- **The immune response of super-shedding cattle to E. coli O157:H7.** The mucosal immune response to E coli O157:H7 was investigated. Preliminary results indicated that super shedders may have a decreased IgA response compared to the control animals. This suggests that E. coli O157:H7 colonization in certain animals leads to mucosal damage and an impairment of local IgA response, that could be associated with high levels of bacterial colonization and the resultant super shedding phenomenon.

- **High Event Periods contamination with E. coli O157:H7.** Processing plants experience sporadic peaks in contamination rates where multiple E. coli O157:H7-positive lots are clustered in a short time frame “High Event Periods” (HEP) of contamination. HEP show little to no diversity of strain genotype. Each HEP surprisingly had one strain type that made up most if not all of the contamination. In addition, it was found that a high proportion of HEP are caused by strain types associated with human illness.

- **Antimicrobial interventions for non-O157:H7 STEC.** Decontamination interventions targeting E. coli O157:H7 were developed and implemented; and the efficacy of these interventions against non-O157 STEC strains was evaluated. The interventions used by the beef industry were demonstrated to be as effective against the top six non-O157 STEC as for E. coli O157:H7. Thus, the beef processing industry would not have to implement additional interventions to control non-O157 STEC's.

- **Efficacy of ultra violet (UV) light and UV+ozone**. The effectiveness of UV and UV-ozone combination on inactivation of STEC, Salmonella, Listeria monocytogenes inoculated fresh beef was found to be at least equivalent, or more efficacious than many currently used chemical interventions. The effects of UV on quality issues were minor with some color effects occurring during retail display. These could be addressed through modified atmosphere packaging and/or other novel packaging technologies.

- **Novel beef trim sampling methodology.** The procedure for testing beef trimmings is the [N=60] sampling method. However, this approach leaves much of the trim in a given combo bin un-sampled, leading to a recent OIG audit recommending improvements in sampling regimes. A sampling device for beef trim combo's was developed that provides samples representative of the entire trim lot. FSIS has agreed to a [Letter of No Objection] for testing this method in a commercial setting. Two commercial beef processing companies have agreed to side-by-side evaluation with their N-60 program. A CRADA is in place with a commercial testing company to develop a commercial device for further testing.

- **STEC contamination and veal calves.** FSIS data unexpectedly indicated that veal samples had higher levels of non-O157 STEC's (O111, O103, O45) relative to E. coli O157:H7 in beef samples. Veal barns did not appear to be as heavily contaminated, thus lairage and transport are the most likely contributors to this contamination. In addition, it was demonstrated that hide directed interventions at veal processors significantly reduce the contamination. Further studies are needed to examine the E. coli O157:H7 and non-O157 STEC colonization cycles of young calves.

Outcome and Impact

An important factor in the success of this work was obtaining key industry collaborations to conduct high priority research under industry relevant conditions. Research provided important new information from applied research evaluating near to market technologies incorporating feed additives generally recognized as safe (GRAS) such as organic acids, essential oils, and other natural products. Although studies have shown the GRAS compounds to be generally modest in reducing pathogen colonization, their use could be warranted where readily available and low in cost. Moreover, in some cases results have revealed that certain GRAS strategies as well as other technologies may be modified to enhance efficacy. Accordingly, research resulted in the discovery of potentially new mechanistic interventions targeting specific metabolic and cellular targets. These included the development of a conjugated form of thymol able to bypass absorption in the proximal gut; and the reduction-to-practice of a previously developed chlorate-

based feed additive for preslaughter reduction of foodborne pathogens as a non-antibiotic alternative to reduce E. coli and Salmonella diarrhea in young pigs and calves. Additionally, building on knowledge obtained under CRADA research has helped reduce-to-practice new bactericidal nitro-compounds able to inhibit pathogen survivability while concurrently reducing economic and environmental costs associated with ruminal methane production. Work was also completed reducing to practice an existing chlorate-based technology, previously developed by the Program as a terminal feed additive fed in the last meal or two before slaughter; again for application as an antibiotic alternative to control E. coli and Salmonella infections in growing piglets and calves. While the chlorate and nitro-compound technology are efficacious when each are administered individually, subsequent research demonstrated that when applied together as a combined technology they acted synergistically yielding a product more than 100-times better at controlling pathogens. Overall, the research helped respond to the needs of industry to have available a variety of cost effective interventions.

Research provided strong evidence that diet can affect the shedding of E. coli O157:H7 by finishing cattle, and that shedding can be manipulated. However, when interpreted in combination with other research, the environmental load may play a role in sustaining E. coli O157:H7 prevalence in feedlot cattle. Nonetheless, it was evident that producers can feed high levels of WDGS through a significant period of the finishing phase and then reduce the WDGS level to 15% or less prior to harvest to reduce E. coli O157:H7. This provided a strategy for cattle producers to use this economical and nutritious feedstuff, while limiting the risk of E. coli O157:H7 at harvest. There was some progress on identifying factors resulting in super shedder animals, but there were a number of challenges and lessons learned. As an example, the studies on E. coli O157:H7 super shedder isolates interacting with the bovine intestinal lining and immune response were performed studying the effects of diet on cattle and environmental contamination. The prevalence of super shedder cattle in these experiments was much lower than anticipated, thus prolonging the study to include additional feeding seasons for a valid number of supper shedding cattle to be identified and enrolled in our analysis.

In many studies, incremental improvements were made rather than great leaps. For instance, although the Shiga-toxin and intimin genes are not the best targets for the identification of STEC containing samples, these are the easiest and most relevant targets available. By addressing the subtype of the intimin gene, this incremental change could reduce the number of false positive testes by over 50%. The finding that there were significant STEC's present throughout the production system and that high event contamination occurs which was sporadic and unpredictable, poses a potential challenge to the current model for finished product contamination during beef processing. Consequently it should initiate new hypotheses' relative to beef contamination, and guide research to help elucidate the sources of these pathogens entering the beef supply, and devise more specific targeted interventions to be developed and applied.

The efficacy of UV light, and UV + ozone was examined and identified as novel pathogen reducing interventions. Common beef processing interventions were found to be equally effective on non-O157 STEC as on E. coli O157:H7 which assists processors. Interventions for cattle hides, beef and veal carcasses, and cheek meat were identified.

Examples of Relevant Publications

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Introduction

Swine

Dietary antimicrobials are often fed to production swine, particularly young piglets in the nursery after weaning, to improve health, reduce pathogen load, and, as a result, improve production. Dietary antimicrobials may also influence the composition of bacteria in the intestinal tract, but their impact on foodborne pathogens has not been clearly defined. Previous research indicated that pathogen shedding from production swine can vary over time and may be related to type of dietary antimicrobial used in the diet. Fecal shedding of pathogens was influenced by the phase of production, with some pathogens higher earlier in the growing phase and other pathogens higher near the end of the finishing phase. Previous efforts to replace antimicrobials with GRAS compounds have not been positive since few natural alternatives have been identified.

The populations of pigs reared for meat in the U.S. can be delineated into those which are raised indoors under certification programs where the environmental impact on the pig population is strictly controlled and monitored; and those where access to pasturage and wildlife is either reduced (partial confinement) or uncontrolled (pasture-raised). Animals with access to the outdoors and have exposure to wildlife are at increased risk for infection by Trichinella and/or Toxoplasma. As the demand for pasture-raised, organic pork increases, a complete understanding of infection risk to pasture-raised pigs, and development of strategies to eliminate or mitigate the risk of animal exposure to these zoonotic pathogens are needed. Reducing the risk of foodborne human infection from parasites in meat requires adherence to livestock production practices that prevent exposure of animals to the parasites at the farm level, the development of new treatments which can be used in pasture-raised pigs to prevent infection, implementation of appropriate pre- and post harvest surveillance procedures, and post harvest meat treatments that reduce the risk of infection. Research focused on: evaluating the impact of different management and production practices as they relate to foodborne risk; assessing the effectiveness of on-farm interventions to prevent enteric infection; evaluating the impact of anthelmintic and antiprotozoal treatments on parasitic foodborne infections and the residual foodborne risk, and the interaction between foodborne bacterial infections and foodborne parasitic infections during co-infections and changes in immune effectors and the swine gut microbiome following antiparasitic treatments.

Examples of Accomplishments

- **Dietary growth promoting antimicrobials.** Dietary chlortetracycline in the growth phase reduced Campylobacter prevalence and Shiga-toxin gene incidence in swine feces. In contrast, dietary bacitracin in the finishing phase resulted in higher prevalence for Campylobacter and higher incidence of Shiga-toxin genes. Shiga-toxin producing E. coli O26, 0103, O121, and O145 were detected in swine feces, particularly in the growth phases, and were positive for genes associated with ability to cause human disease.

- **Reducing pathogens.** A commercial lysozyme product was evaluated as a replacement for dietary antibiotics in 10-day old early-weaned piglets. Application reduced Campylobacter and Shiga pathogens and pathogen indicators in feces compared to diets without lysozyme or supplemented antibiotics. Studies in older weaned piglets confirmed the ability of lysozyme to reduce pathogen shedding an improve performance. Bacteriophages, when given in the feed to pigs for five days prior to slaughter, significantly reduced Salmonella populations in the living animals. Although phage treatment shows promise as a potential viable strategy in swine before harvest, actual implementation of this technology requires further research.

- **Trichinella genotypes and infection.** The potential role of immunity in the epidemiology of Trichinella was examined to determine if primary infection with a sylvatic, non-persistent Trichinella genotype protects against a secondary infection with T. spiralis. T. nativa, T. murrelli, and Trichinella T-6, have significant protective effects when given as primary infections prior to a challenge infection with T. spiralis. T. pseudospiralis offers little protection against a challenge infection with T. spiralis.

- **Trichinella and Toxoplasma infection in feral pigs.** The prevalence of Trichinella and Toxoplasma infections in feral pigs was determined through collaboration with USDA-APHIS Wildlife Services. Overall seroprevalence of antibodies to Trichinella spp. and T. gondii, indicating infection, was 3.06% and 17.7%, respectively. A small proportion of feral pigs (0.7 %) were seropositive for both parasites. Tissue analysis of feral swine for isolation and genotyping of Trichinella muscle larvae revealed that all tissue positive pigs were infected with the species T. spiralis; no other Trichinella species were detected. Feral pigs serve as the only major reservoir of infection for T. spiralis in North American wildlife, presenting a risk to both sylvatic carnivores and domestic swine.

- **Trichinae Certification Program**. Lack of a wildlife surveillance program in the U.S. has hindered acceptance of U.S. certified pork by trading partners with respect to Trichinella, as is required by World Organization for Animal Health (OIE) and Codex. Prevalence estimates established feral swine as an appropriate indicator population for Trichinella in the U.S. In partnership with a consortium of 1890 University institutions, a training program and a network of surveillance laboratories to monitor Trichinella prevalence in feral swine was established.

- **Regulations for Trichinella.** Collaborative research with laboratories in France, Italy, Germany and Canada identified standardized production conditions and methodology for evaluation, and demonstrated the suitability of serology as a tool for surveillance testing and assessing risk. Results have been used by the World Organization for Animal Health in developing new rules on acceptable surveillance methods for wildlife populations and for swine raised in controlled and uncontrolled management systems, and serve as a scientifically-based reference for regulatory authorities when developing legislation or interpreting requirements for food safety. ARS administered the USDA-AMS Analyst Training and Check Sample Program for trading fresh pork originating in the U.S.

Outcome and Impact

Research demonstrated that dietary antibiotic growth promoters could influence pathogen shedding, and that the use of dietary antibiotics prior to harvest needed to be further/fully evaluated for their effects on pathogen load. The use of lysozyme in diets of young piglets could maintain a safe food supply and reduce the use of prophylactic antibiotics typically used for swine production, providing the industry with a desperately needed alternative to antibiotics. Phage treatment could be potentially part of an integrated multi-hurdle system, but studies have to be undertaken to determine the most efficacious dosing and application strategies, and the most effective combinations of phages that target the most significant Salmonella serotypes that contaminate swine and impact human health.

There are readily available interventions for reducing the foodborne risk to consumers from Trichinella and improving the image and acceptance of pasture-raised pigs as a safe food product and potentially increasing market share. Sylvatic encapsulated genotypes of Trichinella provide significant protection against a challenge infection with Trichinella spiralis. Further, protection against infection with T. spiralis may be an achievable vaccination strategy using non-persistent sylvatic strains to generate immunity. Strong potential exists for introduction of Trichinella and

Toxoplasma into domestic herds of non-biosecure domestic pigs as a result of increasing overlap of the range of feral pigs with non-biosecure domestic production facilities.

The ARS driven standardization of international protocols for surveillance demonstrated the negligible risk from Trichinella posed by U.S. conventionally raised pigs, provides a basis for increased market share. The USDA-AMS/ARS training program preserves an international market for U.S. exporters exceeding 5 billion dollars annually. These efforts support export marketing efforts as requested by USDA regulatory agencies and maintain access to export markets for U.S. pork producers.

Examples of Relevant Publications

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Introduction

Poultry

The colonization of poultry flocks with bacterial pathogens in particular Campylobacter spp., Salmonella spp., Listeria monocytogenes and CPE-producing Clostridium perfringens Type A is pervasive. Despite many of these organisms being commensal and non-pathogenic in poultry, their presence during production, although diminished but maintained during processing, enables conveyance onto the processing facility, onto raw poultry product, and onto the final product supplied to consumers. Identifying on-farm management and rearing practices that perpetuate and/or disseminate in poultry flocks combined with implementation of novel alternatives or interventions should reduce pathogen presence. Specific preharvest interventions are also especially important as the trend to remove sub-therapeutic antibiotics in poultry feeds continues and will help address the need for reducing antibiotic usage during commercial poultry production. There were various approaches examined which included for example: evaluating the impact of every-day feeding compared to skip-a-day feeding programs for pullets during rearing; appraising litter sampling time points, as litter could serve as a potential indicator of flock colonization persistence. Novel biocontrol intervention strategies were also studied, for example: bacteriophage therapy; probiotics to reduce pathogen GI tract colonization; natural alternatives to antibiotic use; or products for example (peptides and lytic enzymes) that could readily be incorporated into chicken feed.

The processing plant is a post-harvest site in which numerous manipulations are made to poultry

carcasses and meat, many of which impact the microbial safety of the end products. Research studies were designed to examine the distribution and dispersion of bacterial pathogens in and around poultry processing plants and poultry products. Studies provided a molecular systems approach to foodborne pathogens and their movement in the poultry processing environment. Additional research on the host-microbial interaction of protozoa and foodborne pathogens, and other biological populations may provide important interventions at the animal production or processing level. Intervention techniques and processing modifications to lessen microbiological contamination of poultry meat were designed and tested. Research was undertaken into the development of alternative sanitizers that could be used in commercial processing operations.

Examples of Accomplishments

- **Colonization persistence in pullets.** The most widespread feed restriction program (skip-a-day) used for broiler breeder pullets during rearing was demonstrated to contribute to persistently higher Salmonella prevalence after environmental challenge compared to shorter persistence (4 weeks sooner) in pullets subjected to every-day restrictive feeding programs. Campylobacter recovery was more than 50% lower for pullets in the every-day restrictive feeding programs. Alternatively restrictive feeding every-day for broiler breeder pullets by broadcasting on the litter promotes a shorter persistence of both.

- **Isolation of bacteriophage.** Animal and environmental samples were screened for virulent bacteriophages that could lyse Clostridium perfringens. Bacteriophage genes were identified that encoded lysins that could be used as alternatives to antibiotics. Two putative phage lysin genes were cloned (in E. coli and yeast) and the resultant recombinant proteins were capable of lysing both parental phage host strains of Clostridium perfringens but did not lyse other bacteria beyond the species. Another phage gene was identified and cloned; the expressed protein lysing L. monocytogenes strains, both a free cells and in biofilms.

- **Antimicrobial peptides (AMP).** AMPs are regarded as a potential source of future alternatives to antibiotics owing to a unique set of properties ranging from molecular simplicity to low-resistance kill of a broad range of bacterial pathogens. Campylobacter jejuni is highly susceptible to killing by chicken host defense peptides. A set of chemically synthesized AMPs were assayed for their effectiveness; and all lysed C. jejuni. AMP genes were clones in yeast for potential commercial use.

- **Examples of additives as alternative interventions**
  - **Caprylic acid.** Prophylactic and therapeutic supplementation of caprylic acid in feed significantly reduced S. enteritidis and C. jejuni populations in the cecum. Body weight, feed intake, pH, or endogenous cecal bacterial population was not affected.
  - **Trans-cinnamaldehyde, eugenol, carvacrol, and thymol.** A 2-log reduction of Campylobacter jejuni was observed with the combination of 0.5% thymol and 0.5% carvacrol. Treatments did not always produce consistent reduction in between trials.
  - **Yeast culture (YC).** Intermittent addition of YC to feed prior to transport events significantly improved FCR of transport- stressed turkeys. The YC also tended to decrease isolation of Salmonella and Campylobacter from the ceca of treated birds.

- **Contamination of carcasses during processing.** Leakage of gut contents during automated feather picking causes an increase in Campylobacter levels. Neither, hanging the carcasses in a vent side down orientation or plugging the vent with spray foam were reliable means to prevent gut content contamination. Changing processing order to eviscerate prior to scalding and feather picking was effective to significantly moderate the increase in Campylobacter that is co-incident with feather removal. With a commercial processor, chlorine dioxide was applied in automated feather picking machines as a spray. The treatment significantly moderated any increase in Campylobacter, E coli and Salmonella detected on treated carcasses.

- **Airborne transfer of Listeria monocytogenes during wash-down.** Studies examined the dangers associated through an accidental discharge of a water hose into a contaminated floor drain if it could result in airborne transfer of Listeria monocytogenes to other surfaces. After a two second spray Listeria was detected settling out of the air as far away as 4.0 m (13 ft) on the floors and even 2.4 m (8 ft) high on the walls. This stresses the need for care during cleaning and sanitation of processing facilities.

- **Ultraviolet light killing of Listeria on raw poultry meat.** Treatment at 800 micro-Watts/cm^2 for times as little as 5 seconds is adequate to significantly reduce numbers of L. monocytogenes on raw chicken breasts.

- **Cross-contamination of poultry during carriage to the processing plant.** Most processors do not wash poultry transport cages between uses. When cages are washed, the conventional spray method is only partially effective. Allowing feces deposited on coop flooring during transport to dry out was more effective to eliminate detectable Campylobacter but also more time consuming. Two potential solutions to the time problem were examined: the utility of cornstarch as a desiccant and hot air as a drying aid. Both methods showed promise, significantly lowering contamination in 15 minutes or less.

Outcomes and Impact

Studies indicated that skip-a-day restrictive feeding programs (pervasive throughout the broiler industry) for pullets extenuate the persistence of flock infection. Alternative daily feeding programs should be refined and implemented. The two-day cyclic feed availability for pullets on skip-a-day restrictive feeding programs apparently facilitates Salmonella and Campylobacter ingestion with environmental pecking resulting in colonization, proliferation, spread, and persistence throughout the rearing period. In contrast, feeding pullets restricted amounts of feed every day appeared to reduce alimentary tract exposure and susceptibility to Salmonella colonization. The daily consumption of feed from the litter surface also facilitates a natural competitive exclusion and thereby limits colonization by Salmonella from natural exposure. The challenge for daily feeding programs is to maintain flock body weight uniformity as well as fertile egg production and hatchability, especially for the overweight and underweight hens.

The potential for bacteriophage lytic enzymes and antimicrobial peptides to reduce levels of the highest priority bacterial food-borne pathogens found in poultry was demonstrated. Significant genomic diversity exists even among closely-related bacteriophages. Holins and endolysins

represent conserved functions across divergent phage genomes and this project demonstrated endolysins can have significant variability and host-specificity even among closely-related genomes. These findings may have important implications for potential biotechnological applications of phage gene products. Cloning and enhanced expression of lytic enzymes and antimicrobial peptides was accomplished in yeast which can be readily incorporated into chicken feed, providing a practical preharvest intervention product.

Research continues at the state-of-the-art organic/pasture poultry research facility in Arkansas, one of the very few organic certified poultry research facilities in the U.S. The development of antibiotic alternatives is a need for the entire spectrum of the poultry industry; however, the organic poultry market has an immediate and critical need for this research. The probiotics discovered were licensed to an Arkansas based start-up company in cooperation with the University of Arkansas. The commercial product (FloraMax-B11™) is marketed in 16 countries with ~ 300 million birds dosed/year. In an effort to improve the efficacy of probiotic cultures, a novel in vitro screening technique was developed, a rigorous selection of poultry bacterial isolates based on motility and flagella characteristics. Extrapolating current data, which indicates chickens and turkeys treated with this probiotic, results in increasing meat yields which translates to a greater than $6 million increase in production yields for every 300 million birds treated in the US/year. A Patent application on the Motility-Enhanced Probiotic Technology has been filed. Research with caprylic acid supplementation to broiler chickens demonstrated consistent reductions of both Salmonella and Campylobacter with both prophylactic and therapeutic efficacy. Natural plant extracts have promise as a strategy to reduce pathogen contamination, however, the most consistently effective concentrations and combinations still need to be determined.

A key effort was to test intervention strategies for controlling Campylobacter and Listeria contamination of poultry products. Research included testing advancements in pathogen culturing methods, testing of physical interventions during poultry processing such as altering poultry processing procedures (vent plugging, upside-down processing), chemical treatments such as a proprietary additive to enhance the effectiveness of chlorine treatment, and investigation of pathogen reservoirs in a processing plant such as monitoring long term residence of L. monocytogenes. Incremental progress has been made that will aid in improving the microbiological quality of poultry products by helping industry design intervention strategies that fit their needs and reporting initial data that will form the basis of further investigations. Specific impacts include the impact/role of defeathering on expelling cloacal contents leading to carcass contamination. Studies demonstrated that interventions at that critical point would significantly lower the load of Campylobacter on the poultry product. New processing intervention treatments are frequently being introduced and are likely to provide incremental improvements that will augment current methods. A long-term future goal is to evaluate new methods and optimize protocols that producers can incorporate into their processing regimes.

A major source of Listeria contamination of further-processed poultry products is from long term-resident bacteria that can be found in floor drains. However, the numbers of Listeria in a given drain maybe small, making it difficult to measure the effectiveness of intervention techniques. Also, current data does not show that drains are the primary source of contamination or just an indicator of the presence of the organism in the environment.

Studies on bacterivorous protozoa did not progress as far as planned. Most such protozoa are highly motile and thus are difficult to isolate alive. Isolation is necessary to compare the ability to kill the pathogens of concern. The current most effective methods to isolate motile protozoa are quite expensive but new methods are in development that may assist.

Examples of Relevant Publications

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Introduction

Seafood

When adjusted for per capita consumption, seafood is responsible for more cases of foodborne illness than any other food category. Some work was undertaken at the request of the USDA Food Safety Inspection Service (FSIS) after this agency assumed its role regarding catfish inspection as part of the 2008 Farm Bill. This was a major collaborative study to determine the microbiological and chemical safety of retail catfish. In addition, separate studies were requested to examine the microbiological safety of catfish nuggets, which are made from the belly flaps of catfish fillets. These survey studies were conducted in concert with microbial risk assessments by the FSIS and GAO. Studies were focused on developing and validating models to simulate pathogen behavior under both growth and inactivation conditions; developing and validating non-thermal and advanced thermal intervention technologies to inactivate pathogens and spoilage microorganisms in raw and ready-to-eat seafood and aquaculture products, in particular, catfish: and defining the impact of non-thermal and advanced thermal intervention technologies on food quality and chemistry.

Molluscan shellfish, like oysters, clams and mussels, are common causes of seafood-associated illnesses and occasional deaths in the US. Illnesses are commonly from viruses, like norovirus, which is the principle cause of foodborne illness in the U.S., and from bacteria of the Genus Vibrio. Norovirus is associated with fecal pollution and causes an estimated 20 million illnesses in the U.S. annually, while Vibrio are naturally-occurring in the marine environment. Two Vibrio species (V. parahaemolyticus and V. vulnificus) are particularly problematic because they cause the highest numbers of bacterial-induced illnesses and deaths among shellfish consumers, respectively. Another Vibrio, V. tubiashii, causes high mortalities in larval shellfish in U.S. shellfish hatcheries and has reduced the availability of seed oysters and clams which are needed by the commercial shellfish industry. Studies were focused on identifying the mechanisms by which bivalve shellfish become contaminated with pathogenic viruses and Vibrio's and to identify, and validate in laboratory and field situations processing interventions to reduce illnesses and losses to the shellfish and associated industries.

Examples of Accomplishments

- **Collaborative study on the safety of retail catfish fillets. A** multi-agency collaboration between USDA (FSIS, ARS, and AMS), and academia: Delaware State University, Cheyney University, Mississippi State University and Auburn University examined the microbiological and chemical safety of retail catfish fillets in the Northeastern U.S. The research was completed and the final (confidential) report was transferred to FSIS Administration by Cheyney University.

- **Gamma radiation inactivation of Shiga-toxin producing O157:H7 and non-O157:H7 Escherichia coli in catfish fillet meat.** The ability of gamma radiation to inactivate non-O157:H7 serovars O26:H11, O45:H2, O103:H2, O11:NM, O121H19, and O145:RM and three O15:H7 serovars suspended in catfish fillet meat at refrigeration temperature (4^{o}C) were compared. The radiation doses needed to inactivate the non-O157:H7 STEC's were similar to those needed to inactivate E. coli O157:H7.

- **Vibrio inhibitor.** A Vibrio inhibitor was identified as a naturally-occurring predatory bacterium from the Genus Bacteriovorax. Salt-water Bacteriovorax effectively regulate the levels of pathogenic Vibrio in oysters and may have practical application as a post-harvest processing technology in market shellfish. A survey of Bacteriovorax spp. showed its presence nearly year round in seawater from the Mid-Atlantic Coast of Delaware, the Gulf Coast of Alabama, and Hawaii, suggesting that this bacterium may also be important in modulating pathogenic Vibrio levels in the environment worldwide.

- **Vibrio tubiashii.** Vibrio tubiashii is responsible for high mortality of larval shellfish in U.S. hatcheries causing shortages in seed oysters needed by commercial shell-fishermen. At industry request, a plan was developed and implemented to control V. tubiashii in shellfish hatcheries using bacteriophages. Studies isolated/characterized the first phages against V. tubiashii. ARS received a Phase I Small Business Innovative Research (SBIR) grant and developed a CRADA with Intralytix Inc., to commercialize the intervention. Proof-of-principle testing demonstrated that a 15 phage cocktail reduced larval oyster mortalities by 70 percent for both Eastern and Pacific oyster larvae.

- **Differentiating norovirus**. Norovirus cannot be replicated in the laboratory and a suitable animal model has been elusive; therefore, a means of extracting or separating potentially infectious virus from inactivated virus was needed. Studies developed a procedure to extract human noroviruses from shellfish and to differentiate infectious from inactivated viruses. The extraction method is based on the concept that a virus binds to a cellular receptor to initiate an infection. If it cannot bind, then it is not infectious. Thus using porcine gastric mucin, which mimics the norovirus receptor, it is possible to separate potentially infectious norovirus from intact capsids that are inactivated.

- **Pathogen intervention**.
    - High Pressure Processing (HPP): HPP can inactivate human norovirus in uncooked oysters. Inactivation was enhanced when performed at 6°C as opposed to 22°C. Taste panels showed that oysters treated with 400 (MPa) at 6°C, are as readily accepted as oysters processed under current commercial conditions (300 MPa at 22°C).
    - E-beam: Application at levels currently approved by the FDA inactivated about 90% of norovirus and hepatitis A virus within oysters. E-beam irradiation penetrates oyster shell and may be useful as a processing intervention.
    - Freezing and thawing: viruses were highly resistant, with no reduction in virus titers or apparent virus infectivity after 14 cycles of freezing and thawing.

Outcomes and Impact
Submission of the Catfish report; however, the consequences from the report's conclusions are unknown to the Program. The growth rates (refrigerated and temperature abuse storage) and inactivation kinetics for intervention technologies (thermal and irradiation) for both non-O157:H7 and O157:H7 Shiga-toxin-producing Escherichia coli (STEC's) in catfish fillet meat were conducted and the information transferred. Regulatory agencies, the seafood processing industry and consumers will benefit from this research as they will not need to expend additional resources to control non-O157:H7, over O157:H7 STEC's in seafood/finfish products.

Studies identified a role for predatory bacteria in the reduction of pathogenic Vibrio in shellfish and suggested an application for Bacteriovorax in the reduction of Vibrio and other pathogens in other food types/products. The identification of predatory bacteria (Bacteriovorax spp.) in seawater and their ability to inactivate Vibrio in shellfish is a milestone in the quest for a better understanding of the mechanisms by which Vibrio are naturally regulated in the marine environment. Bacteriovorax isolates have been provided to collaborators in Italy and internally who are interested in developing methods to eliminate Vibrio in mussels, and E. coli and Salmonella in produce.

Studies characterized and demonstrated a proof-of-principle that bacteriophages against the larval shellfish pathogen V. tubiashii have the potential to significantly reduce larval oyster mortalities, and led to a CRADA with an industry partner to commercialize these phages. The goal of the CRADA is now to market phage intervention for domestic and international shellfish hatcheries to reduce shellfish mortalities so that they can provide a steady supply of larval shellfish for commercial use. Its success to date suggests that phage intervention may have practical applications in the disinfection of not only the shellfish pathogen V. tubiashii, but also human pathogens like V. parahaemolyticus and V. vulnificus from shellfish. Other studies [*accomplishment not shown*] demonstrated environmental and genetic factors that influence the survival, growth, and invasiveness of V. parahaemolyticus and identified factors involved in the immune response to V. parahaemolyticus infection in mammals.

Noroviruses are the number one cause of foodborne illness in the U.S. Studies demonstrated a means of discriminating infectious from non-infectious (inactivated) norovirus negating the need for expensive and cumbersome human challenge studies to investigate inactivation parameters. This discovery will dramatically reduce the use of human norovirus surrogates in food safety studies since the pathogen itself can now be evaluated. The finding has already led to research defining inactivation temperature, ultraviolet and high pressure inactivation parameters and a means to evaluate chemical sanitizers. The sanitizer work has shown that human norovirus are remarkably resilient. It is also anticipated that this will open other major areas of research. The development of second generation tests in combination with the infectious virus (porcine mucin magnetic bead assay) for human norovirus shows promise as a means of automating shellfish testing. Currently, due to sewage treatment, many inactivated viruses are released into estuaries, so it is unclear how much virus detected in shellfish is infectious vs. inactive. Current water quality standards are based on fecal coliform testing of water or shellfish meats, which is not a direct or determinative test for viruses. Automated, hemocyte-based testing of live shellfish could make it possible to streamline testing of shellfish and potentially change how shellfish growing waters are classified and regulated by State Health Departments and the FDA.

HPP was shown to be effective against human norovirus and hepatitis A virus within shellfish. Results from a recent taste panel indicate that HPP-treated oysters are well accepted by consumers when treated under conditions known to inactivate these viruses. Although high pressure can inactivate norovirus and hepatitis A virus, achieving widespread use of high pressure processing will be a considerable challenge due to the high capital expense of the equipment and some general resistance to high pressure processing by the shellfish industry. While e-beam irradiation was effective in eliminating one-log of hepatitis A virus and murine norovirus, it is not considered sufficient to provide a substantial reduction in oyster-transmitted viral illnesses.

The mechanisms of enteric virus uptake and persistence in oysters were determined [*accomplishment not shown*]. Virus uptake was through the oysters hemocytes (primitive blood cells), and explains why depuration protocols for live viruses are ineffective. Hepatitis A virus persist the longest from among the enteric viruses tested to date. This suggests that regulators who conduct epidemiological investigations of hepatitis outbreaks should consider closing shellfish beds for longer periods after the virus is detected.

Examples of Relevant Publications

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Introduction

Plant Crops/Produce/Nuts

Foodborne pathogens on fresh produce accounts for 9.5 million (12%) of the 76 million U.S. foodborne illnesses annually at a cost of ~$39B in medical and productivity losses. Outbreaks from fresh produce have increased from 0.7% of all reported outbreaks in the 1970s to 6% in the 1990s. A March 2013 report from the CDC shows that almost half [46%] of all foodborne illnesses that led to hospitalization and or deaths between1998-2008 were attributable to fresh produce. Two major 2006 E. coli O157:H7 outbreaks with spinach and cut-lettuce were a major impetus. Research on contamination pathways has shown the complexity of the situation, and the need for more specific data on sources, pathways of contamination, and persistence of enteric pathogens on fresh produce. Also, the scientific-basis for practices/criteria in existing GAPs (Good Agricultural Practices), FDA, and industry commodity-specific guidance documents to reduce risk of pathogen contamination from various sources require specific evaluation to support development of national standards by action agencies. There were a number research studies within this sub-objective, both pre- and post-harvest: Studies investigated sources of contamination of fresh produce at the farm level namely, irrigation water, compost, soil amendments, insect as vectors, and other issues. The transfer of pathogens from these sources to foliar surfaces was investigated and the effect of contamination level was evaluated. Intervention strategies to reduce contamination at the farm level were developed and evaluated at the field level.

Studies were conducted to research plant-microbe model systems: specifically to identify and characterize the microbial genes that are involved in the attachment, colonization and survival of enteric pathogens on produce; to determine the genetic and biochemical factors in plants that effect the attachment, growth and survival of human pathogens in/on plants; to assess the role of other microflora and aerosols in survival and transmission of enteric pathogens in agricultural environments; and to develop methods for the detection of enteric viral and bacterial pathogens from produce and soil (found in Problem Statement 1.C).

Studies were conducted to identify the sources and prevalence of parasites and to prevent illness from infection. These parasites can be spread by fecal contamination, all are waterborne, and all are identified as contaminants of fresh produce. Improve recovery of the parasites from fresh produce using new reagents to dissociate the parasites from plant material, enabling an accurate determination of the extent of contamination of fresh produce and the sources of contamination as well as a superior washing process that removes parasites and possibly other pathogens that strongly adhere to fresh produce

Post-harvest studies were conducted to develop and validate new and effective chemical and physical decontamination interventions for produce and/or improve the performance of current interventions to reduce pathogens on fresh produce implemented at fresh-cut packing facilities; understand ecological factors that influence treatment decontamination efficacy; and develop and evaluate process models, including economic analysis models, in order to facilitate technology transfer and commercial adoption of interventions and intervention combinations.

Examples of Accomplishments

- **Dissemination of E. coli O157:H7 from cattle**. Studies evaluated the impact of proximity of cattle feedlots on produce crop contamination. E. coli was recovered on leafy greens at low rates, but was found in samples at all sites up-to 600 feet. Analysis of bioaerosols and cattle pest flies indicated their potential roles in the transport of E. coli O157:H7. The risk for pathogen transport is increased in situations where feedlot pen surfaces are very dry, and this when combined with cattle management or movement activities can generate substantial airborne dust. The findings suggest that the current (Leafy Greens Marketing Association) guidelines of 400 feet may not be adequate.

- **Persistence of Salmonella and E. coli.** Avian pathogenic E. coli isolated from poultry broilers and manure survived for longer durations on basil, spinach and lettuce compared to E. coli O157:H7 when simultaneously inoculated on to these leafy green commodities. Salmonella isolated from fresh produce persisted at significantly higher numbers on spinach plants when sprayed with contaminated water compared to Salmonella isolated from poultry. Salmonella was recovered for up to 6 weeks from spinach leaves when sprayed with a high inoculum load. Longer persistence of Salmonella on spinach leaves when contaminated at a high inoculum level reinforces need for water quality standards such as those in the California Leafy Greens Marketing Agreement.

- **Use of zero-valent iron column.** Zero-valent iron (ZVI) filtration is an effective means of purifying irrigation water contaminated with E. coli O157:H7. In field trials, ZVI filtration significantly reduced E. coli O157:H12 populations compared to control or sand filtration and was able to reduce E. coli counts exceeding the California LGMA irrigation water standards for fresh produce irrigation.

- **Chlorine stabilizer.** The chemical washing aid T128 (patented by ARS) significantly increased the efficacy of chlorine wash against bacterial cross contamination while maintaining the quality of leafy green vegetables under real world fresh-cut processing conditions. New Leaf Food Safety Solutions Inc. in collaboration with industry partners is currently optimizing the T128 application protocol.

- **Almond safety.** California supplies 80% of the world's almond crop valued at > $4.3B. The Almond Board of California mandates a 4-log reduction of Salmonella; however, current almond roasting with hot air technology involves long processing times, high energy input, and does not efficiently pasteurize almonds. A sequential infrared and hot air roasting technology was developed that reduces contamination by 7-logs in one third the time compared with traditional hot air roasting alone. This new technology provides industry with a combined roasting and nonchemical dry pasteurization process that is cost-effective and has high decontamination efficacy. Recommendations for integration of an infrared heater into the existing commercial equipment and processing parameters for various roasting levels were transferred to the Almond Board of California.

- **Cold plasma.** Industry requested a waterless, zero-contact, chemical-free method for removing pathogens from foods and food-contact surfaces. Research tested cold plasma technology for its ability to remove biofilms from food-contact surfaces. Treatment for

15 seconds reduced E. coli O157:H7 biofilms by 99.9% and Salmonella biofilms by 99%. In pathogen-inoculated almonds, cold plasma treatments of 10 or 20 seconds inactivated both Salmonella and E. coli O157:H7 (> 95% reduction).

- **Chlorine dioxide.** Pilot scale chlorine dioxide gas treatment of green tomatoes and cantaloupe to eliminate inoculated pathogens were conducted. Treatments consisting of 6 h fumigations at 0.4 or 0.8 mg/l (tomatoes) or 1.0 mg/l (cantaloupes) reduced Salmonella by 99.997% on tomato and 99.999% on cantaloupe. These reductions persisted after 7 days of storage, and served to increase the shelf life by reducing spoilage microorganism populations. A shorter treatment of Mung bean seeds and sprouts (15 minutes at 0.5 mg/l) reduced 99.999% of inoculated Salmonella.

- **Safer, cleaner cantaloupes and tomatoes.** A direct-from-field surface pasteurization treatment for whole cantaloupe and green tomatoes was developed which can reduce Salmonella by 99.999%. Optimal treatment for cantaloupe is a 45 second immersion in 70°C, (160°F) water, while a 3.5 minute treatment is suitable for green tomatoes. Ongoing collaboration with an industry partner is underway to evaluate a scaled-up commercial version of this chemical-free process on microbial quality, shelf-life, and sensory quality.

- **Integration of UV-C light and ionizing radiation.** Integrated treatments of low dose UV-C and 0.25 kilogray (kGy) irradiation reduced the population of key pathogens (E. coli O157:H7 and S. enterica) by ~ 99.9%. More than 99.999% reduction of the two pathogens was accomplished by the combined UV-C treatment with 0.75 kGy or higher irradiation. The effects of this combined treatment on tomato firmness (texture), lycopene content and color were minimal. The current FDA regulations allow the commercial application of radiation at doses up to 1 kGy for fresh fruits and vegetables. The dose applied in this study is within the FDA permissible limit. The integrated strategy can provide 99.999% reduction of pathogens on tomatoes which meet the recommendations of the National Advisory Committee on Microbiological Criteria for Foods.

Outcomes and Impact

The incidence of foodborne illnesses associated with the consumption of fresh produce has increased on a global basis including developed and industrialized countries. Increased consumption of fresh produce, changes in production and distribution systems, growing awareness among consumers, and active surveillance by health agencies have been cited as factors contributing to increase in outbreaks linked to fresh produce. Further, the FDA-Food Safety and Modernization Act required FDA to promulgate regulations for the growing, harvesting, packing and holding of produce for human consumption. Studies herein investigated mechanism(s) of introduction and transference of Shiga-toxigenic E. coli (STEC) and Salmonella to fresh produce during growing, harvest, and postharvest handlings. The transfer of pathogens through insect vectors, contaminated composted manure, or irrigation water to fresh produce were investigated, as was the persistence and survival of pathogens on fresh produce leaves. The role of specific surface appendages and virulence factors demonstrated biofilm formation and subsequent interventions were evaluated to reduce pathogen attachment to produce surface.

Novel post-harvest treatments were evaluated and existing treatments were modified to enhance pathogen reduction on leafy greens.

Research revealed clear risks in current industry practices, and further provided the quantitative data for the FDA to develop science-based food safety regulations for preventive controls against bacterial contamination. The research findings helped fill knowledge gaps that have been identified by regulatory agencies to provide food safety guidance and recommendations to the produce industry and regulatory agencies, for example, in establishing and validating standards for irrigation water and soil amendments. Work with emphasis on identification of sources of contamination at the farm level, mitigation of these sources, and development of new intervention strategies, is being utilized by FDA to develop/validate proposed regulations. Some accomplishments have also been utilized by trade organizations and producers with respect to development, evaluation and implementation of GAPs, GMPs (Good Manufacturing Practices) to minimize the potential for contamination of fresh and fresh-cut produce, and effective pathogen inactivation.

Technologies for post-harvest intervention have been shown to be effective and amenable to scale-up to commercial levels. They are expected to lend themselves to incorporation into existing commercial equipment and practices via a development pathway that uses existing engineering expertise. These interventions will have a wider applicability beyond the specific commodities evaluated during the R&D phase. The reduction of human pathogens on fruits and vegetables, while preserving and/or enhancing quality and freshness, suggests that these technologies will have a substantial impact on safety. Furthermore, implementation of the technology may meet the requirements of the Food Safety Modernization Act. In addition, because of simplicity and low cost, the technologies are especially suitable for small processers/packers.

A CRADA was established titled, "Development of Produce Wash for Microbial Decontamination of Fresh Fruits and Vegetables." A provisional Patent for effective combinations of antimicrobials is being filed with the CRADA partner. A 5-year NIFA-funded project in conjunction with the University of Delaware was awarded, entitled "Inactivation of Enteric Foodborne Viruses in High Risk Foods by Non-Thermal Processing Technologies."

Examples of Relevant Publications

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- Gurtler, J., Bailey, R., Geveke, D.J., Zhang, H.Q. 2011. Sodium benzoate, potassium sorbate, and citric acid induce sublethal injury and enhance pulsed electric field inactivation of E. coli O157:H7 and nonpathogenic surrogate E. coli in strawberry juice. Food Control. 22(10): 1689-1694.
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- Ingram, D.T., Callahan, M.L., Ferguson, S.E., Hoover, D., Shelton, D.R., Millner, P.D., Patel, J.R., Kniel, K., Sharma, M. 2012. The use of Zero-valent iron biosand filters to reduce E. coli O157:H12 in irrigation water applied to spinach plants in a field setting. Journal of Applied Microbiology. 112(3): 551-560.
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- Nou, X., Luo, Y., Hollar, L.A., Yang, Y., Feng, H., Millner, P.D., Shelton, D.R. 2011. Chlorine Stabilizer T-128 enhances efficacy of chlorine against cross contamination by E. coli O157:H7 and Salmonella in fresh-cut lettuce processing. Journal of Food Science. 76(3): M218-M224.
- Sharma, M., Lakshman, S., Ferguson, S.E., Ingram, D.T., Luo, Y., Patel, J.R. 2011. The effect of modified atmosphere packaging on the persistence and expression of virulence factors of Escherichia coli O157:H7 on shredded iceberg lettuce. Journal of Food Protection. 74(5): 718-726.
- Ukuku, D.O., Geveke, D.J., Chau, L.I., Bigley Jr, A.B. 2014. Inactivation of natural microflora and Escherichia coli K-12 on cantaloupe rind surfaces using wet steam treatments. Journal of Food Protection. (submitted)
- Yang, J., Pan, Z., Takeoka, G.R., Mackey, B.E., Bingol, G., Brandl, M., Garcin, K., McHugh, T.H., Wang, H. 2012. Shelf-life of infrared dry-roasted almonds. Journal of Food Chemistry. 138(1): 671-678.
- Yang, Y., Luo, Y., Millner, P.D., Turner, E.R., Feng, H. 2012. Assessment of Escherichia coli O157:H7 transference from soil to Iceberg Lettuce via a contaminated harvesting knife. International Journal of Food Microbiology. 153(3): 345-350.
- Yossa, N., Patel, J.R., Millner, P.D., Ravishankar, S., Martin Lo, Y. 2013. Antimicrobial activity of cinnamaldehyde and sporan against Escherichia coli O157:H7 and Salmonella on lettuce. Foodborne Pathogens and Disease. 10(1): 87-96.

Introduction

General Foods and Alternate Interventions

Pathogenic microbes exhibit a variety of responses to foods, the handling of foods before processing, the sequence of food processing steps, and the conditions under which foods are processed, prepared, and/or stored. Determining the ecological niches, persistence, and physiological responses that are initiated by pathogens under the conditions particular to foods and food processing, storage, and/or preparation is an essential prerequisite for the development of directed detection and intervention methods for human pathogens in foods. In light of existing and pending regulatory policies and the paucity of available literature, research is needed to better quantify the association and fate of select food borne pathogens in higher risk foods. Studies were aimed at increasing our understanding of pathogen persistence in foods and, in turn, developing and evaluating effective interventions to enhance the safety and security of our food supply: determining the prevalence, levels, types, and locations of pathogens at various points from production through to consumption of raw, further processed, and/or RTE foods, developing, optimizing, and validating processing technologies for eliminating pathogens; and to develop and/or validate strategies to deliver antimicrobials to raw and packaged foods from production through to consumption.

The implementation of the intervention technologies has been hampered due to limited effectiveness or adverse effects of these technologies on product quality and shelf-life. Chemical and physical intervention technologies need to be developed/modified/optimized to maximize their effectiveness in inactivating foodborne pathogens. Furthermore, the impact of effective chemical and physical intervention technologies on sensory properties, nutrient quality, shelf-life and accumulation of undesirable chemical by-products need to be evaluated for effective treatment intensity (time, concentration, dose, etc.). Integrated approaches involving combined interventions/treatments (hurdles) which are capable of disrupting one or more of the homeostasis mechanisms in microorganisms can produce additive or synergistic effect on pathogen reduction and survival at a much lower intensities of individual treatments. Traditional methods of preserving foods for the purpose of controlling microbes include thermal processing, drying, freezing, refrigeration temperatures, modified atmosphere packaging, and adding antimicrobial agents. Unfortunately, some of these techniques cannot be applied to certain food products, such as fresh produce and other ready-to-eat products.

Antimicrobial packaging is a packaging system that is able to kill or inhibit growth and survival of spoilage and pathogenic microorganisms on foods that are post-process contaminated due to mishandling. The antimicrobial function can be achieved by adding antimicrobial agents in the packaging system and/or using antimicrobial polymers that satisfy conventional packaging requirements. When the packaging system acquires antimicrobial activity, it limits or prevents microbial growth. An integrated approach by combining process interventions with packaging technology can be more effective than individual interventions. Studies were directed to determine sensory, nutritional and/or product quality impacts of efficacious food processing interventions and combinations of interventions; to develop antimicrobial coating and packaging for various foods and enhance the safety and extend the shelf life of various foods; to identify compounds of potential concern formed by novel non-thermal food processing interventions, and to develop/optimize treatment processes and combinations to control pathogens and to minimize

loss of product quality and value. Efforts in this work were directed in a slightly different way, and involved the combined research efforts of food engineers and food scientists/technologists.

Examples of Accomplishments

- **Listeria monocytogenes market basket survey.** At the request of the FDA and FSIS, ARS organized and implemented a multi-agency, multi-disciplinary study to establish the relative occurrence, levels, and subtypes of L. monocytogenes (L.m) in 13 ready-to-eat, higher-volume, higher-risk food categories purchased at retail that are regulated by FDA or FSIS. Over a 2-year period the ARS team tested >23,000 samples purchased at large and small grocery stores within California, Connecticut, Maryland, and Georgia. The overall L.m prevalence (ca. $\leq$1.0%) was appreciably lower that known/expected from previous studies. The estimated prevalence was noticeably higher for foods made/sliced in stores compared with otherwise similar foods that were pre-packaged by the manufacturer. Moreover, pathogen levels in foods testing positive ranged from <0.03 to >110 MPN/gram. These data represent the most comprehensive study on the association of L. monocytogenes with RTE foods and confirmed that changes in policy and processing by regulators and manufacturers, respectively, over the past decade have made an appreciable impact on lowering the occurrence, and the risk from the food supply.

- **Inactivation of STEC in ground beef.** At the request of FSIS studies quantified the thermal inactivation of E. coli (ECOH) and non-O157 strains of Shiga-toxin-producing E. coli (STEC) strains in high fat and low fat ground beef patties before cooking on commercial gas or electric grills to internal temperatures of 60.0-76.6°C. In general, there were no differences in lethality based on fat level, storage/thawing regimens, or grill types; and ECOH and STEC displayed similar lethality. Cooking ground beef patties that were previously refrigerated, frozen, or frozen/thawed to internal temperatures of 71.1° and 76.6°C was effective for eliminating ca. 5.1-7.0 logs CFU/g of ECOH and STEC. Related studies quantified the effect of heat on the fate of ECOH and STEC in flattened ground beef wafers inoculated separately with a single strain of E. coli STEC serotypes. D-values ranged from 13.5-32.6 min, 0.7-1.2 min, and 0.05-0.2 min at 54.4°, 60.0°, and 65.6°C, respectively. Thus, cooking times/temperatures effective for inactivating ECOH in ground beef were equally effective against the seven STEC strains investigated.

- **Translocation and thermal inactivation of STEC in blade tenderized and chemically enhanced steaks.** Studies quantified the translocation of STEC into beef sub-primals following enhancement via blades or injection, and determined the effect of grilling on the fate of the internalized STEC. Regardless of the enhancement treatment, E. coli O157:H7 strains and non-O157 STEC behaved similarly relative to translocation and to thermal inactivation when steaks were cooked to target internal temperatures of 120°F to 160°F. Although cells were recovered throughout tenderized/enhanced sub-primals, most of the cells remained in the topmost 1 cm. As expected, higher temperatures resulted in greater lethality; reductions of 0.3- to 4.4-log CFU in pathogen numbers were observed. In general, results revealed that STEC-8 translocated into the deeper tissues of the sub-primals and cooking was effective to reduce appreciable levels of STEC.

- **Validation of food grade chemicals and manufacturing processes to control Listeria monoctyogenes in RTE specialty/ethnic red meats, poultry, and dairy products.** Collaborations with stakeholders, technology providers, regulators, and manufacturers validated interventions and processes to control L. monocytogenes, STEC and Salmonella, in soudjouk, jerky, scrapple/goetta, cured and uncured ham, chicken breast, and frankfurters. Chemicals blends of levulinate, propionate, lactate, acetate/ vinegar, and/or diacetate acted synergistically to provide reductions of 2-5 logs and inhibited outgrowth of L. monocytogenes during extended refrigerated shelf life. In combination with lauric arginate surface applied [via Patent-pending] Sprayed Lethality In the Container (SLIC) delivery method, the above mentioned blends provided better coverage at lower inclusion rates/cost with less impact on taste and texture attributes.

- **Nanoparticles to inactivate foodborne pathogens**. Antimicrobials incorporated into packaging materials are typically subject to a heating process during formulation. Although inorganic compounds such as ZnO or MgO can survive this heating process, conventional preparation methods are minimally effective against foodborne pathogens. When tested the antimicrobial activities of precision-formulated nanoparticle preparations of ZnO and MgO against E. coli O157, Salmonella spp., and Campylobacter jejuni, these nanoparticles effectively inactivated all three pathogens. These inorganic compounds along with the nanoparticle formulation protocols provide for an active antimicrobial strategy that can potentially be added or incorporated into packaging materials.

- **Intervention technologies for shell eggs.** A radio frequency (RF) process was developed to quickly pasteurize shell eggs through penetrating energy to quickly heat the yolk of the egg. As the egg is rotated, RF energy and streams of cooling water are simultaneously applied to the egg. This initiates pasteurization of the yolk while maintaining a low temperature in the heat-sensitive egg white, thus preventing hazing. Immediately after the RF heating process, the egg is placed in hot water to pasteurize the egg white and to complete pasteurization of the yolk. In studies the yolks of fresh eggs were inoculated with Salmonella and then processed using the novel RF technology; a 99.999% reduction was achieved. When compared to current hot water immersion process, RF pasteurized eggs retained their fresh like appearance and significantly more functionality, while reducing the processing time by nearly >65%.

Outcomes and Impact

The Market Basket Study increased the understanding of Listeria persistence in RTE foods. For some processors, it provided the requisite data to achieve Alternative 2 and possibly Alternative 1 status which, in turn, increased the safety of their products and lowered their cost of doing business. In some instances, Program data were also used by FSIS and the FDA to develop risk assessment models and to develop regulatory policies to enhance and enforce product wholesomeness. The data will be used to update the 2003 Inter Agency Risk Assessment for Listeria monocytogenes on RTE foods. The Listeria team was recognized for their efforts and the impact of their findings to date with the (2012) DHHS Secretary's Honor Award: "for exceptional collaboration in the development and implementation of research to characterize Listeria monocytogenes prevalence and levels in foods".

The STEC studies filled a critical FSIS data gap, and were a major research effort in supporting the historic USDA-FSIS-STEC initiative. The results were used in support of rulemaking for labeling tenderized products for both the U.S. and Canada, as well as to update the non-intact beef risk assessment, including for declaring on-O157 STEC as an adulterant. The present and potential impact of this research was recognized by a (2012) USDA Secretary's Honor Award to the USDA STEC Team "for excellence in leadership to protect the U.S. food supply, through timely development and implementation of a robust science-based program for controlling non-O157:H7 Shiga-toxin-producing E. coli in beef".

Research provided food manufacturers and processors with increased understanding of how intervention technologies and antimicrobial packaging can be used to ensure the safety of various foods and extend their shelf life. Several technologies were developed and tested, while some are approved to be promising for commercial application. One U.S. Patent has been granted; two are pending; and 4 collaborative research agreements (CRADA's) with food industry and a number of material transfer agreements to explore the feasibility of commercial application.

Antimicrobial packaging and interventions will yield economical and environmental advantages for producers. Biodegradable polymers such as PLA, renewable agricultural by-products such as pectin, and natural antimicrobials such as organic acids and essential oils would provide sustainable alternatives for synthetic fossil fuel-based polymers and potentially harmful antimicrobials. Pectin, sugar beet pulp and lignin are by-products from juice, sugar and ethanol production. Utilization for packaging materials will open new byproducts markets. Additionally, incorporation of bio-byproducts into packaging materials could significantly reduce their cost. Consumption of fossil fuel-based raw materials is significantly reduced, complete biodegradability and compostability are achieved, "green-cycling" of packaging materials is simplified and the overall packaging waste-stream volume is reduced.

Research on antimicrobial packaging using nanoparticles led to a new research area that would have high potential for a wide variety of foods, such as juice, beverage, milk and liquid egg products. The U.S. National Science Foundation has estimated that the global nanotechnology market could be worth U.S. $1 trillion by 2015. This work was the first report for the application of nanoparticles in food safety and provides the first molecular evidence that the antimicrobial mechanism of ZnO-nanoparticles is likely due to the induction of oxidative stress in bacteria.

Pathogens on the surfaces of fresh fruits and vegetables may reside in protected sites such as crevices, stomata, or cracks that aqueous sanitizers cannot reach; therefore, non-aqueous antimicrobials may be an option to enhance the microbial safety of fresh produce, such as tomatoes. Gaseous antimicrobials may be more effective in reaching sites where pathogens hide. Gaseous treatments such as in-packaging ozonation and fumigation with vaporous essential oils can be used to enhance the safety of tomatoes in a sealed container without adversely affecting quality parameters. The treatments avoid possible post-treatment contamination.

Research on production of possible carcinogens due to non-thermal processing showed that low dose irradiation was unlikely to generate significant levels of furan. The information has been extensively used for two recent U.S. FDA regulations (irradiation of poultry products, and

uncooked meat products), and Food Standards Australia New Zealand (FSANZ) regulation (irradiation of tomatoes and capsicums).

The radio-frequency (RF) pasteurization process for shell eggs has filed for Patent protection and a CRADA has been signed for commercialization. The RF process kills > 99.999 percent of Salmonella, and when commercialized, would provide an alternative to an hour-long hot-water-immersion process currently used. Unlike conventional heating, this (RF) heating warms the egg from the inside out. This is critical to the success of the process because the dense, heat-tolerant yolk at the center of the egg receives more heat than the delicate, heat-sensitive white (albumen).The comparatively brief hot-water bath helps the yolk retain heat, and to complete the pasteurization. The bath also pasteurizes the egg white without over-processing. From start to finish, the treatment takes approximately 20 minutes, making it about three times faster than the hot-water-immersion technique.

Examples of Relevant Publications

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- Luchansky, J.B., Porto-Fett, A.C., Shoyer, B.A., Call, J., Schlosser, W., Shaw, W., Bauer, N., Latimer, H., 2012. Fate of O157:H7 and non-O157:H7 Escherichia coli cells within blade-tenderized beef steaks after cooking on a commercial open-flame gas grill. Journal of Food Protection. 75(1): 62-70.
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1.E Predictive Microbiology and Data Acquisition

Goals

The goals of the research under this Problem Statement were to generate data on the responses of microorganisms to both defined and changing environmental conditions, and to translate these data into mathematical models, and user friendly tools such as the Pathogen Modeling Program (PMP) or related modeling software. The software applications had to be readily usable by national and international regulatory and public health agencies, industry, and academia to assist in ensuring the safety of the food supply. Of particular importance was that these microbial predictions were [within limitations] acceptable for use in the development and/revision of microbial risk assessments. It was also critical that before translation for use by external entities, that any models, or modeling software be validated by external sources. Internet-based database construction and development, for example Combase, was continued to be coordinated internationally with the aim to ensure the acquisition of new data from varied sources, and the ability to readily mine databases. Database and bioinformatics efforts have become increasingly important so that biologists have the ability to gain information that will foster technological innovation, and an understanding of the genetic basis of foodborne microorganisms. Although not a research project, the Food Safety Research Information Office (FSRIO) based at the USDA-National Library was a critical and integral part of the Food Safety Program. Continued development and expansion of the FSRIO capabilities was a major goal.

Introduction

Environmental

The presence of pathogenic microorganisms in irrigation waters was and still is considered to be a potentially important factor in the preharvest contamination of fresh produce. Many of the essential pathogen fate and transport processes associated with irrigation are currently not well understood or modeled. Studies were focused on; elucidating and quantifying mechanisms and factors controlling pathogen and indicator bacteria fate and transport from animal sources to irrigation water sources via irrigation water delivery systems, and; developing models and computer-based tools to recommend and implement site-specific diagnostics, monitoring, and prediction of the fate and transport of pathogen and indicator bacteria in irrigation water sources and via delivery systems. The integrated approach included laboratory research, field research on irrigation systems, and mathematical modeling.

Examples of Accomplishments

- **Developed new models/databases**
  - The first numerical model of microorganism fate and overland transport (STWIR) as an add-on to ARS model KINEROS2 were developed, validated and applied.
  - Numerical model APEX specifically to simulate the effect of most common management practices on water quality was developed. The model also provided evidence that neglecting the wildlife input in stream bacterial populations can distort conceptualization of potential pathogen sources in the watershed and can mislead mitigation efforts.

- Derived the first comprehensive experimental data/database on sediment E. coli population which was converted into an add-on to the ARS water quality model SWAT. EPA adopted this model for microbiological risk assessment of recreation waters as affected by the agricultural activities.

- **Biofilms in irrigation equipment as a bacterial reservoir.** The propensity of biofilms in pipes in overhead irrigation systems to serve as reservoirs of indicator and pathogenic organisms was demonstrated. Biofilms can substantially modify the microbiological quality of irrigation waters.

Outcome and Impact

A major outcome was the development of a conceptual model incorporating processes and factors that affect microbial water quality. The model became the foundation for mathematical models that are capable of providing regional and site-specific evaluations of existing trends in microbial water quality and their changes under different climatic and management scenarios. A systematic effort is still needed to further validate these models, to evaluate the uncertainty in their predictions, and to establish relationships between model parameters and easily measurable data available from monitoring and surveys. This is critical since there is a paucity of available data on microbiological quality of irrigation waters. This work will be valuable in designing irrigation water quality data collection protocols. The computer code was made available via the WWW. The developed computer tools can be used to improve existing microbial water quality assessment in the variety of regulatory programs, such as TMDL and QMRA.

One of the impacts of this work was that it addressed issues for the developing Produce Rule within the FDA-Food Safety and Modernization Act. FDA is required to promulgate regulations for produce production, including standards for irrigation water. However, the parameters affecting microbial quality of surface waters (that may be used for irrigation) are poorly understood. In addition, there was limited data on how biofilms in irrigation delivery systems may impact the actual microbes that are applied to the produce plants. Material and pipe-age affect biofilm formation, and microorganisms can survive and/or grow and then be released from the irrigation equipment. Monitoring microbiological quality of irrigation water should be done after water passes thought the irrigation system, and the exploitation of irrigation systems should include measures preventing biofilm effects on water quality. Providing fundamental information on variables affecting irrigation water is critical to accurately assess the risks associated with irrigation water, to ensure an adequate level of produce safety for consumers without imposing an unwarranted economic hardship on farmers.

Examples of Relevant Publications

- Cho, K., Pachepsky, Y.A., Kim, J., Kim, J., Park, M. 2012. The modified SWAT model for predicting fecal coliform in the Wachusett Reservoir Watershed, USA. Water Research. 46(15): 4750-4760.
- Pachepsky, Y.A., Garzio-Hadzick, A., Shelton, D.R., Hadzick, Z., Hull, R. 2011. Survival of E. coli O157:H12 in creek sediments after inoculation and re-inoculation. International Journal of Environment and Pollution. 46(3-4): 234-245.

- Pachepsky, Y.A., Morrow, J., Guber, A.K., Rowland, R.A., Shelton, D.R. 2012. Effect of biofilm in irrigation pipes on the microbial quality of irrigation water. Letters in Applied Microbiology. 54(3): 217-224.
- Pan, F., Pachepsky, Y.A., Guber, A.K., Hill, R. 2012. Scale effects on information theory-based measures applied to streamflow patterns in two rural watersheds. Journal of Hydrology. 414-415: 99-107.
- Yakirevich, A., Pachepsky, Y.A., Gish, T.J., Guber, A., Shelton, D.R., Cho, K. 2013. Modeling transport of Escherichia coli in a creek during and after artificial high-flow events: Three year study and analysis. Water Research. 47(8): 2676-2688.

Introduction

Regulatory/Industry

Predictive microbiology is a discipline based on the premise that microbial growth, survival and inactivation can be quantified and expressed through mathematical equations, and that under a specific set of environmental conditions, microbial behavior is invariably reproducible. Models that describe the combined effects of multiple factors on the growth, survival and inactivation kinetics of foodborne pathogens are developed to assist risk managers in addressing the impact of emerging pathogens on our food supply are needed. In addition, food industries and associated regulatory agencies need methodologies to validating new and existing so that they can be safely applied across food systems to effectively manage food safety risks. The models provide regulatory agencies and food industry with an objective means of assessing the microbial risk of a particular food and ensuring that the public is not at risk of acquiring food poisoning. The goal of this work was to produce valid and robust predictive models that describe the growth, survival and inactivation of high priority pathogens in raw and ready-to-eat foods, as a function of intrinsic and extrinsic environmental conditions. Furthermore, the research would produce tools for risk assessors and food companies to aid in designing more effective pathogen interventions targeted at higher risk pathogen-food combinations. Modeling tools are the ARS-Pathogen Modeling Program (PMP) [desktop] and the ARS-Predictive Modeling Information Web Portal (PMIP) [online]. A goal was to incorporate raw data from ARS and other researcher's into Combase, which is an international free database used by microbial modelers and overseen by the ARS; UK-FRI; and UTas (ARS; Food Research Institute, Norwich, UK, and U. Tasmania, Australia).

Examples of Accomplishments

- **Developed new mathematical models.**
  - For survival, death, and growth of bacteria on chicken meat. Data were used in an exposure assessment model that predicts changes in prevalence, number, and type of Salmonella on chicken parts as they move from retail to consumption.
  - Mechanistic primary (Huang): model determines the lag phase duration, exponential growth rate, and maximum population density of microorganisms in foods.
  - Two secondary (Huang square-root, and Arrhenius-type) to evaluate the effect of temperature on microbial growth.

- Many kinetic models - example; Cronobacter sakazakii in reconstituted infant formula; Listeria monocytogenes in fresh-cut cantaloupes, ready-to-eat ham, deli salads, smoked seafood, fermented sausage, and fresh produce; non-O157:H7 Shiga-toxin-producing E. coli (STEC) in spinach leaves, E. coli O157:H7 and Salmonella in fermented sausage.
- Growth models: of acid-stressed L. monocytogenes and E. coli O157:H7 and in foods with competitive microflora.
- Predictive, estimating microbial growth during cooling of cooked products.

- **USDA Integrated Pathogen Modeling Program (IPMP 2013).** A new IPMP Program was made available in 2013. This easy-to-use integrated data analysis and model development tool can be used by students and scientists, without any programming knowledge, in order to develop accurate mathematical models for microbial shelf-life prediction and risk assessments. The software package is offered as a free tool to scientists and risk modelers globally, and can be downloaded from http://www.ars.usda.gov/Main/docs.htm?docid=23355.

- **ComBase** can be found at http://www.combase.cc/index.php/en/about-combase. The *ComBase Database* consists of thousands of microbial growth and survival curves that have been collated in research establishments and from publications. The *ComBase Predictive Models* are a collection of software tools based on ComBase data to predict the growth or inactivation of microorganisms.

Outcome and Impact

The USDA-FSIS routinely use the developed models to set priorities in relation to inspection efforts. The data serve as the scientific basis to establish standards/regulations on performance standards for the production of processed meat and poultry products; in risk assessments; and in food inspection programs. The food industry uses the concepts to identify potential new approaches for the safe production of cooked foods to avoid bacterial food poisoning. The newly developed product, IPMP, allows food scientists to easily develop accurate mathematical models for use in shelf-life prediction and risk assessments, and can be used by educators in colleges and universities to train students for studies in predictive microbiology.

The continued expansion of the USDA-ARS Pathogen Modeling Program (PMP), the Predictive Microbiology Information Web Portal (PMIP) was critical. Complex underlying mathematics of the predictive models were transformed into easy-to-use interfaces that can be successfully used by food microbiologists, regulatory staff members and industrial professionals to explore the predictions of these models on scenarios relevant to food processing operations. Since small and very small food processors generally lack food safety resources, the outcomes of this project are particularly helpful to these producers to improve food safety of their products.

Raw data from this and other research was submitted to ComBase**,** an international microbial modeling database. During this past cycle the Combase collaboration with associated partners (ARS-IFR-Utas) as an international data resource continued to grow, as did the size of the database. The *Predictor* developed is a modified, augmented and improved web-version of the

Growth Predictor, a stand-alone program. It is used for predicting the response of pathogens and spoilage microorganisms to key environmental factors (temperature, pH, salt concentration, etc) characterizing the food environment. Combase is now currently funded through various sources; appropriated funds, and by a long-term National Capability Grant from the BBSRC – Biotechnology and Biological Science Research Council in the UK. Combase is also now available in several languages including English, Spanish, Japanese and Chinese.

Examples of Relevant Publications

- Huang, L. 2012. A simplified method for numerical simulation of gas grilling of non-intact beef steaks to eliminate Escherichia coli O157:H7. Journal of Food Engineering. 113(3): 380-388.
- Huang, L. 2013. Optimization of a new mathematical model for bacterial growth. Food Control. 32(1): 283-288.
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- Juneja, V.K., Marks, H.M., Huang, L., Thippareddi, H. 2011. Predictive model for growth of Clostridium perfringens during cooling of cooked uncured meat and poultry. Food Microbiology. 28(4): 791-795.
- Oscar, T.P. 2011. Development and validation of a predictive model for survival and growth of Salmonella on chicken skin stored at 4 to 12 deg C. Journal of Food Protection. 74(2): 279-284.
- Oscar, T.P. 2013. Validation of a predictive model for survival and growth of Salmonella Typhimurium DT104 on chicken skin for extrapolation to a previous history of frozen storage. Journal of Food Protection. 76(6): 1035-1040.

Introduction

Fermentation

Ensuring the control of bacterial pathogens that may be present in refrigerated, fermented, and bulk acidified pickled vegetable products remains an important issue for industry and the FDA. Granted outbreaks are now rare with this food product category, however, deaths and major outbreaks of illness have resulted from acid foods such as un-pasteurized apple cider and commercial orange juice. Furthermore, a large number of acidified vegetable products have similar pH and acid conditions. This left a regulatory gap for FDA and was a major concern for producers of acidified foods, particularly as the FDA-Food Safety Modernization Act (FSMA) was being implemented. Definitive scientific data was lacking to show that acid resistant pathogens, such as E. coli O157:H7, Listeria monocytogenes, and Salmonella enterica die off over the range of conditions under which commercial processing and fermentations occur. Studies have shown that acid resistant bacterial pathogens have the ability to survive in selected acidified vegetable products for one month or more, particularly in refrigerated products. Research was undertaken to fill the knowledge gaps and help establish the parameters for safe production of acid and acidified processed vegetables and related products such as dressings, sauces, and others.

Examples of Accomplishments

- **Thermally processed-acid and acidified food products.** The times and temperatures needed to assure the destruction of pathogens using appropriate acid and pH formulations for a wide variety of acidified foods were determined. Subsequently, the FDA required that linear models for heat process data were to be used with electronic process filing forms. Processing parameters were adapted for a linear model that met or exceeded the heat processing conditions needed. Recommendations were adopted by the acidified foods industry, and used for the required FDA process filings.

- **Non-heated acid and acidified food products.** For the FSMA requirements: studies determined how common ingredients, such as acetic acid and preservatives, alone or in combination, can result in an appropriate reduction cell numbers of acid resistant pathogenic bacteria in the absence of a post-packaging pasteurization step.

- **Refrigerated acidified food products.** Refrigerated acidified vegetable products are exempt from the acidified foods regulations, production relying primarily on GMP's. Studies developed a brine formulation with reduced acetic acid, but containing fumaric acid, that could assure an appropriate reduction in cell numbers of E. coli O157:H7 without a heat process.

- **Fermented vegetable products.** The fermentation conditions needed to kill acid resistant pathogens such as E. coli O157:H7 in cucumber fermentation brines were determined, While pH was found to be the main factor affecting the die off of pathogenic bacteria during fermentation, other factors such as salt concentration and the presence of preservatives also played a role in achieving a safe fermentation process.

Outcome and Impact

In response to concerns by the FDA, and in conjunction with the principal trade association for the acidified foods industry (Pickle Packers International), research determined the conditions needed to assure the destruction of vegetative bacterial pathogens in acidified foods.
Applied research determined the preservative and acid formulations and holding times and temperatures needed to assure safe processing while basic research addressed how acid resistant pathogens can survive in the presence of acids, and determined the efficacy of different food acids and preservative for killing the pathogens. For many acidified food products the time, temperature, salt and acid conditions needed to achieve a safe process were not known, this has now been addressed. The impact of this work is that the data was used by both industry and FDA, and established a scientific basis for determining safe production practices for acid and acidified foods. The data is also being used internationally by producers of acidified foods to help assure the safety of products shipped to and sold in the US.

Example of Relevant Publications

- Breidt, F., Sandeep, K.P., Arritt, F. 2010. Use of Linear Models for Thermal Processing Acidified Foods. Food Protection Trends. 30(5): 268-272.
- Breidt, F., Caldwell, J.M. 2011. Survival of Escherichia coli O157:H7 in cucumber fermentation brines. Journal of Food Science. 76(3): M198-M203.
- Breidt, F., Kay, K., Cook, J., Osborne, J., Ingham, B., Arritt, F. 2013. Determination of 5-log reduction times for Escherichia coli O157:H7, Salmonella enterica, or Listeria monocytogenes in acidified foods with pH 3.5 or 3.8. Journal of Food Protection. 76(7): 1245-1249.
- Hosein, A.M., Breidt, F., Smith, C.E. 2011. Modeling the effects of sodium chloride, acetic acid and intracellular pH on the survival of Escherichia coli O157:H7. Applied and Environmental Microbiology. 77(3): 889-895.

Introduction

FSRIO:Database

The USDA-ARS Food Safety Research Information Office (FSRIO) based at the National Agricultural Library was mandated by the United States Congress to support the needs of the food safety research community. This Office focuses on: (a) providing a Website as a central place to access current food safety research efforts, outcomes, and the latest up-to-date scientific information (b) providing a publicly accessible and searchable research projects database that showcases food safety research projects funded by both U.S. and International government agencies, as well as educational institutions and other private or non-government organizations (c) showcasing research publications from peer-reviewed journals on important food safety topic areas (d) providing food safety information tools and products that assist the research community to assess research needs and priorities , explore current research activities, and prevent duplication of efforts allowing for efficient use of research dollars (e) fostering increased cooperation among individuals and agencies engaged in food safety research (f) tracking research initiatives by academia, industry and government at the national and local levels (g) bringing awareness to these services by presenting at scientific conferences and other food safety related meetings

Examples of Accomplishments

- **Food Safety Research Office web site/database.**
  - Provides ready access to the largest searchable collection of food safety research projects conducted nationally and internationally. The database has grown to 8,000 projects by 2013: ~4000 projects indexed with key words and food safety categories. ~2400 projects tagged with farm-to-table categories. An automated feed of the latest food safety research publications released from more than 50 peer-reviewed journals, including publications ahead of print was developed.
  - Other food safety information tools that have been developed and enhanced based on results of stakeholder surveys: Outreach: Presentations given at conferences nationally and internationally. Internships: Food Science Internship was offered to eight UMD NFSC undergraduate students to earn semester credit hours. Staff mentored students and helped them complete independent projects.

Outcome and Impact

The FSRIO Website and key information products have shown continued growth each year. From FY2010 to FY2013, the Website page-views increased 75% (to 700,000) and the Research Projects Database page-views increased 80% (to 116,000). Journal publication feeds for 2013 (a new initiative implemented that year) were 25,000. FSRIO has a global impact since ~40% of its visitors are international with the UK, EU, Philippines, Canada and India being the top countries outside the U.S. The FSRIO Website provides a central place to access global food safety research initiatives. Unique information products such as the Research Projects Database and Peer-reviewed Research Publication Feeds are publicly accessible and showcase current research projects and publications. This information: assists in assessing research needs; impacts action plans, regulations, and policy decisions; and reduces the duplication of research. Potentially this information could be used to coordinate the development of an interagency research agenda to use research monies more efficiently.

1.F Chemical and Biological Contaminants: Detection Methodology, Toxicology/Toxinology

Goals

The goal of the research under this Problem Statement was the development, validation and successful implementation of technologies which provide a more effective and efficient means of monitoring the food supply and environment where food is grown. Better technologies assist researchers conducting toxicological and toxinological studies to provide basic and applied knowledge on the effect of exposure to biological toxins. This research also provides data for better scientific and regulatory decision-making, reducing the likelihood of tolerance limit-errors, protection of consumers, and prevention of economic losses resulting from inappropriate regulatory actions.

Studies were a special challenge requiring a multidisciplinary approach. Coordinated efforts within ARS, and between other agency's both nationally and internationally were required as the results may have far reaching effects regarding biosecurity, regulations and trade issues. Similar to Problem Statement 1.D, this was a research area where promising technologies needed to be advanced quickly, and where possible, and appropriate, would undergo validation through national or international bodies (for example: FERN, AOAC). Research was divided into three sub-sections: Sensing, Chemical Contaminants and Biological Toxins.

Sensing technology: Regulatory agencies have placed more of the burden of inspection responsibility on the producers and processors. Plants are also responsible for meeting other consumer protection 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 is difficult. To reduce these issues studies are focused on developing automated, low cost, accurate, on-line and hand-held, computerized inspection [sensing] systems. These automated systems operate with minimum human intervention and are able to function despite changes in physical plant structure, and environmental conditions. A specific need was to continue development and validation where appropriate with the goal of commercial implementation. On-line, computerized sensing-systems placed or used strategically will assist and improve the regulatory and in-house inspection system; minimizing the problems of human error and variability; and increasing commercial processing productivity and profitability.

Chemical contaminants: The regulation and control of veterinary drugs, chemical residues, heavy metals, and persistent organic pollutants was critical to the program's principal stakeholders (FSIS and FDA). To protect public health and the environment, regulations set limits on contaminants in edible agricultural products. Compliance and enforcement of these regulations continues to be a critical role of the Program's stakeholders that requires the availability of practical detection and characterization methods for chemical residues (dioxins, pesticides), veterinary drugs (antibiotics, beta-agonists), heavy metals (As, Cd), and organic pollutants (polybrominated diphenyl ethers). In addition to regulatory monitoring there was a need to understand the biological effects of any inadvertent human or animal contamination. Included in

this section was the development of specific sensing technologies. The use of nondestructive spectral sensing technologies, for example, whole-surface [in-line] hyperspectral imaging is one option for reducing the risks of contamination or food adulteration.

Biological Toxins: (Mycotoxins/ Bacterial/Plant): Research was directed towards developing methods for the detection and identification of mycotoxins; and evaluation of mycotoxin toxicity and mechanism of action. Research on the development of biocontrol technologies, and crop/fungal/toxin relationships is part of this area, and may also impact Problem Statement 1.B (System Biology). Research on production practices, expert systems, and breeding resistant crops, was limited. Toxins derived from bacteria and plants are an integral component of any food safety/food biosecurity program. Research was directed towards developing methods for the detection and identification of toxins. In addition we examined the relationship between dose and its effects on the exposed organism (*toxicology*); and the properties and their biological significance for the organisms involved (*toxinology*).

Introduction

Sensing Technology

Generally, illness outbreaks result from foods becoming contaminated at some point during processing, handling, and distribution. Two routes by which to address this problem are identifying/removing individual contaminated food stuffs, and ensuring the effectiveness of efforts to clean and sanitize processing surfaces. Studies focused on the development of optical sensing technologies; including spectroscopy, imaging, hyperspectral imaging, and nanotechnology for the detection of contaminants and defects on food products and processing surfaces. Nondestructive imaging technologies can be used to identify and facilitate removal of contaminants and contaminated foods at all stages of processing from farm to table. The effective outcome of this applied engineering research is commercialization of the technologies developed both nationally and internationally. A specific focus was the development of hand-held imaging inspection devices for use in for example, the effectiveness of sanitation and cleaning operations in food processing environments.

Examples of Accomplishments

- **Automated online poultry inspection**. Two online inspection systems were developed: a line-scan spectral imaging system for wholesomeness/systemic disease, and a system for surface fecal contaminants. In collaboration with industry a commercial prototype version was developed using a common imaging platform combined with hyperspectral imaging instrument customized for poultry inspection and configured as a multispectral imaging instrument. The application software developed in-house for fecal detection was expanded to add an image analysis module for disease detection. The system has been tested extensively for real-time image-based inspection at a processing speed of 140 birds per minute. Implementation of the automated line-scan imaging inspection system is underway. Two U.S. Patents were issued in 2012 and 2014.

- **Online whole-surface produce inspection.** An online imaging systems for produce for fruits and leafy greens that included separate presentation methods and processing conveyor systems was developed. The whole-surface imaging methods coupled with the patented multitask-imaging technology allowed thorough safety and quality inspection of round fruits and leafy greens on commercial processing lines. Validation with an industry partner is underway. Two U.S. Patent applications for sample presentation and imaging methods were submitted in 2013.

- **Food adulterant detection.** To detect adulteration/contamination of food ingredients a point-scan non-destructive Raman chemical imaging system was developed. The technology allows for acquisition of high resolution hyperspectral Raman image data– i.e., spatial and spectral measurements of relatively large quantities of minimally-prepared sample materials. A U.S. Patent was issued in May 2013.

- **Handheld imaging devices for monitoring efficacy of sanitation and cleaning.** An inexpensive fluorescence-based handheld imaging device with Wi-Fi capabilities to display live inspection images on smart-phone or tablet devices was designed and developed. These imaging devices through in-plant testing demonstrated that existing sanitation and safety surveys can be greatly enhanced providing an objective means to assess the effectiveness of sanitation procedures and/or determine potential problem areas within a processing environment. A U.S. Patent was granted in 2012 and a commercial licensing agreement was signed in 2013.

- **Chemical composition of eggshell microstructure and automated egg crack detection.** A Laser Induced Breakdown Spectroscopy (LIBS) method was developed to correlate the chemical composition of the eggshell with microstructural features that can lead to eggshell damage. The method will aid in determining chemical characteristics that are implicated in the formation of micro-cracks. An egg hairline crack detection system developed by ARS for USDA-AMS was upgraded resulting in a more uniform illumination of all eggs, enhancing the overall coverage of the detection system, and improving accuracy, especially for brown-shell eggs.

Outcome and Impact

The development of various rapid, non-destructive imaging technologies for identifying, detecting, or quantifying adulterants in foods, or damage on foods, with the direct adoption by the food industry and/or regulatory agencies and their laboratories for high-volume food sampling and testing. The Raman chemical imaging technology shows great promise as a rapid and nondestructive method for adulterants in food ingredients, with potential for adoption by industry groups (such as U.S. Pharmacopeia) as a standard testing method. Handheld inspection tools can be used to improve the efficacy of cleaning and sanitation procedures in processing plants with no additional labor costs. The technologies were patented by ARS and licensed to U.S. commercial partners. Expanded commercial systems are under ARS/partner development.

Examples of Relevant Publications

- Ahn, C., Baek, I., Mo, C.Y., Kang, S., Kim, M.S., Cho, B. 2012. Development of non-destructive quality measurement technique for cabbage seed (Brassica campestris L) using hyperspectral reflectance imaging. Food Engineering Progress. 16(3): 257-262.
- Chao, K., Yang, C., Kim, M.S. 2010. Spectral line-scan imaging system for high-speed nondestructive wholesomeness inspection of broilers. Trends in Food Science and Technology. 21(3): 129-137.
- Kim, M.S., Delwiche, S.R., Chao, K., Lefcourt, A.M., Chan, D.E. 2012. Visible to SWIR hyperspectral imaging for produce safety and quality evaluation. Sensing and Instrumentation for Food Quality and Safety. 5(5): 155-164.
- Lawrence, K.C., Jones, D.R., Yoon, S.C., Heitschmidt, G.W., Anderson, K.E. 2011. Improved hairline crack detector and poor shell-quality eggs. Applied Engineering in Agriculture. 28(1): 153-158.
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- Park, B., Yoon, S.C., Windham, W.R., Lawrence, K.C. 2011. In-plant test of in-line multispectral imaging system for fecal detection during poultry processing. Applied Engineering in Agriculture. 27(4): 623-630.
- Windham, W.R., Yoon, S.C., Ladely, S.R., Haley, J.A., Lawrence, K.C., Park, B., Narang, N., Cray Jr, W.C. 2013. Hyperspectral imaging of Shiga-toxin-producing Escherichia coli serogroups O26, O45, O103, O111, O121, and O145 on Rainbow Agar. Journal of Food Protection. 76(7): 1129-1136.

Introduction

Chemical Contaminants

Chemical contaminants in foods concern not only the American consumer, but also producers since residues impact their ability to market food products worldwide. Global food trade is a $1.4 trillion market, and chemical contaminants are commonly monitored worldwide to better ensure food safety and compliance with regulations, and meet several other governmental, industrial, and academic needs. Several food crises have occurred with respect to chemical contaminants leading to even greater concerns. In response, food testing by 3rd party labs has grown about 50% over the past 5 years into a $3 billion market worldwide. The implementation of the FDA-Food Safety Modernization Act in the U.S. will likely increase the monitoring rate even further. To meet needs, the detection methods used by monitoring labs must be fast, simple, inexpensive, rugged, and provide very low detection limits for a wide scope of chemicals in all types of foods. Development and validation of analytical methods to meet acceptable regulatory performance criteria was a major challenge. Heavy metal accumulation may exceed levels considered to comprise risk to consumers of several crops, and further some crops have limits for exports which require careful management by growers and shippers to satisfy markets. In an effort to assure the American public and export markets that the U.S. food supply is safe, both the USDA's-ARS, the -FSIS, and the DHHS-FDA have made a serious, long-term commitment to fund research that addresses these vital national interests in food safety. Research addressed problems/needs in several areas.

To address the lack of rapid, automated, cost-effective, waste-minimizing, safe, and high-quality analytical methods to detect multiple chemical residues and other toxic compounds in foods. This work was undertaken to meet the needs of the USDA-FSIS, the FDA, and other organizations that monitor chemical residues in food, which also includes industry, consumer groups, and academic scientists worldwide. The goals were to develop, validate, and transfer improved analytical methods to real-world monitoring labs for regulatory food safety and other purposes.

Examples of Accomplishments

Detection Technologies:

- A screening method using ultrahigh performance liquid chromatography – tandem mass spectrometry that improved screening logistics and capabilities for analyzing 120 of the most important drugs of regulatory concern was developed, validated, optimized. The technology was transferred to FSIS National Residue Program, where an analyst can do 60 samples/8-hours. Technology replaces the 7-plate microbial growth inhibition assay.

- Levels of inorganic arsenic (iAs) in rice is a major issue, and trace-level speciation and quantification is challenging. The sum of iAs species was detected using a newly developed, validated method involving microwave digestion, solid-phase extraction for cleanup, and quantification by hydride-generation atomic fluorescence spectrometry.

- FSIS was required to perform a risk assessment of nitrosamines in bacon [OIG audit 9 CFR 424.22]. At the request of FSIS, ARS developed a new method for ultra-trace quantification and identification of the 5 most important nitrosamines. The method and data provided the science to guide the FSIS decision, and close out this OIG audit.

- Other:
  - Based on ARS-developed QuEChERS technology: combined with low-pressure gas chromatography-tandem mass spectrometry, a simultaneous multi-class, multi-residue method for 200 targeted environmental contaminants and pesticides in seafood.
  - A new fast and simple lab-based method for the analysis of polyphenolic compounds.
  - Detection of lipid A analysis by supercritical fluid chromatography coupled to an ion trap mass spectrometer. (food defense application)
  - A portable time-resolved fluorometer for fluoroquinolone antibiotics, after dispersive liquid-liquid micro-extraction (DLLME) cleanup.
  - Oxytetracycline residues in catfish using dispersive liquid-liquid micro-extraction and europium-sensitized luminescence.

Outcomes and Impact

This research addressed the development of rapid, automated, cost-effective, waste-minimizing, safe, and high-quality analytical methods to detect multiple chemical residues and other toxic compounds in foods. Studies met the needs of the USDA-FSIS, FDA and other organizations including industry, consumer groups, and academic scientists worldwide who monitor/examine chemical contaminants in food. Examples are described above, and these accomplishments have

led to higher sample throughput and faster sample turnaround time at lower cost for more drug residues, while achieving more reliable results. FSIS has used the savings to restructure its National Residue Program to increase the number of samples analyzed in surveillance monitoring of additional chemicals that have been missed in the past due to lack of resources. The impact of this research has been the successful development, validation, and transfer of faster, simpler, and more accurate monitoring technologies for a wider range of pesticides, veterinary drugs, environmental contaminants, toxins, nitrosamines, arsenic, mercury, and other chemicals of concern in foods and beverages. Beyond the direct, tangible benefits to government and industry monitoring labs, this research helps to promote better food production practices and protect human health through better food safety enforcement, more effective control of food trade, and higher quality of data for risk assessment and other purposes.

Examples of Relevant Publications

- Geis-Asteggiante, L., Lehotay, S.J., Lightfield, A.R., Dutko, T., Ng, C., Bluhm, L. 2012. Ruggedness testing and validation of a practical analytical method for > 100 veterinary drug residues in bovine muscle by ultrahigh performance liquid chromatography – tandem mass spectrometry. Journal of Chromatography A. 1258: 43-54.
- Kmellar, B., Abranko, L., Fodor, P., Lehotay, S.J. 2010. Routine approach to qualitatively screen for 300 pesticides and quantify those frequently detected in fruits and vegetables using liquid chromatography tandem mass spectrometry. Journal of Chromatography A. 27(10): 1415-1430.
- Lehotay, S.J., Mastovska, K., Lightfield, A.R., Gates, R.A. 2010. Multi-analyst, multi-matrix performance of the QuEChERS approach for pesticide residues in foods and feeds sing LC-MS/MS analysis with different calibration techniques. Journal of Association of Official Analytical Chemists International. 93(2): 355-367.
- Lehotay, S.J., Koesukwiwat, U., Van Der Kamp, H., Mol, H.G., Leepipatpiboon, N. 2011. Qualitative aspects in the analysis of pesticide residues in fruits and vegetables using fast, low-pressure gas chromatography - time-of-flight mass spectrometry. Journal of Agricultural and Food Chemistry. 59(14): 7544-7556.
- Lehotay, S.J., Lightfield, A.R., Geis-Asteggiante, L., Schneider, M.J., Dutko, T., Ng, C., Bluhm, L., Mastovska, K. 2012. Development and validation of a streamlined method designed to detect residues of 62 veterinary drugs in bovine kidney using ultrahigh performance liquid chromatography - tandem mass spectrometry. Drug Testing and Analysis Journal. 4(S1):75-90.
- Sapozhnikova, Y.V., Lehotay, S.J. 2013. Multi-class, multi-residue analysis of pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers and novel flame retardants in fish using fast, low-pressure gas chromatography-tandem mass spectrometry. Analytica Chimica Acta. 758:80-92.

Introduction

Food and Environmental Contaminants

To provide data that can be used to understand the broad impacts that chemicals play in influencing food and environmental safety. Three broad classes of chemicals were targeted for study: veterinary drugs or feed additives administered to food animals under extra-label use conditions; endogenous steroid hormones; and novel developmental chemicals of potential utility for mitigating the risks of human pathogens from animal or vegetable products. Regardless of the chemical class being investigated, the development of sensitive and accurate analytical tools to detect chemical residues in food animals or food products was of paramount importance. Therefore, a significant portion of the project was devoted to developing the analytical tools for either laboratory or field use.

Examples of Accomplishments

- **Fate of estrogen and estrogenic activity in farm settings.** The effects of estrone, and the estrogen 17α-estradiol, were assessed in/from surface waters. Animal waste management systems including composting, lagoon digestion, and constructed wetlands, effectively reduce estrogen activity of processed wastes to below the levels of environmental concern. Application of treated animal wastes to croplands does not increase the estrogenic activity of surface waters in those fields. Consequently, application of treated wastes to fields used for crop production poses minimal risk.

- **Residues of animal health drugs in food animals.** Studies requested by FSIS determined that penicillin residues in sows took much longer to deplete from kidney than previously believed. A new withdrawal period of 51 days was recommended to FSIS and pork producers. A commercial rapid screening assay could be employed pre-slaughter using urine to predict those animals that contained violative penicillin G residues.

- **Novel treatments to reduce pathogen loads of livestock and produce.** Sodium chlorate consistently reduces fecal shedding of pathogenic Enterobacteriacea in cattle. Residue and metabolism studies showed that chlorate residues are not the limiting factor for the development of a viable commercial product. Residues are well under safe tissue concentrations established by federal regulators. Studies have been included as a portion of a registration dossier submitted to U.S. regulatory agencies.

Outcome and Impact

Studies investigated the disposition of potent steroid hormones on farms and in the environment; the fate of veterinary drugs that are often detected as violative residues by regulatory agencies; and chemicals being developed to mitigate the risks of food-borne pathogens on food products. An important component was the development of rapid detection tools that can be used by regulators or producers to detect pathogens or chemicals in, or on, food products. Impacts range from basic to applied: for example, mechanisms of steroid hormone release from farms and transport into surface waters have been proposed; residue data on new chemical entities have been submitted to regulatory agencies as portions of product dossiers; and specific recommendations for maintaining residue free products have been proffered to regulatory

agencies and industry. Research conducted will provide context to support or refute both the concepts of "proper use" or "imprudent risk", that is, ultimately it is the data that will support or refute the use of a chemical for a given purpose.

Examples of Relevant Publications

- Shappell, N.W., Elder, K.H., West, M.S. 2010. Estrogenicity and nutrient concentration of surface waters surrounding a large confinement dairy operation using BMP for land application of animal wastes. Environmental Science and Technology. 44(7): 2365-2371.
- Shelver, W.L., Smith, D.J. 2011. Immunochemical-based zilpaterol measurement and validation in urine and tissues. Food and Agricultural Immunology. 22(3): 247-258.
- Shelver, W.L., Tell, L.A., Wagner, S., Wetzlich, S.E., Baynes, R.E., Riviere, J.E., Smith, D.J. 2013. Comparison of ELISA and LC-MS/MS for the measurement of flunixin plasma concentrations in beef cattle after intravenous and subcutaneous administration. Journal of Agricultural and Food Chemistry. 61(11): 2679-2686.
- Smith, D.J., Taylor, J.B. 2012. Kinetics and disposition of orally dosed sodium chlorate in sheep. Journal of Animal Science. 90(6): 2026-2034
- Zitnick, K.K., Shappell, N.W., Hakk, H., DeSutter, T.M., Khan, E., Casey, F.X.M. 2011. Effects of liquid swine manure on dissipation of 17ß-estradiol in soil. Journal of Hazardous Materials. 186(2-3): 1111-1117.

Introduction

Other Contaminants

To investigate the sources, levels, metabolism, elimination, and depletion kinetics of some of the most persistent and toxic food contaminants present in U.S. meats including dioxins, furans, brominated flame retardants (BFRs), and perfluorinated hydrocarbons (PFOS, PFOA). To develop rapid screening methods capable of detecting multiple contaminants with sufficient sensitivity to meet regulatory limits.

Examples of Accomplishments

- **Survey of U.S. meats.** USDA-ARS/FSIS conducted a statistically-based survey of dioxins, furans, PCBs, and BFRs in the U.S. meat supply providing data on halogenated chemical contaminants. Allow regulatory agencies to propose daily consumption guidelines, and establish action levels for contamination. Data indicate that dioxins, furans and PCBs continued to display a steady decline, demonstrating the effectiveness of intervention measures. Polybrominated diphenyl ethers (PBDE) levels also showed a strong decline.

- **Metabolism studies.** The perfluorinated surfactant PFOS exhibits a very long half-life in beef cattle, while its very close relative, PFOA, was quickly and quantitatively eliminated. Furthermore, PFOS distributed principally to edible tissues. Differing half-lives and metabolic pathways were observed among three closely related hexabromocyclododecane (HBCD) isomers present in a commercial BFR product of environmental concern.

- **Development of rapid screening assays.** Antibodies towards numerous BFRs and other contaminants were generated and tested for sensitivity, selectivity, applicability to high throughput systems, and suitability for use in various food and environmental matrices. Kits were commercialized for PBDEs, a major production volume BFR, and triclosan, in addition to streamlined sample preparation. The reagents/tests have found utility both nationally and internationally (China, Europe).

Outcome and Impact

Studies measured the levels, sources, and biological impacts of chemical residues in meat products. Surveys of the U.S. meat supply have documented a systematic decline in toxic dioxins, furans, and PCBs over the past 15 years in beef, pork, chicken, and turkey. These declining trends illustrate the effectiveness of regulations on the environmental release of dioxins and dioxin-like compounds and will likely result in corresponding declines in human exposures. The surveys also indicate that current estimates of contaminant daily intake from US meats are within safe amounts. An additional source of brominated flame retardant (BFR) exposure has been identified in addition to those previously described through this project; that is, household dust. The studies demonstrated the bioavailability, persistence, metabolism, and tissue targets of numerous BFRs and fluorinated contaminants. These data are vital for both regulators and risk assessors and are valuable for biomonitoring. Antibodies critical to the development of rapid, economical, portable, and sensitive detection tools were produced for the classes of chemical contaminants that are of major food safety concern. The studies impact's range from basic to applied. They included data needed by regulators and risk assessors for establishing regulations and dietary guidelines; residue data necessary to assure consumers and industry of food safety; expansion of international markets for U.S. food products; and commercialized chemical screening kits.

Examples of Relevant Publications

- Hakk, H., Huwe, J.K., Murphy, K., Rutherford, D. 2010. Metabolism of 2,2',4,4' Tetrabromodiphenyl (BDE 47) in Chickens. Journal of Agricultural and Food Chemistry. 58(15): 8757-8762.
- Huwe, J.K., Archer, J.C. 2013. Dioxin congener patterns in commercial catfish from the United States and the indication of mineral clays as the potential source. Food Additives and Contaminants: Part A. 30(2): 331-338.
- Lupton, S.J., Huwe, J.K., Smith, D.J., Dearfield, K.L., Johnston, J.J. 2012. Absorption and excretion of 14C-perfluorooctanoic acid (PFOA) in Angus cattle (Bos taurus). Journal of Agricultural and Food Chemistry. 60(4): 1128-1134.
- Szabo, D.T., Diliberto, J.J., Hakk, H., Huwe, J.K., Birnbaum, L.S. 2011. Toxicokinetics of the flame retardant hexabromocyclododecane alpha: effect of dose, timing, route, repeated exposure and metabolism. Toxicological Sciences. 121(2): 234-244.
- Wang, J., Li, H., Shelver, W.L., Wang, Z., Li, Q.X., Li, J., Xu, T. 2011. Development of a monoclonal antibody-based, congener-specific and solvent-tolerable direct enzyme-linked immunosorbent assay for the detection of 2,2',4,4'-tetrabromodiphenyl ether in environmental samples. Analytical and Bioanalytical Chemistry. 401(7): 2249-2258.

Introduction

Heavy Metal Contaminants

Studies were conducted to better understand the soil-plant relationships for cadmium, lead and arsenic in several crops where sale or consumption of the crop might comprise health risk depends on soil contamination, and on soil and crop management practices. Besides crop accumulation of trace elements, the bioavailability of elements in crops may vary widely and the nature of a crop, or the conditions needed to produce a crop may prevent excessive transfer to bioavailable levels of soil elements into crops.

Examples of Accomplishments

- **Reduce Cd accumulation in leafy vegetables grown on Cd-mineralized soils.** Leafy vegetables grown on Cd-mineralized soils in California accumulate high levels of Cd which threaten marketing of these crops. Testing of fertilizer Zn applications to inhibit Cd showed that Zn additions alone would not reduce crop Cd; it requires a combination of making the soil calcareous with lime, along with Zn fertilization to reduced accumulations to below 4 mg Cd/kg DW, the CODEX limit.

- **Testing of bioaccessible soil Lead.** Some root crops accumulate Pb in edible crop tissues, and the low-growing leafy vegetables and herbs may accumulate Pb or become contaminated with fine soil particles rich in Pb stuck on leaf surfaces. Most common garden fruits and seed crops accumulate very little Pb from soils. A rapid, convenient, and inexpensive Pb soil test was developed using common soil laboratory approaches and calibration to human feeding test. New feeding tests are being conducted by cooperators to validate the correlation of these bioaccessibility methods.

Outcome and Impact

Research identified needs for improving management alternatives to limit heavy metal concentrations in crops, or their bioavailability in crops, to aid public decisions. Studies showed that while soil amendment with Zn reduced Cd accumulation by leafy vegetables, it was only sufficiently effective if soils were made calcareous. New concern about As accumulation in rice led to retrospective analysis which showed that fully aerobic management could remarkably reduce grain As, but also increase grain Cd and reduce grain yields by 50%. Based on FDA concerns about Pb in carrots grown on old orchard soils, research found a strong accumulation of Pb within the xylem inside carrot storage roots. Concerns about Pb in urban gardens from multiple sources, including ingestion of soil or dust by children was shown as the major risk from such soils, rather than crop accumulation of Pb. Some root crops and low-growing leafy vegetables and herbs can become high enough in lead to require warning urban gardeners. Cost-effective soil analysis methods were evaluated to assess Pb chemical bioaccessibility correlated with bioavailability.

The impacts of this work are far-reaching. U.S. rice producers are faced with difficult choices regarding potential limits on (As) in rice grain. Integrated information about soil-plant-animal aspects of soil As has significantly assisted growers to better understand the choices they might make to address concerns about inorganic As. Concerns about Cd in mineralized soils required

communication of existing knowledge and advice for field testing of management alternatives. Understanding of crop accumulation of Pb from contaminated orchard and urban garden soils has identified a few crops which should be avoided for contaminated soils/gardens.

Examples of Relevant Publications

- Centofanti, T., Sayers, Z., Cabello-Conejo, M.I., Kidd, P., Nishizawa, N.K., Kakei, Y., Davis, A.P., Sicher, R.C., Chaney, R.L. 2013. Xylem exudate composition and root-to-shoot nickel translocation in Alyssum species. Plant and Soil. 373(1-2): 59-75.
- Gu, H., Qui, H., Tian, T., Zhan, S., Deng, T., Chaney, R.L., Wang, Z.-T., Tang, Y.-T., Morel, J.-L., Qiu, R.-L. 2011. Mitigation effects of silicon rich amendments on heavy metal accumulation in rice (Oryza sativa L.) planted on multi-metal contaminated acidic soil. Chemosphere. 83(9): 1234-1240.
- Khaokaew, S., Chaney, R.L., Landrot, G., Ginder-Voget, M., Sparks, D.L. 2011. Speciation and release kinetics of cadmium in an alkaline paddy soil under various flooding periods and draining conditions. Environmental Science and Technology. 45(10): 4249-4255.

Biological Toxins (Mycotoxins)

Introduction

Detection/Modeling

Certain fungi that infest crops produce toxins [mycotoxins], are hazardous to animals and humans. These toxins such as aflatoxins, trichothecenes and fumonisins are secondary metabolites and include compounds that are potent carcinogens, hepatotoxins, nephrotoxins, and neurotoxins. The Food and Agriculture Organization has estimated that each year 25% of the world's food crops are affected by mycotoxins. Mycotoxins in commodities and products cause extensive economic losses to growers, processors, livestock and poultry producers and food and feed processors. Resultant, there are significant efforts associated with keeping contaminated agricultural commodities out of the U.S. food and feed supplies. These efforts include those addressing development of detection methods for mycotoxins and derived toxins (masked mycotoxins), monitoring, various control (intervention) methods, genomics, and endophytes. Some accomplishments also have impacts within Problem Statement 1.B, Systems Biology.

Examples of Accomplishments

- **Development of novel detection methods.** A rapid, reusable biosensor for detecting deoxynivalenol (DON) via biolayer interferometry (BLI). A fluorescence polarization immunoassay assay to detect DON in bran and whole-wheat flour. Monoclonal antibodies to bind "masked" mycotoxins. Ambient ionization mass spectrometry for mycotoxin analysis directly from grain, bypassing grain grinding, extraction with organic solvents, and sample clean-up.

- **Masked mycotoxins.** A method for the production of the masked mycotoxin [T-2 toxin glucoside] was developed. Production of the toxin was shared with labs in Italy, UK, Singapore, and Japan to aid in development of analytical methods for detection.

- **Synthetic polymers**. Computational methods were used to develop synthetic polymeric materials to detect and/or bind to various mycotoxins: ochratoxin A (OTA), fusaric acid, patulin, T-2, and citrinin. With collaborators in Japan, developed a simple way to isolate patulin from apple juice/sauce, supporting efforts to ensure the safety of baby foods.

- **Bioactive metabolites.** Novel metabolites were discovered: phomalevone that exhibits anti-fungal and anti-bacterial activity, and a potentially life-saving bis-naphthopyrone that inhibits the action of botulinum neurotoxin.

- **Fumonisin toxicology**
  - Model studies determined which organs were damaged by fumonisin intoxication.
  - Animal model studies, transferrable to humans found that Neural Tube Defect (NTD) induction by Fumonisin B1 is significantly reduced with folate supplementation.
  - The oral dose of fumonisin B1 for induction of NTD, maternal toxicity, and elevated sphingoid bases and sphingoid base 1-phosphates were determined. Results predicted the urinary level of FB1 likely to indicate toxicity.
  - Hydrolyzed fumonisin B1 produced during alkaline cooking does not induce NTD.

Outcome and Impact

Research focused on selective binding materials: the development of improved toxin-binding materials, which have produced improved toxin detection methods. Materials that have been developed include antibody fragments (scFv) and synthetic materials that selectively bind economically important mycotoxins. Mass spectrometry-based methods for fumonisins and other mycotoxins, allowed for detection of some mycotoxins directly from intact grain.

Studies identified and characterized gene clusters responsible for biosynthesis of fumonisins and two other mycotoxins, and identified processes that affect distribution of these clusters among Fusarium species. Studies examined the genetic basis and ecological significance of variation of type of mycotoxins produced by Fusarium and other fungi. Genes were characterized that are critical in determining the type of trichothecene produced; studies identified organisms that can detoxify trichothecenes, and genes that confer resistance to trichothecenes. Tools were developed to produce model trichothecene metabolites, some of which are useful for development of antibodies, high throughput screening of libraries, or other analytical methods. Studies discovered of yeast species that biotransforms trichothecenes and has enabled production of large quantities of T-2 toxin glucoside, a masked mycotoxin for determination of its relative toxicity and threat to public health.

In endophyte studies, efforts to induce/produce more of the plant's defensive compounds were unequivocal. Results suggested a reduction of Fusarium graminearum disease incidence and severity in wheat with the B. mojavensis treatments; however, in-field implementation is a major concern.

Urinary fumonisin B1 was a potential exposure biomarker for intake and sphinganine 1-phosphate in blood is a potential biomarker for ceramide synthase inhibition in humans. Lack of biomarkers to conduct human studies was a major gap in the fumonisin risk assessment. NTD is a major birth defect worldwide and folate supplementation reduces NTD risk in humans. The mouse model developed in this work is the model of choice for studying NTD causation and intervention. The model provides a research tool for assessing the threshold for disruption of sphingolipid metabolism in humans and for designing epidemiological studies to evaluate the potential of fumonisin exposure as a contributing factor to human disease. Nixtamalization (alkaline cooking) effectively reduces fumonisin exposure, and dose-response findings contributed significantly to hazard characterization and recommendations for tolerable daily intakes (exposures) published by the Joint FAO/WHO Expert Committee on Food Additives in 2012.

Examples of Relevant Publications

- Alexander, N.J., McCormick, S.P., Waalwijk, C., Van Der Lee, T., Proctor, R. 2011. The genetic basis for 3-ADON and 15-ADON trichothecene chemotypes in Fusarium graminearum. Fungal Genetics and Biology. 48(5): 485-495.
- Bacon, C.W., Hinton, D.M., Mitchell, T.R., Snook, M.E., Olubajo, B. 2012. Characterization of endophytic strains of Bacillus mojavensis and their production of surfactin isomers. Biological Control. 62(1): 1-9.
- Cardellina, J., Roxas-Duncan, V., Montgomery, V., Eccard, V., Campbell, Y., Hu, X., Khavrutskii, I., Tawa, G.J., Wallqvist, A., Gloer, J.B., Phatak, N.L., Hoeller, U., Soman, A., Joshi, B.K., Hein, S.M., Wicklow, D.T., Smith, L.A. 2012. Fungal bis-naphthopyrones as inhibitors of botulinum neurotoxin serotype A. ACS Medicinal Chemistry Letters. 3(5): 387-391.
- Gelineau-Van Waes, J., Rainey, M.A., Maddox, J.R., Voss, K.A., Sachs, A.J., Gardner, N.M., Wilberding, J.D., Riley, R.T. 2012. Increased sphingoid base-1-phosphates and failure of neural tube closure after exposure to fumonisin or FTY720. Birth Defects Research Part A: Clinical and Molecular Teratology. 94(10): 790-803.
- Maragos, C.M. 2011. Detection of deoxynivalenol using biolayer interferometry. Mycotoxin Research. 27(3): 157-165.
- McCormick, S.P., Price, N.P., Kurtzman, C.P. 2012. Glucosylation and other biotransformations of T-2 toxin by yeasts of the Trichomonascus clade. Applied and Environmental Microbiology. 78(24): 8694-8702.
- Riley, R.T., Torres, O., Showker, A.J., Zitomer, N.C., Matute, J., Voss, K.A., Maddox, J.R., Gelineau-Van Waes, J., Gregory, S.G., Ashley-Koch, A.E. 2012. The kinetics of urinary fumonisin B1 excretion in humans consuming maize-based diets. Molecular Nutrition and Food Research. 56(9):1445-1455.
- Van der Westhuizen, L., Shephard, G.S., Gelderblom,, W.A., Torres, O., Riley, R.T. 2013. Fumonisin biomarkers in maize eaters and implications for human disease. World Mycotoxin Journal. 6(3): 223-232.

Introduction

Aflatoxins/Biological Control

Research focused on prevention of mycotoxin contamination, with emphasis on prevention of aflatoxins in tree nuts, one of the U.S.'s highest valued crops. Tree nuts and raisins are major California specialty crops with an annual production value of over $5.5 billion. The main efforts involved studies on pre- and post-harvest fungal control, disruption of mycotoxin biosynthesis and catabolism, and mycotoxin detection in storage and stockpiles. Because insect-feeding damage creates wounds and avenues for fungal infection, this also included the use of natural host plant volatiles for insect control. Certain fungal infections can result in highly invasive diseases of animals and humans; thus efforts to apply fungal control technology to these pathogenic fungi were investigated. Ecological studies determined the attributes and changes in the microbial communities in order to understand the dissemination of pathogens and toxins in/on tree nuts and raisins and the interactions and relationships within these communities. Although alternative interventions were studied, the most effective method for preventing aflatoxin contamination is a type of biological control that utilizes Aspergillus flavus isolates that do not produce aflatoxins to competitively exclude aflatoxin producers. ARS has investigated this concept and studies continue to advance biological control through increased understanding of the population biology of A. flavus and continued engagement of stakeholders. Efforts were directed to development of criteria for selection of optimal biocontrol strains based on phenotypic, genetic, and geostatistical analyses, as well as assessment of adaptation to cropping systems. Multi-season influences of biocontrol were characterized and rationale for the design of strain mixtures with greatest value in target rotations, regions, and environments. Stakeholders, collaborators and industry assisted both with funding and opportunity for the continued improvement of practical implementation of biocontrol. In order to make possible application of management procedures in poor communities in developing nations a partnership was formed with International Institute of Tropical Agriculture [Africa] and funds were provided by the Bill & Melinda Gates Foundation, Meridian Institute, USAID, United Kingdom Department for International Development, Austrian Development Agency, and USDA-FAS.

Recent work revealed that A. flavus has a complicated evolutionary history that includes a history of genetic recombination. One aspect studied was to understand A. flavus population dynamics in agricultural environments, and to provide additional insight into the evolution of diversity within A. flavus. Developing new competitor strains was needed with improved properties for tracking their dispersal after introduction onto crops and with improved ability to over-winter in order to decrease the need for annual reapplication. Optimal candidates should be unable to produce the neurotoxin, cyclopiazonic acid, (as well as other harmful secondary metabolites) without altering their competitive ability. The potential for restoration of both AF- and CPA-producing ability of the atoxigenic strain in the laboratory or the field would be assessed. The ability to produce hydrolases by the biocontrol strain is important for its competitive ability, and with these studies, it is expected to be able to either develop new biocontrol strains or improve the design of currently used biocontrol strains.

Examples of Accomplishments

- **Natural compounds as antifungal chemosensitizers.** Several natural products were discovered to act as potent antifungal chemosensitizing agents. Salicylaldehyde when combined with the antifungal itraconazole in the gas phase inhibited the growth of Aspergillus fumigatus, a pathogenic fungus that can cause aspergillosis.

- **Plant volatiles to attract agricultural insect pests.** Damage by the navel orangeworm (NOW) is directly associated with contamination from Aspergillus flavus and/or A. parasiticus. A blend of host plant volatiles for use in monitoring and mating disruption was developed. The system is currently undergoing studies in treated orchards.

- **Biocontrol agent antagonistic to Aspergillus flavus.** Pichia anomala WRL-076 a yeast stain inhibits the growth of Aspergillus flavus and aflatoxin production in both the lab and field situations. This yeast has broad biological control activities as stated in the U.S. Patent No. 7,579,183 and can be used for a variety of crops. A stable liquid formulation for P. anomala WRL-076, allows the biocontrol product to be easily dispersed in water and delivered by spraying or dipping. A second U.S. Patent No. 8,206,972. Pichia anomala WRL076 has now been licensed by Verdesian Life Sciences LLC as a biological control agent antagonistic to mycotoxigenic fungi.

- **Contemporary aflatoxin management.** Aflatoxin management through the accepted use of competitive exclusion (use of atoxigenic strains) was adapted to agronomic practices in the U.S. Work was undertaken in partnership with the pistachio, corn, and cotton domestic industries. Materials were submitted to the EPA to support industry applications for biopesticide registrations and expansions of registrations for atoxigenic strain use. Timing of applications and compatibility with agronomic practices were developed in order to provide recommendations for commercial use.

- **Partnerships with food aid communities.** In collaboration with the International Institute of Tropical Agriculture (IITA)**,** and numerous national partners across sub-Saharan Africa, atoxigenic strains native to Senegal, Nigeria, Zambia, Mozambique, Kenya, and Tanzania were identified and incorporated into biological control formulations (competitive exclusion). In several nations, farmer's field testing of the biological control products is underway. Advanced, low-cost manufacturing methods and formulations were developed and adapted for use in Africa and a full scale manufacturing plant was constructed at the IITA campus in Ibadan, Nigeria. Scientists from African nations were trained to work with aflatoxins, aflatoxin-producing fungi, and in the implementations of biological control.

Outcomes and Impact

Volatiles from fungal spores can act as early warning signals of contamination and in conjunction with portable detector will help detect and remove fungal hot spots from tree nut containers. A blend of host plant volatiles was found to attract the tree nut insect pest navel orange worm more effectively than the current monitoring standard. Consistent and effective monitoring will allow growers to apply better-timed sprays. Moreover, the developed host plant

volatile blend may play a large role in monitoring for IPM strategies where mating disruption treatments are used. Natural products were shown to serve as potential and potent antifungals/ chemosensitizers against fungal pathogens. The biggest impact of chemo-sensitization is its capacity to lower the dosage levels of fungicides necessary for effective control of fungi. A surprising result [*not described above]* was the discovery that carbon dioxide was the biomarker that signaled aspergilli response to stress and was associated with an increase in aflatoxin production. If an increase in carbon dioxide levels within the sphere of aflatoxigenic aspergilli results in an increase in aflatoxin production, this may result in negative impact on food safety issues for tree nuts or other aspergilli-infected crops.

No clear differences between bacterial and fungal populations were observed following organic vs. conventional (chemical) interventions which was unexpected. Technologies were developed to identify and quantify bacterial and fungal species on almond nuts and raisins. These indicated that properly dried and stored almonds are not conducive to fumonisin contamination, and that ochratoxin contamination is sporadic, and possibly caused by other Aspergillus species. These findings point out future research efforts for solving ochratoxin contamination in almonds. Investigation of mycotoxin cross-contamination to processing equipment indicated limited transfer of aflatoxin to the "clean" almonds. As hypothesized the amount of aflatoxin correlated strongly with the number of "hot nuts" in each sample. The discovery and patenting of the biocontrol yeast Pichichia anomala WRL-076 provided a viable method to control populations of A. flavus in tree nut orchards for reduction of aflatoxin contamination. Biological control provides a good alternative to the use of fungicides in agriculture to reduce harmful fungi while having a minimal adverse impact on the environment.

Biological control of aflatoxin contamination was confirmed as a highly effective method for limiting contamination of many crops in diverse environments. Future aflatoxin management strategies will likely include initial use of biological control for 2 to 3 years followed by maintenance treatments at reduced rates/frequencies with reduced costs. Studies suggested effective endemic atoxigenic strains adapted to target regions and crop rotations can be selected from natural populations and have ability to provide area-wide benefits that extend over multiple years. The future of biological control will be best served by optimizing area-wide and long-term benefits and developing economic models for maintenance of reductions in aflatoxins across regions after economic impacts from contamination have been ameliorated.

The research provided new information about the limitations as well as the advantages of the biocontrol strategy for aflatoxin reduction in contaminated crops. It definitively proved that outcrossing of the biocontrol A. flavus is a frequent occurrence and, therefore application of biocontrol A. flavus is likely to be necessary on a repetitive basis. This will help in knowing how biocontrol works in field conditions, and will assist in defining the right conditions and strains for use in the biocontrol strategy. AflaGuard displayed lower levels of pectinase P2c than other tested atoxigenic isolates which could affect its ability to outcompete aflatoxin-producing A. flavus strains.

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Introduction

Aflatoxins/Genomics-Breeding

The biosynthesis of aflatoxins (AF) has been extensively studied by ARS, and the genetics have been elucidated in great detail in the genomes of A. flavus and the related aflatoxin-producing species A. parasiticus. A. nomius as well as the non-aflatoxigenic species A. oryzae have been sequenced, with their genome sizes being about 36.3 Mb on eight chromosomes, encoding about 12,000 proteins. However, much less was known about what causes the fungi to produce AFs under certain environmental conditions and only on certain plants. Also, it was not fully understand how toxin formation was transcriptionally regulated during conidial, sclerotial, and mycelial development although a strong association with these processes has been proven. Information was still lacking concerning how external stress factors affect aflatoxin formation although much evidence suggests that there was a connection. Furthermore, a better understanding of how interactions with susceptible crops affect transcription of fungal genes involved in aflatoxin formation was needed to improve on intervention strategies. Studies were undertaken to: determine the dynamics of interaction among the key nutritionally and environmentally induced transcription factors necessary for production of AF in order to develop novel inhibitors to one or more of these factors to prevent AF formation in crops; examine the effects of known natural (plant-derived, such as volatile aldehydes) inhibitors of AF production on key components of the AF transcription machinery to ultimately design safe,

inexpensive chemicals that inhibit proteins unique to fungal secondary metabolite biosynthesis; and to identify safe and effective procedures for use on crops intended for consumption by humans or animals.

A most practical solution to the contamination problem would be to prevent the contamination process in crops before harvest. One of the easiest technologies to implement by growers would be to utilize germplasm with enhanced resistance to Aspergillus flavus growth and aflatoxin contamination. ARS studies had identified a number of genes in corn associated with aflatoxin-resistance that have been used to develop molecular markers to transfer resistance to agronomically desirable corn lines. These lines are currently being field tested in the U.S. and Central and West Africa. Unlike in corn, there is no described natural resistance to A. flavus present in cotton plants. Therefore, transgenic approaches have been used to introduce resistance genes into cotton. A number of synthetic antifungal peptides have been found to be inhibitory to A. flavus growth and the gene encoding one of these, D4E1, has been introduced into cotton and transgenic lines expressing D4E1 are being evaluated from field trials in Arizona. RNA interference (RNAi) techniques are also being used to develop transgenic corn and cotton lines that demonstrate improved resistance to A. flavus growth and aflatoxin production. Allied to this was the need for development of rapid, non-destructive detection methodology based upon hyperspectral imaging technology that could be used remotely, by satellite or drone, or in processing facilities to exclude aflatoxin-contaminated corn from the food stream before harvesting or prior to packaging.

Examples of Accomplishments

- **Sequencing different strains of Aspergillus (A.) flavus and A. parasiticus.** Two different format whole genome microarrays were designed, and used to identify over 100 genes involved in fungal response to various environmental factors favoring mycotoxin production. Sequencing data is being analyzed to provide insights into what genes are involved in the interaction between the fungus and the crop, and which genes provide a competitive advantage to the toxigenic A. flavus and A. parasiticus.

- **Function of other gene clusters**. Comparison of the Aspergillus (A.) flavus/parasiticus genomes indicated that they contain several gene clusters predicted to produce a variety of secondary metabolites. Collaborations with Ghent University, Belgium, evaluated compounds such as ditryptophenaline, a diketopiperazine dimer with potential anti-inflammatory and anticancer properties.

- **Sources of maize germplasm resistant to aflatoxin contamination.** New ARS-IITA developed inbred maize lines demonstrated resistance to aflatoxin contamination in field trials in Central and West Africa. Several crosses have been made using other ARS-IITA lines to obtain lines with drought tolerances and to aflatoxin accumulation. Screening of ARS-IITA lines with dual resistance to A. flavus and F. verticillioides was completed.

- **Hyperspectral imaging**. A whole corn ear hyperspectral imaging system was developed under a grant from the Bill & Melinda Gates Foundation Grand Challenge Exploration, to develop portable (practical) technology to detect aflatoxin contamination in single corn

ears for farmers in the developing countries such as Africa. A grant from USAID was awarded to develop "AflaGoggles for screening aflatoxin contamination in maize". Technology for both grants is based on U.S. Patent 20120061586 A1.

Outcomes and Impact

One current strategy for reducing AF accumulation on agricultural fields continues to be the introduction of non-aflatoxigenic A. flavus as bio-competitors. An additional/alternate strategy that is emerging is to transform susceptible crop plants resistant to AF contamination either by breeding for resistance or by introducing resistance genes into the plant. For both these strategies to be effective it is very important to know how and why the fungus produces aflatoxins, how the fungus reacts to environmental and nutritional signals in its habitat, and how the fungus responds at the genetic level when it invades the crop and produces aflatoxin. This last parameter is significant especially because the physiology of crops such as corn, peanuts, cotton and tree-nuts that are susceptible to preharvest contamination is very different, and the only common factor for toxin contamination is the producing fungus.

Studies concluded that transcriptional regulation of the AF biosynthesis gene cluster in A. flavus involves many different proteins that are subject to developmental, environmental and nutritional controls. Current and continuing work will identify protein targets for inhibition in A. flavus that would prevent aflatoxin production on susceptible crops. The most appropriate initial strategy involves the identification of low cost inhibitors of regulatory proteins, unique to A. flavus, which can safely be applied to susceptible crops to prevent A. flavus from producing AFs.

Whole genome sequence comparison of A. flavus L- and S-strains and atoxigenic strains has allowed identification of as yet poorly understood components of the pathogenesis machinery and the regulatory machinery involved in secondary metabolism. This will lead to the identification of potential gene targets for inhibition to reduce fungal infection and AF formation by either of these strains. Volatile plant-derived or synthetic low-molecular-weight inhibitors of AF biosynthesis could be easily applied to agricultural fields. If the inhibitors are produced by an AF-resistant plant, inter-cropping might be a safe and effective strategy to prevent AF contamination of the susceptible crop. Inhibitors of this type (plant phytoalexins, aldehydes) have been identified in some of our previous studies. New safe and effective inhibitors could be obtained by analysis of plant's resistant to AF contamination. Current and future studies will seek to identify inhibitors known to act on key regulatory proteins unique to fungi and which are required for transcription of AF pathway genes.

At this point it is difficult to make firm conclusions regarding plant breeding studies. Research has made great strides in identifying and developing maize germplasm that demonstrates enhanced resistance to aflatoxin contamination but further field trials in the U.S. and Africa are needed to validate the potential of these lines. Development of binary vectors for expression of antifungal genes and RNAi-based gene knockouts in maize are in early stages of analysis. We are also awaiting analysis of Arizona field trial data of antifungal peptide D4E1-expressing cotton lines for inhibition of A. flavus contamination.

Current and future research will continue to analyze the antifungal efficacy of novel synthetic peptides and methods to improve their production and half-life in the plant cell. Development of maize and cotton germplasm's with superior anti-A. flavus/aflatoxin traits using either marker-assisted breeding or transgenic approaches, in combination with improved management practices and biological control should provide a formidable defense against infection of the crop by A. flavus and subsequent aflatoxin contamination.

Finally, development of faster, non-destructive, hyperspectral instrumentation can (a) exclude aflatoxin contaminated corn from the food stream in a cost-effective manner, and (b) measure physical and biochemical attributes associated with resistance to aflatoxin contamination in corn kernels and, therefore, identify resistant corn genotypes demonstrating relatively less fungal infection and aflatoxin contamination.

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Introduction

Bacterial and Plant Toxins

Foodborne bacterial disease may be caused by toxins produced by bacteria or in some cases by plants. Most toxins are proteins (exotoxins or endotoxins) that act enzymatically or through direct action upon the host, stimulating a variety of responses, for example, vomiting, diarrhea, or neurological disorders often resulting in death of the host. Just as evolution has provided bacteria and plants with survival mechanisms to evade host defenses, their toxins have gained enhanced stability, permitting their activities to persist after [mild] food processing interventions that are often adequate for killing the bacteria/plant. Many of these toxins exhibit oral bioavailability sufficient for significant absorption and can cause disease following ingestion. Some toxins are listed as Select Agents (42CFR73) because of their importance not only to food safety but also to U.S. national security. Although toxin-producing bacteria are almost ubiquitous and, for the most part, readily detected to certain levels, sensitive toxin assays are critically needed since often the amount of toxin required to elicit a response may be at the nanogram level. Within this research area there were several goals: to develop and validate new assays for toxins and their variants; to develop new data on the bioavailability of the toxins; animal assay systems would be used to calibrate the new in vitro methodology; to characterize the impact of food processing on toxin activities; and finally to study antibody-mediated clearance of toxins, especially via the oral route of intoxication.

Examples of Accomplishments

Bacterial Toxins/Detection/Inhibition/Activity

- New monoclonal antibodies for Clostridium botulinum serotypes B and E, complementing [our] previously produced and recently patented mAbs for BoNT/A (U.S. Patent No. 7,732,579) were developed. Sensitive tests were developed for serotypes A, B, and E, including sandwich ELISAs detecting amounts as low as 2 pg/mL BoNT/A or BoNT/B in food matrices. Studies of the epitopes (Ab-binding sites) of BoNTs were conducted for vaccine development. The test is 50x more sensitive than the current [standard] mouse bioassay. A lateral flow diagnostic test that detects and distinguishes between BoNT/A and B was developed that could be used by minimally trained personnel in the event of a foodborne outbreak or a bioterrorist threat.

- Three assays were developed in collaboration with a commercial kit manufacturer for the detection and quantification of BoNT serotype A, B, and F proteolytic activities in complex matrices in less than 24 hours. The limits of detection were below 1 pM for BoNT/A and BoNT/F and below 10 pM for BoNT/B in most tested matrices using 0.2 mL samples and as low as 10 fM for BoNT/A with an increased sample volume. The rapid, robust, and high-throughput assays are compatible with a wide range of matrices.

- The monoclonal antibodies specific for BoNTs were tested for their ability to provide protection against botulism exposure in a mouse model system. Following intravenous and oral exposures to lethal levels of toxin, the timing of antibody neutralization of the toxin was determined. The results provided new information on the toxicity of BoNTs and revealed windows of opportunity for therapeutic treatment with antibody. In studies of BoNT serotype B, some mAbs were shown to confer strong protection against toxin.

This protection was correlated with the antibody-potentiated depletion of BoNT/B toxin in the blood of intoxicated mice.

- A recombinant Stx toxoid, Stx2E167Q, was used as an immunogen to prepare a polyclonal antibody. This antibody binds specifically to the A-subunit of Stx2 and is capable of neutralizing Stx2 toxicity in a cellular assay. A simple, rapid, and sensitive ELISA capable of detecting all subtypes of Stx2 was also developed using this polyclonal antibody, with a limit of detection between 10 and 100 pg/mL for Stx2a. The ELISA/Lateral Flow Device is the first immunoassay that can detect all STx2 subtypes.

- Staphylococcal enterotoxins (SEs) generate a "superantigenic" immune response and gastrointestinal disease at very low concentrations. SEA or SEB can be detected by measuring this superantigenic response. New assays were developed using this response that detect and quantify cell surface markers and secreted effector molecules (cytokines) of mouse immune cells. One new assay measures the responses of mouse immune cells exposed to SEA or SEB using a flow cytometer. This response provided the basis for a test for SEs that proved effective in spiked samples of milk and other food matrices.

Toxin Stability

- The bioavailability of BoNT, crude and complexed BoNT/A were about 17-fold more toxic orally compared to purified toxin. Factors such as the size of the toxin complex, the presence of non-toxin components of complexes, and the type of food matrix impacted toxicity and bioavailability. Determining the fate and persistence of toxin following oral or systemic exposure permit more successful treatment of botulism.

- Orally ingested Stx damages kidney, spleen, and thymus tissues in mice. Stx survives passage through the digestive system while Stx2 activity was not destroyed by pasteurization. Stx2 was however, inhibited by components of fresh and processed apple juice from Red Delicious apples [also Staphylococcal enterotoxin SEA]. Inactivation varied among juices from different varieties of apple. It was hypothesized polyphenols were the inhibitory compounds. Further, demonstrated that components of grape seed pomace had similar Stx2-inactivating activity. 4-hydroxytyrosol from olives inactivated S. aureus enterotoxins; olive powder inhibited multiple toxins; and reconstituted milk powder inhibited the activity of ricin (similar activity to Stx).

Biosecurity Related

- The potential use of ricin as a bioweapon highlights the necessity for developing detection methods that work well for food samples. A new method for the detection of ricin in food matrices was developed using electrochemiluminescence. The assay enabled measurement of about 1 billionth of a gram of this toxin per 25g [in ground beef].

- Studies were conducted on the fate of ricin following oral intoxication in a mouse model system. Such data were previously unavailable. Studies quantified ricin in sera and feces, providing information on the time course of ricin distribution in an exposed system.

Outcome and Impact
The development of new antibodies, immunoassays, activity assays, lateral flow devices, and bead array assays that enhance our ability to monitor for various toxins in foods, providing enhanced food safety and biosecurity. Studies in mouse models of botulism demonstrated that components found in the crude bacterial lysates contributed to BoNT toxicity and structural studies revealed features that protect toxin from degradation in the intestine and facilitate its absorption. In mouse model systems, some mAbs for BoNT and Stx can protect animals from toxin, providing guidance for further efforts that will have clinical application.

The newly developed technologies were disseminated to stakeholders: federal and state agencies (FDA, FSIS, Food Emergency Response Network, Military, FBI, DHS etc). Several Patent applications were filed and one Patent was issued. The project transferred the antibody technology to the food and environmental diagnostics industries via two royalty-bearing Patent licenses and several licenses of unpatented monoclonal antibodies.

Examples of Relevant Publications

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