

***SALMONELLA* IN AN OYSTER PRODUCTION AND
SMALL FEEDLOT ENVIRONMENT, USE OF
NOVEL PROTEINS EXPRESSED BY AN
ATTENUATED *SALMONELLA* VECTOR FOR THE
REDUCTION OF *CAMPYLOBACTER*
COLONIZATION IN BROILER CHICKENS.**

by
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ABSTRACT

The CDC estimates that 48 million illnesses, 128,000 hospitalizations, and 3,000 deaths annually are attributable to foodborne illnesses, making their impact significant in terms of both human health and economic losses (3). Estimates vary, but it is frequently stated that *Campylobacter* species affect 2.4 million people annually (28). Among bacterial foodborne pathogens it is second in the US only to *Salmonella*, which in recent years has consistently been the most frequently reported, most likely to cause hospitalization, and deadliest foodborne bacterial illness in the US (3, 106).

In order to reduce the burden of illness caused by these pathogens and improve the safety of our food supply, continued investigation of the epidemiology, transmission and interactions of these organisms with their environments is necessary. Additionally, prevention of colonization within natural reservoirs of these bacteria which contribute to contamination of foods is an important step in the reduction of the burden of foodborne illness. This work examines the relationship of *Salmonella* to oysters and the aquatic environment in which they are raised, the interactions of *Salmonella* in a small feedlot environment, and the reduction of colonization of broiler chickens by *Campylobacter jejuni* through vaccination with recombinant attenuated *Salmonella* vectors into which novel *Campylobacter* genes had been cloned.

It was found that while *Salmonella* is still sporadically present on the West Coast of the US, an area where oysters were previously found to be positive for the organism, the strain which predominated in the last study of that area is reduced in prevalence. Additionally, it was found that that strain does not possess special fitness in oysters or the aquatic environments in which they are raised, though *Salmonella* survives in oysters and water samples longer than a representative coliform. *Salmonella* is also present in the small feedlot environment sampled, and animal stress appears to play a role in the shedding of the organism in that environment, leading to the potential contamination of beef carcasses during processing. Reduction of colonization by *C. jejuni* in broilers was achieved in the case of both vaccines, with a maximum reduction of four logs as compared to controls.

CHAPTER 1 - INTRODUCTION

1.1 *Campylobacter*

Campylobacter is a genus of Gram-negative, spirally curved rods which are highly motile through the use of polar flagella. They are members of the class Epsilonproteobacteria, being most closely related to the human pathogen *Helicobacter* and the bovine commensal *Wolinella* (129). *Campylobacter* is a microaerophilic commensal colonizer of poultry and other avian species which has an optimal growth temperature of 42 degrees Celsius indicating it is evolutionarily adapted to life within the intestines of these hosts (7, 111). *Campylobacter* strains often colonize at high levels and are not known to cause disease in avian species, possibly because elements of the mucous found in their intestinal tracts assist in protection of their epithelial cells (25). The organism may be shed in avian feces at levels higher than 10^9 CFU/gram (91). Eighteen species of *Campylobacter* are currently recognized, and though only half cause disease in humans, others may be pathogenic in animals (129).

1.1.1 Clinical Significance

The infectious dose of *Campylobacter* varies, but is often reported as being less than 500 organisms (though in some cases it may theoretically be as high as 10^6) depending largely on the strain, and to a lesser extent on individual

susceptibility or as yet undetermined factors (112, 129). One species, *Campylobacter jejuni*, is responsible for the vast majority of cases of campylobacteriosis, though other species including primarily *C. coli*, followed by *C. fetus*, *C. lari*, and *C. upsaliensis* may cause disease in humans (20, 28).

There may be as many as 2.5 million cases of campylobacteriosis in the US, making it more common than salmonellosis, though the latter continues to be responsible for more hospitalizations and deaths. *Campylobacter* infection, however, should not be underestimated as it is responsible for an estimated 17% of foodborne illness related hospitalizations and 5% of such deaths (84). Underreporting of campylobacteriosis is common because of the normally self-limiting nature of the disease, an inflammatory enterocolitis, which generally presents as loose stools or watery diarrhea. Commonly reported symptoms in laboratory confirmed cases are fever and abdominal cramping and up to half of these may include bloody diarrhea or dysentery. Rarely, the disease may progress to bacteremia, septic arthritis, endocarditis, meningitis or death, though serious complications are rare outside very young, old, or immune compromised populations (7, 88, 89). Chronic sequelae may occur, falling into the categories of either peripheral neuropathies or rheumatic diseases. Disease presents somewhat differently in developed and developing countries, with infection in the latter more likely to occur early in life (<2 years of age) and result in watery rather than bloody diarrhea (34).

1.1.2 Guillain-Barre Syndrome

One potentially serious consequence of infection with *Campylobacter* is Guillain-Barre Syndrome (GBS), which occurs following roughly 1 in 1,000 cases of campylobacteriosis. Though other conditions including viral and other bacterial infections also contribute, infection with *Campylobacter* is the most common antecedent to onset of GBS (88, 89). Because of the availability of polio vaccines and their use in many parts of the world, GBS is now the most common cause of acute flaccid paralysis (87). GBS is a serious autoimmune disorder attacking the peripheral nervous system and causing loss of reflexes and muscle weakness in both the limbs and the respiratory system. While most patients begin to recover after 2-3 weeks, up to a fifth of patients are left with permanent neurological damage. Death occurs much less frequently when treatment is available (2-3% of cases) but mortality rates remain high in developing countries (89).

Campylobacter spp. possess lipooligosaccharide (LOS) which is similar to the lipopolysaccharide found in most Gram-negative species in that it is composed of Lipid A and a core oligosaccharide, but different in that it lacks O-antigen repeats (129). The LOS structures of some strains appear to be more similar to human peripheral nerve gangliosides. Host produced antibodies to these structures may then be responsible for axonal damage, demyelination and the resulting neuropathy (92).

1.1.3 Reactive Arthritis (Reiter Syndrome)

Reactive arthritis generally refers to arthritis which follows an infectious disease without the joints having been directly affected by the microbe. Despite the breadth and potential ambiguity of the term, it is the currently used and accepted medical term for what had previously been defined as a variety of individual syndromes attributable to infection by a variety of organisms (119). Like GBS, reactive arthritis is believed to be an autoimmune disease (7). The sterile arthritis which follows infection with bacterial pathogens such as *Campylobacter* and *Salmonella* may be accompanied by additional inflammation of other tissues including tendons, skin, the eyes and the genitourinary tract. It is probable that a significant portion of the roughly \$300 billion spent annually in the US on medical costs associated with rheumatic diseases could be saved by preventing the infectious diseases which give rise to them (119). The disease previously referred to as Reiter syndrome was in practice synonymous with today's reactive arthritis, though technically it required conjunctivitis and urethritis in addition to symptoms of arthritis. Reiter syndrome (a term still in use in some medical practices) is somewhat more common than GBS, likely affecting more than 10 times as many individuals (23, 119, 129).

While it is widely agreed that reactive (or postinfectious) arthritis is associated with human leukocyte antigen (HLA)-B27, much else, including diagnostic criteria, is debated. Because of these disagreements, it is difficult to

properly assess the incidence of the disease or its relationship to individual pathogens. Additionally, no outbreak investigations within the US have focused on the connection between *Campylobacter* infection and reactive arthritis. That said, a Finnish study reported that 39% of patients experienced some form of musculoskeletal symptom following *Campylobacter* infection, and 4% experienced diagnosable reactive arthritis (107). Additionally, a Danish study found that patients were substantially more likely to experience reactive arthritis following *Campylobacter* infection than enterotoxigenic *Escherichia coli* infection (16% vs. 6%) (80).

1.1.4 Vehicles

Campylobacter is generally considered to be transmitted via the fecal-oral route. The organism may be found in the feces of humans and many common animals including poultry and birds, cattle, domestic animals such as dogs, and pests such as rodents and flies (14). Direct contact with these animals' feces or consumption of contaminated food and water may contribute to infection and disease in humans (32). The majority of human illness caused by *Campylobacter* is sporadic rather than outbreak associated. In 2005 and 2006, only 1.2% and 0.7% of European campylobacteriosis was outbreak associated (93). Within the US, the four year period between 1998 and 2002 saw only 61 *Campylobacter* outbreaks (disease in two or more people originating at a common source) sickening 1,440

individuals as compared to 585 *Salmonella* outbreaks totaling 16,821 illnesses in the same period (81). The direct cause of *Campylobacter* infection for most cases is not identified (14, 93). Based on identified sources and sampling of potential sources, the primary risk factor for campylobacteriosis is consumption of poultry and poultry products, with lesser risk factors including consumption of unpasteurized milk, contaminated water and dirt (potentially while involved in recreational activities), and contact with infected humans and pets. Among 2005 and 2006 cases of campylobacteriosis in which a source could be identified, nearly a third were directly related to consumption of chicken (93). One study used multi locus sequence typing to draw the conclusion that 97% of sporadic *Campylobacter* infections were attributable to contaminated meat (130) and half of all cases of human infection are attributable to chicken (109, 130).

1.1.5 Poultry Colonization

Not all poultry flocks become colonized with *Campylobacter jejuni*. While no one factor is responsible for introduction of the bacterium into a flock, much effort has gone into investigating the most common route of introduction into poultry houses. Contaminated feed and litter have been found in several studies of colonized flocks, however it appears that these play a role in transmission of *Campylobacter* through a broiler house rather than initial infection (19, 63, 91). Contaminated water (a source of human campylobacteriosis) has not been found

in all studies of colonized flocks and thus may be a contributing, but not the sole factor, in introduction of the organism, despite the ability of *Campylobacter* to enter a viable but non-culturable (VBNC) state and integrate into biofilms (19, 55, 74, 91). Like feed and litter, other studies have found that water becomes contaminated only after the flock is positive, though chlorination of the water supply may be a valuable tool in slowing dissemination of the organism within the flock (9, 42, 67).

Other avian species including wild birds are capable of functioning as reservoirs of *Campylobacter*, as are mammalian species including dogs, cats, cattle and sheep (4, 108). While these are generally not directly present in a poultry production environment, feces and the organisms present within it can easily be transferred from fomites such as workers' shoes. Additionally, pests such as rodents and flies, which may easily gain access to the interior of poultry houses, are also known reservoirs of the bacterium (14). In support of these theories of initial infection, strains from mammals have been shown to infect poultry (136) and wild animal genotypes have been shown to match those of strains isolated from poultry houses (91, 115). A recent study of multiple potential sources of *Campylobacter* found the most likely reservoir in the farm environment to be other animals, and also found colonization of broilers to be with the same strain present in cattle before the broilers were placed (42).

The study of the dynamics of flock colonization is further complicated by the diversity of chicken rearing operations. The size, layout, and management practices of individual operations are important factors to consider, but a more important variable is probably the genetics of the host – in this case, the chicken. Differences in host susceptibility have been considered a factor in human infectious dose, and have been established in studies of poultry (15, 114). In a 2011 study, two commercial broiler lines available in the US were inoculated with 1.8×10^5 CFU/bird, however only 27.5% of one line were colonized as compared to 70% of the second line (75).

A generally accepted series of events in the colonization of an initially negative broiler flock is sporadic colonization of a few birds at 10-21 days. This is followed by the spread of the organism to other birds in the house, by feed, water, litter and direct contact with feces. Some birds may remain negative, and spread of the organism throughout the house may take several weeks. Multiple strains of *Campylobacter* may be present, and non-poultry strains will tend to be replaced by poultry strains over time, if they are present (31). The later a bird is initially colonized, the shorter the duration of shedding, however, chickens have been shown to shed *Campylobacter* for up to 42 weeks, well past the seven week average lifespan of a production broiler (66, 78). Much is still unknown about *Campylobacter's* interaction with the avian immune system, however, antibody production may play a role in the reduction or elimination of *Campylobacter* over

the natural lifespan of a chicken. Unfortunately, this natural response also does not occur in a relevant timeframe. This response to *Campylobacter* appears to play a role in the reduced shedding of chickens colonized at an older age, and resistance to initial colonization through the use of maternal antibody (39, 85, 104). In contrast it has also been recently reported that the chicken's mucosal immune response may lead to tolerance of the organism and persistent colonization (60).

Though up to 90% of flocks in both the US and Europe become positive (39, 91, 113), it is important to remember that meat from flocks with no *Campylobacter* may still become contaminated by the time it reaches the consumer. High level shedding of *Campylobacter* at the time of slaughter is a critical step in the contamination of carcasses, but also the cross contamination of poultry from negative flocks at processing (64, 96). Mathematical modeling has suggested that with low levels of initial *Campylobacter* contamination on carcasses at the beginning of processing, post processing prevalence will not increase. However, high level, low prevalence initial contamination results in nearly 100% prevalence at post processing (58), underscoring the importance of reducing the amount of *Campylobacter* entering processing facilities.

1.1.6 Current Intervention Strategies and their Effect

Interventions and prevention strategies designed to reduce illness caused by *Campylobacter* in the consumer can be divided into five main approaches: 1) those designed to reduce the number of colonized flocks, 2) those that attempt to reduce the amount of bacteria in colonized flocks, 3) those that aim for reduction of cross contamination in processing, 4) screening of processed meats for lower risk applications such as cooked products, and 5) those that target consumer behaviors directly (125). Because it is difficult to control consumer behavior, it is critical that the level of microbial contamination at retail sale of food products be as low as possible.

In response to the long standing association between *Salmonella enterica* and eggs, the Food and Drug Administration (FDA) announced in 2009 a plan to reduce contamination of US eggs not destined for cooking or pasteurization (1). Many of the regulations are *Salmonella* specific; however, under the plan producers must also take measures to reduce hen colonization, including rodent and pest control, biosecurity measures, and house disinfection. While this legislation in and of itself will likely have little effect on rates of campylobacteriosis because it specifically applies to laying hens, the success of these measures may inform future decisions on similar legislation for *Campylobacter* in broilers. Current biosecurity and disinfection measures for *Campylobacter* in broiler houses have been found to be insufficient (90, 100).

Additional farm level strategies have also been considered, including probiotic and prebiotic interventions which aim to reduce colonization by competitive exclusion. To date, these have shown little promise for the reduction of *Campylobacter* levels (125).

Control measures at the level of processing are currently considered to be the most important in terms of impact on the level of contamination on broiler meat (58). Poultry processing Hazard Analysis and Critical Control Points (HACCP), a system of controlling a variety of hazards including biological contamination at points which are likely to pose problems, is required in all Food Safety and Inspection Service (FSIS) inspected poultry plants (120). These processes are plant specific and dependent on individual variables, but generally poultry are processed using a similar set of steps: poultry are brought into the facility, killed, scalded, defeathered, eviscerated and chilled. Each step, with the exception of defeathering, generally reduces numbers of *Campylobacter* present on the carcass. Unfortunately, because it is a process involving many individuals, a great deal of water and potentially high levels of initial *Campylobacter*, there is a high potential for cross contamination (11, 110). Chlorine increases the efficacy of chill water, though this step is not permitted in some countries (12, 83).

Reports of post chill *Campylobacter* vary greatly, with prevalence reported at 35% (US, 2007) 46.4% (Iran, 2010) and 84.7% (Brazil, 2007) (12, 49, 97). These studies are often difficult to compare meaningfully because of differences in

sampling methodology and physical and temporal locations, but likely vary naturally due to factors such as flock colonization levels and order of processing. Because water based chilling procedures have been thought to increase cross contamination and promote uptake of water which is unsatisfactory to the consumer and regulatory agencies, but chilling is such an important microbiological control step, an alternative air chill system has been introduced in some processing plants (133). While air chilling does appear to produce a consistently more attractive retail product, reports are divided on whether it improves or worsens the situation with regard to cross contamination and final *Campylobacter* levels (6, 13, 105).

Commercial chicken processing facilities are designed with pathogen reduction in mind though reports vary greatly on the success of implemented intervention strategies where *Campylobacter* is concerned. Recent estimates indicate that high levels of *Campylobacter* can be found on market chicken. A 1999 study in Spain found a prevalence of 49.5%, while 70.2% of retail samples in the UK were found to be positive in 2011, and studies in the US have found levels as high as 70.7% and 77% (34, 40, 43, 134). Retail prevalence often appears to be higher than levels found at the end of processing, potentially indicating further contamination during packaging or transportation. Though many attempts have been made to improve poultry raising and processing practices, and many new and unproven strategies are being investigated in an effort to reduce levels of

bacteria to acceptable levels, few have met with great success and those that have are not practical for large or cost competitive industry (65, 125). Because of this, interventions which reduce initial flock colonization and colonization levels once again achieve greater importance.

1.1.7 Vaccination

Given what is known about the chickens' antibody response to *Campylobacter*, vaccination is in many ways an obvious choice for the reduction of the pathogen before it has had a chance to enter production facilities or the consumer environment. Certainly, attempts have been made to design an effective poultry vaccine against *Campylobacter*, but to date there have been no marketable successes. A truly successful vaccine would need to exhibit a number of difficult to attain features simultaneously, including safety in both humans and animals, early protection against colonization which will take over where maternal immunity leaves off, protection against multiple strains and ideally species of *Campylobacter*, and finally a cost low enough to be attractive to large scale production industry (39). Killed whole cell vaccines have shown limited or no reduction of colonization despite multiple modifications and attempts, and pose a potential health risk to humans who could potentially develop GBS in reaction to components of the whole cell (39, 99). Administration of mutated non-

colonizing strains of *Campylobacter* has also failed to provide protection against later colonization with wild type strains (135).

Outer membrane proteins (OMPs) have previously been used as antigens for successful vaccination against Gram-negative bacteria and so potentially antigenic OMPs of *Campylobacter* which may prove useful in this way have been investigated (77). Most notably, flagellin, which is both well characterized and known to be important to colonization, has been targeted through several strategies with some success against homologous strains (68, 127, 128). These vaccines, however, give limited protection against heterologous strains because of glycosylation as well as phase and antigenic variation (77, 125). Additional OMPs known to be important to colonization in birds remain promising future targets.

Arguably the most promising results have come from expression of *Campylobacter* proteins by attenuated *Salmonella* vectors. The first greatly successful example of this was the use of a putative ABC transporter protein, CjaA, to reduce cecal colonization of broilers by six logs in nearly all tested birds (132). More recently, the use of CjaD/Omp18 expressed on the surface of a *Salmonella* vector reduced colonization of the ileum to undetectable levels in broilers (72). Other attempts to use *Salmonella* vectored *Campylobacter* antigens have also had some success (18), though issues remain which stand in the way of commercialization of these vaccines. Most notably, previous studies have

utilized genes which are not novel, their studies are not reproducible or did not use adequate controls, or the expression plasmids utilized require antibiotic selection which is not appropriate for use in the poultry production industry.

1.2 *Salmonella*

Salmonella is a genus of facultative anaerobic Gram-negative bacilli. It is a member of the class γ -proteobacteria and the family *Enterobacteriaceae*. Taxonomy of *Salmonella* has historically been heavily debated, though more recently a substantial effort has been made to clarify nomenclature for researchers and physicians. Currently, the genus is divided into two species: *S. bongori* and *S. enterica*, the latter of which is further divided into subspecies either by name or roman numeral (*enterica* I, *salamae* II, *arizonae* IIIa, *diarizonae* IIIb, *houtenae* IV, and *indica* VI – with *bongori* as former subspecies V) (61, 116). A third species, *S. subterranea*, has been proposed, but it is not yet clear whether it represents a third species of *Salmonella* or would be better placed in a closely related genus such as *Escherichia* or *Enterobacter* (129). Salmonellae are further classified using the Kauffman-White scheme which allows recognition of more than 2,500 serovars based on H (flagellar) and O (somatic lipopolysaccharide) antigens. Additional serovars are recognized each year (17, 129). A capsular polysaccharide antigen associated with virulence (Vi) may also be present, as on *S. Typhi* and *S. Dublin*, among others (61). Thus, *S. Newport* represents the full classification *Salmonella enterica* subsp. *enterica* serovar Newport.

Salmonellosis is a reportable illness in the US, and an isolate from any laboratory confirmed case is sent to the appropriate state laboratory for serotyping and analysis by pulsed field gel electrophoresis (PFGE) (52). Because

it is the standard for comparison of clinical isolates, and because the CDC maintains a national database of these profiles (PulseNet), PFGE is the most common genomic comparison tool for *Salmonella* isolates. It has the additional benefit of providing more information about true genetic diversity than serotyping alone; however substantial time and therefore cost is involved in the technique. Alternative genomic comparisons include Restriction Fragment Length Polymorphism (RFLP) analysis or Multilocus Sequence Typing (MLST) and, as it becomes less expensive, whole genome sequencing. Each of these techniques has both applications to which they are best suited and individual drawbacks.

1.2.1 Clinical Significance

S. enterica subsp. *enterica* serovars are responsible for the overwhelming majority of disease in warm blooded animals, though this disease varies greatly in severity and other serotypes may cause illness (129). While nearly 600 of the known serotypes have been found to cause disease in the US (1), only four to five are generally responsible for half the human infections in a given year - *S. Typhimurium*, *S. Enteritidis*, *S. Newport*, *S. Javiana*, and *S. Heidelberg* in 2007 (29). In 2010, the CDC's Foodborne Diseases Active Surveillance Network (FoodNet) reported 2,290 hospitalizations and 29 deaths attributable to *Salmonella*. They additionally reported that rates of infection caused by *Salmonella* have not declined significantly in more than ten years, leading to direct medical

expenditures of \$365 million each year (3). Because of the self-limiting nature of most cases of salmonellosis, it is estimated that for every laboratory confirmed case 29 to 35 people have actually become ill (30) with 1.4 million cases of human diarrheal illness being thought to occur annually in the US (84). Exact global totals are impossible to calculate, however *Salmonella* infection is thought to be responsible for hundreds of millions of cases of illness and hundreds of thousands of deaths globally (129). While the much more serious systemic Typhoid fever is comparatively well controlled in the US, it is still responsible for up to 21.5 million illnesses in other parts of the world (36). Untreated, enteric fever, as it is also known, is lethal in up to 30% of cases and is most often caused by human specific serovars *S. Typhi* and *S. Paratyphi A* (56). Similar invasive disease may occur in other species by infection with other host adapted serovars, *S. Gallinarum* in fowl or *S. Dublin* in cattle are examples (21, 46). Longer term consequences may include postinfectious arthritis similar to that described for *Campylobacter* infection.

1.2.2 Vehicles - Poultry

Poultry and poultry products are the most common sources of infection in large part because *Salmonella enterica* is a commensal microorganism in birds. *Salmonella* does not generally affect the health, growth, or profitability of poultry species and therefore may be overlooked because clinical signs are absent or not

obvious and production is not impacted (59). Artificial infection with some strains may produce some clinical symptoms such as apparent depression, anorexia or reduced egg laying (51). Day-old chicks can be colonized by as few as five *Salmonella* cells, in part because of a lack of competing normal flora in the gut at that age. This makes it unsurprising that environmental contamination of an area where baby chicks are to be raised is a substantial risk factor for their eventual colonization (103). Colonization of poultry is arguably the most important factor in later contamination of poultry meats and eggs, and therefore rates of human infection.

The most common source of *Salmonella* in foodborne infection following eggs is fecal contamination of animal carcasses at processing (47). *Salmonella* strains capable of strong biofilm formation are generally more resistant to killing by macrophages and antibiotics, as are all bacteria protected by the EPS of a biofilm community, but they have also been shown to be more resistant to killing by acetic acid, a common cleaning agent in industrial applications such as abattoirs (57). This may increase the ability of fecal *Salmonella* to survive in processing environments.

1.2.3 Vehicles - Cattle

Cattle are a major reservoir for *Salmonella*, potentially responsible for the introduction of the organism into the food chain at several points (41, 45). A

study examining presence of both *Salmonella* and Shiga toxin producing strains of *E. coli* found seasonal variation in the levels of both pathogens (10). This has also been found in other studies and has sometimes been thought to relate to increased shedding of the organisms in response to the animals' stress, increased by higher summer temperatures. Overall, 4.4% of fecal samples were positive for *Salmonella*, but a staggering 71% of hides were positive, and 12.7% of carcasses were positive following dehiding but before intervention or evisceration (12). This illustrates the danger of hide contamination, a relatively small number of shedding animals can introduce the bacteria onto the hides of other cattle by directly defecating on them in close quarters or by contaminating the living environment of the other animals. In this study it was found that standard interventions significantly reduced contamination by the end of processing to 0.01% for *Salmonella* (10). These results are not inconsistent with the majority of other studies, several of which have reported contamination of 0.1 to 1% of post intervention carcasses (102, 121). Dry aging of beef (more common than wet, gaining popularity in industry because of cost considerations) significantly reduces contamination of carcasses with *Salmonella* (69).

Between 1998 and 2007 the USDA-FSIS found only between 0.76% and 2.44% of cows and bulls to be positive for *Salmonella*, though one very recent study found a *Salmonella* prevalence of 72.6% in pre-harvest feedlot cattle (98). The USDA also found that even with a lower prevalence during that time period,

up to 6.4% of ground beef samples were contaminated (122). Grinding of beef increases risk of all foodborne diseases as it allows the bacteria entry into the center of a meat, which is less likely to reach the necessary temperature to kill microorganisms in the food in real consumer settings. Additionally, it increases the likelihood of multiple animals in a single meal, providing a greater chance for widespread contamination from a single infected carcass. *Salmonella* contamination of ground beef remains a problem despite the continued enforcement of HACCP protocols in beef processing facilities (117).

Though industry interventions in beef appear to be relatively successful in the reduction of *Salmonella*, there remains a risk of introduction of bovine strains from feedlot and dairy cattle into the environment where they may come into contact with produce or aquaculture operations. Another recent study of dairy cattle found that 93 of 831 sample herds contained 576 culture positive cows (of 2,565 tested) (38). As in human cases of salmonellosis, shedding of the organism may continue beyond the resolution of symptoms, in the case of cattle, the median duration of shedding was reported as 50 days, with a maximum shedding period of 391 days (37). These figures are all the more troubling because cattle have been identified as a reservoir for, and possibly a source of, multidrug resistant strains of nontyphoidal *Salmonella* species (53, 123). Use of antimicrobials in livestock production selects for and maintains these strains in dairy and beef cattle (126). One study found that multidrug resistant strains of *S.*

Newport and *S. Typhimurium* were significantly more likely to be of bovine than human origin (62). Multidrug resistant strains of *Salmonella* are associated with increased human morbidity and mortality and are inherently less treatable with commonly used antibiotics prescribed for serious infections which would not resolve on their own (53, 123, 126).

Bovine feces is used as fertilizer and deliberately spread on edible fruits and vegetables to increase their growth. Fecal material from cows and other species is treated, generally composted, to reduce pathogen load. This method is considered very good and reduces pathogen levels more than four logs, with some difference in bacterial species reductions (33, 35). This process may not completely eliminate high levels of bacteria in manure which is then introduced directly to produce likely to be consumed raw. Perhaps more importantly, run off from beef and dairy production areas remains completely untreated and may enter irrigation or aquaculture areas necessitating a greater understanding of the interactions of *Salmonella* in these production environments.

Salmonella may cause gastrointestinal disease in cattle as well as humans and reducing levels of the pathogen in feedlot cattle is important to production as well as human and animal health (26, 84, 98). Pre-harvest interventions are therefore critical to prevention of cattle morbidity and mortality, reduction of *Salmonella* amplification and reintroduction to the environment through the cattle host, and contamination of hides and ultimately beef products responsible for

illness in the consumer (98). A complete understanding of the sources of *Salmonella* in the feedlot environment is important to maximizing the effectiveness of these strategies.

1.2.4 Vehicles - Oysters

Bovine and wild animal feces wash into local streams and channels following rainfall events, leading to contamination of waterways with unprocessed fecal bacteria. Additional fecal bacteria may be present from inadequately maintained septic systems. These waterways, in some areas, empty into larger tributaries and potentially into areas used to raise fish and seafoods or into irrigation water supplies. Filter feeding marine organisms such as oysters may become heavily contaminated as they concentrate particulate material including bacteria from the water.

Salmonella has been found in marine and fresh water worldwide. Water from lagoons in Mexico, which are used for oyster production, was found to be positive for *Salmonella* in 86.7-100% of samples taken over a two year period (27). 30% of water samples and 10% of oyster samples taken off the coast of Brazil in 1998-1999 were found to be positive for *Salmonella*. As an example of fresh water presence, 79.2% of samples taken from the Little River in Georgia were also positive for the pathogen (54). A Spanish study isolated *Salmonella* from sea, river and reservoir samples, finding seawater to be somewhat more commonly

contaminated (94). Presence of *Salmonella* in coastal waters has sometimes been associated with warmer temperatures, however *Salmonella* has been isolated from North Sea water and sediment samples and has been shown to survive as well as enteric viruses in cold water (118, 124).

Two oyster species, *Crassostrea gigas* and *C. virginica* make up the majority of oyster production in the US (2). Though they are generally cultivated on the West and East coasts respectively, both species are widely shipped and commercially available at retailers and restaurants nationwide. All experiments conducted using oysters in this work were performed with *C. gigas*, though both oyster species have been shown to behave similarly in the experimental set up used for the experiments presented here.

Oysters are individually capable of filtering 50 gallons of water daily in an effort to concentrate sufficient particulate food matter. In this process, they are also known to concentrate viral and bacterial pathogens and toxins present in their environments (24). Fecal contaminants may be as much as 62 times higher in an oyster than its surrounding environment (22). More than 400 disease outbreaks have been linked to pathogens as diverse as Hepatitis A Virus, *Vibrio spp.* and *S. Typhi* (101). Up to 8% of total foodborne infections in the US may be attributable to the consumption of oysters (8). *Salmonella* has been found on a number of occasions in oysters and other marine mollusks around the world (16, 82, 131).

Oysters are a particular concern for food safety, as unlike other shellfish they are often consumed raw. Approximately 8% of Americans surveyed routinely eat raw oysters, and about 50% of the more than 69 million pounds of oyster meat eaten in the US is consumed raw (2, 44, 86). Because these animals are frequently eaten raw and *Salmonella* is found within the tissues of the oyster rather than simply on the surface of the prepared food, no interventions aside from prevention of pathogens in the growing environment and post-harvest testing of oyster meat will sufficiently protect consumers.

1.2.4.1 Depuration

Depuration is a practice of the commercial shellfish industry which is intended to reduce or remove low to moderate levels of pathogens from shellfish produced in contaminated growing waters. The process was initially developed as a potential response to large scale outbreaks of disease caused by *Salmonella typhi*. The process involves placing the shellfish in clean seawater, allowing the natural filtering and expulsion of intestinal contents conducted by the animals to separate previously ingested pathogens and toxins from the tissue. This process is known to be variably effective in the reduction of contaminants potentially harmful to the consumer, with marine bacteria such as *Vibrio parahaemolyticus* and *Vibrio vulnificus* showing inconsistent or no reduction (73). A variety of factors influence the effectiveness of the process itself, including duration of

treatment, handling of the shellfish pre-depuration, and the design and implementation of the system. For this reason, the Food and Agriculture Organization of the United Nations (FAO) has developed guidelines for the establishment of a maximally effective depuration system. FAO guidelines were used as a basis for the depuration system used in this research.

1.2.5 Use as a Vaccine Vector

Salmonella is an attractive choice as a potential vaccine vector for a number of reasons. Unlike many current vaccine preparations, the bacterium is stable at room temperature for short periods of time and can easily be lyophilized for longer term storage. Administration of the vaccine may be oral, eliminating the need for sterile individual hypodermic needles and trained healthcare personnel. In the case of animal vaccines, this allows the dosing of entire flocks rather than individual animals, which is critical in large farming operations. Production of the vaccine primarily involves growth of the organism, which is relatively simple and inexpensive (71). As a pathogen, *Salmonella* has the advantage of increased immunogenicity over bacterial strains which are entirely commensal, however this means that in order to be safely administered vaccine strains must be attenuated without losing features important to the generation of immune responses by the host (76). Successful attenuation of this type can be accomplished via the deletion of various virulence genes, notably *fur*, *pmi* and

crp. Attenuated strains of *Salmonella* can be specifically engineered to deliver an extraordinary variety of antigens from other pathogens and induce a broad range of host responses, including serum and mucosal antibody production and cell-mediated immunity for both local and distal protection (70). *S. Typhimurium*, in particular, is a good candidate because of its ability to colonize a broad range of hosts, well studied virulence factors, and its relative ease of genetic manipulation using modified *E. coli* techniques (71). A bonus in the use of *Salmonella* vaccines expressing antigens from other pathogens is the potential to gain secondary immunity to *Salmonella* infection.

Plasmids are generally used to carry the foreign antigenic gene to which an immune response is desired. Because antibiotic selection is not a viable way of maintaining plasmids within a vaccinated host, an alternative method has been devised. Plasmids with functional copies of necessary genes may be used to complement chromosomal mutations, leading to maintenance of the extra-chromosomal element within host tissue (50, 71). Further refinements continue to be made to existing vaccine strains, in an effort to make them as safe and antigenic as possible while retaining their other attractive features. One recent advance is the creation of a *Salmonella* strain which produces a version of Lipid A which more closely resembles the form currently approved for use as an adjuvant in human vaccine preparations than the endotoxin normally produced by the bacterium, potentially reducing side effects in humans (70).

Recombinant attenuated *Salmonella* vaccines (RASVs) have yielded promising results on a number of occasions. Protection or partial protection of mice against an extraordinarily diverse array of microorganisms has recently been achieved through the use of RASVs. The recombinant Lipid A producing strain mentioned above, when also expressing a surface protein of the Gram-positive bacterium *Streptococcus pneumoniae* and delivered orally, significantly reduced death rates following challenge with that bacterium (70). An intranasal vaccination against the nematode *Trichinella spiralis* reduced parasite burden by 61.83% at 8 days post challenge (95). Most relevantly, oral vaccination preceding challenge with the Gram-negative bacterium *Helicobacter pylori* significantly reduced colonization (79).

1.3 Hypotheses and Specific Aims of the Present Study

Because of the importance of *Salmonella* and *Campylobacter* as human pathogens and the diversity of vehicles by which each enters the food supply, this work is concerned with a variety of specific aims which share the common goal of aiding in the reduction of these pathogens in various foods.

Previous work in our laboratory has indicated that the *Campylobacter jejuni* genes *CjLAJ1* and *CjLAJ2* are potential virulence factors important to the colonization of poultry. Colonization studies using a *CjLAJ2* defective mutant support the role of this gene in broiler colonization. Thirty-seven of 40 birds were colonized with the parent strain, *C. jejuni* M129 at an average of 7.35×10^5 CFU/g of feces, whereas 0 of 39 birds (<10 CFUs) were colonized with the *C. jejuni* mutant strain $\Delta CjLAJ2$. Therefore it was hypothesized that expression of these genes by an attenuated *Salmonella* vaccine vector could potentially reduce colonization of boiler chickens when administered orally. Given this information, the aims of the present study were:

- Generate recombinant attenuated *Salmonella* vectors (RASVs) containing the *Campylobacter jejuni* genes *CjLAJ1* and *CjLAJ2*.
- Use these RASVs to vaccinate broiler chicks and assess their ability to provide protection against colonization of the homologous strain *Campylobacter jejuni* NCTC 11168 and, in the case of *CjLAJ2*, against a heterologous clinical isolate.

Salmonella is routinely isolated from cattle, where it is an economic burden to farmers and a health concern for both animals and humans. The initial source and transmission of *Salmonella* in feedlot environments is not fully understood. In an effort to gain additional information on *Salmonella* in a small, closed feedlot environment, the aims of the present study were the following:

- Assay potential sources of *Salmonella* in the feedlot environment for presence of the organism.
- Test cattle for *Salmonella* shedding prior to arrival at the feedlot, following transportation to the feedlot, throughout their time there and immediately before processing.
- Use multiple sample sites to determine the most appropriate method for assessing the presence of *Salmonella* on beef carcasses.
- Serotype and determine antimicrobial susceptibility of all isolates. Use this information to gather information about transmission within the feedlot if possible.

Finally, *Salmonella* has been found in oysters, filter feeding marine bivalve mollusks which are frequently consumed raw. A previous study found an overall *Salmonella* prevalence in oysters of more than 7%, the majority of which was *S. Newport* of a single PFGE profile (LAJ160311). In order to determine whether this particular strain has a special fitness in oysters or their environment

and to determine whether it continues to be found in oysters several years after the initial survey, the aims of the present study were:

- Determine whether *S. Newport* LAJ160311 is vertically transmissible in oysters.
- Inoculate fresh and brackish water samples (obtained from an oyster growing area and an upstream tributary) with three isolates of *Salmonella Newport*: TEB9356 a human clinical isolate, TEB3082 a bovine isolate, and LAJ160311 as well as *E. coli* ATCC 25922 representing a typical fecal coliform. Monitor the survival of each strain to determine relevant environments in which the oyster isolate may have increased fitness.
- Use the above strains to inoculate oysters which are then subjected to depuration conditions in order to assess increased fitness of the oyster strain under these conditions and differences in *Salmonella* and *E. coli* reduction during this treatment.
- Sample oysters, clams, and water from areas previously found to be positive for LAJ160311 in oysters over a period of three years in order to determine if this strain remains in the environment and potentially identify factors associated with *Salmonella* contamination of the estuarine environment.

CHAPTER 2: PRESENT STUDY

All pertinent methods, results and conclusions of the appendices are briefly presented here. Detailed information is presented in each appendix.

Dissertation format

This dissertation contains two main sections 1) an introductory section in which the problem is defined, research objectives stated and a comprehensive literature review provided and 2) four appendices describing details of materials and methods, results, and conclusions, all in manuscript format. The dissertation was written and prepared by the degree candidate, Alexandra E. Armstrong. Included manuscripts were reviewed by coauthors. In the case of Appendix C, of which Ms. Armstrong is not the first author, a section is included in Chapter 2 detailing her contribution to the research presented.

2.1 Summary of Appendix A

Appendix A presents the investigation of the potential use of two *Campylobacter jejuni* genes as potential antigens in a recombinant attenuated *Salmonella* vaccine intended for use in broiler chickens.

Vaccines were constructed by cloning two genes: *CjLAJ1*, a putative periplasmic protein, and *CjLAJ2*, a protein of unknown function with partial homology to a hemolysin of *Brachyspira hyodysenteriae* into the expression vector pYA3493. This plasmid was amplified in *E. coli* and electroporated into the recombinant attenuated *Salmonella* Typhimurium strain χ^{9992} .

Vaccine strains were grown overnight and administered to Cornish X Rock broiler chicks at 10 and 16 days post hatch. Challenge with the homologous *C. jejuni* strain NCTC 11168 or a heterologous clinical isolate M129 followed at day 26. Animals were sacrificed at day 36 and cecal contents were assayed for *C. jejuni* levels.

In all cases, vaccination reduced cecal colonization at day 36. The *Salmonella* vaccine was detected in broiler feces at days one and three, but was no longer present at day seven post vaccination. Vaccination with χ^{9992} pLAJ1 reduced cecal colonization with *C. jejuni* NCTC 11168 by 2.5 logs, while χ^{9992} pLAJ2 performed similarly with an overall two log reduction across three replicates. No significant differences were observed between unvaccinated control groups and those which received the Empty Vector vaccine (χ^{9992} pYA3493). Vaccination with χ^{9992} pLAJ2 reduced colonization by

C. jejuni M129 two logs as compared to unvaccinated controls and nearly four logs as compared to Empty Vector vaccinates in a single trial.

These results indicate that *CjLAJ1* and *CjLAJ1* hold promise for the vaccination of poultry to reduce colonization with *C. jejuni*, ultimately reducing the risk of introduction of this pathogen into the poultry production system and potentially reducing cases of human illness.

2.2 Summary of Appendix B

Appendix B presents a longitudinal study of *Salmonella* spp. present in a feedlot environment, including wild birds and flies and a cohort of 36 cattle at the range, that feedlot, and finally processing.

Each of 36 steers in the cohort were individually assayed for the presence of *Salmonella* prior to their arrival at the feedlot, at arrival, and periodically until processing. Samples were also taken within the processing plant and after dry aging of the meat. Within the feedlot environment, wild birds, flies, feed, water, feed bunks and drag swabs were assayed for the presence of *Salmonella* both before the arrival of the cattle and periodically while they were present. *Salmonella* serotypes, PFGE profiles and antibiotic resistance patterns were analyzed to provide insight into the spread of *Salmonella* within the feedlot environment and the acquisition of this organism by cattle in such an environment. An additional cohort of nine steers were assayed only at the range and following transportation to a large feedlot in Kansas, US.

Although *Salmonella* was detected in the feedlot environment prior to the arrival of cattle it could not be definitively determined that cattle were infected in this environment and not at an earlier point such as the range or transport vehicle. *Salmonella* was only detected in cattle feces at arrival to either the Arizona (17%) or Kansas feedlots (100%) indicating that the stress of transportation is likely a factor in the shedding of this organism.

Salmonella was also detected from three carcass swabs taken from two animals, but not from the feces of these animals immediately prior to transportation to slaughter. The *S. Alachua* serotype isolated from the carcass swipes of one animal had an antimicrobial resistance profile which matched the isolate recovered from that animal's feces at arrival, but not the profile isolated from drag swabs taken in that pen. This further supports the idea that transport stress is a factor in shedding. No *Salmonella* was isolated from carcasses or ground beef following dry aging. While few carcasses were contaminated during processing, this data none the less supports previous findings that dry aging reduces *Salmonella* on beef carcasses.

S. Alachua, the predominant strain isolated in Arizona, is uncommon and has not previously been isolated from cattle. All but one of these isolates and the majority of other serotypes recovered in the study (92%) were resistant to at least one tested antimicrobial supporting the previous finding that feedlots may be reservoirs of drug resistant strains of *Salmonella*.

2.3 Summary of Appendix C

Appendix C presents the results of an investigation into the prevalence of *Salmonella enterica* serovar Newport in oysters as found by a previous study (16). The only sections of this appendix discussed here are those to which Ms. Armstrong was the primary contributor to the research.

A 2003 study by Brands, et al. found an overall prevalence of 7.4% for *Salmonella* in market oysters from bays across the US. Curiously, this study found that the majority (77%) of *Salmonella* isolates from oysters were *S. Newport* and nearly all (98%) of those were of a single PFGE type (LAJ160311). They also found that coliform levels were not predictive of *Salmonella* presence. The work presented in Appendix C seeks to determine whether the consistent isolation of this PFGE type is related to an increased fitness of that strain in either the oyster or the estuarine environment in which oysters are cultured.

Initially the enrichment process used in (16) was confirmed not to be artificially selecting for LAJ160311 over other genotypes of *S. Newport*. Each enrichment and plating media was seeded with LAJ160311 and TEB9356 a human clinical isolate or TEB3082 a bovine isolate. Media were placed in appropriate growth conditions for the length of time specified in Brands' methods. Total and individual strain plate counts were obtained by utilizing the varied antibiotic resistance profiles of the strains. No significant differences were found between the growth of any of the strains in any of the tested media.

In order to assess any particular fitness of LAJ160311 to survive in fresh or brackish water (upstream of or in oyster growing waters), water samples were obtained from an environment in which *Salmonella* was previously isolated from oysters. Water samples were divided into aerated containers and inoculated with cultures of human, bovine, or oyster isolates of *Salmonella* Newport or *E. coli* ATCC 25922, with all strains having an initial concentration between 1.0×10^7 and 5.1×10^7 CFU/mL for the river water experiments and between 1.8×10^6 and 2.5×10^6 CFU/mL for the bay water experiments. Negative controls remained uninoculated. Samples were taken, filtered, and enumerated at three day intervals to day 12. No differences were observed between strains of *S. Newport*, all of which survived significantly longer than *E. coli*.

In order to test the possibility that *Salmonella* could be transmitted to oyster larvae by vertical transmission following chronic or acute *Salmonella* infection, oysters were exposed to *Salmonella* LAJ160311 at 31 and 3 days prior to the induction of spawning by temperature fluctuation. Female gametes and adult oyster tissues were assayed for levels of *Salmonella*. Adult tissues contained 5.6×10^4 CFU/g *Salmonella*; gametes were negative.

To assess the ability of depuration to reduce levels of LAJ160311 and additional genotypes of *S. Newport* as compared to *E. coli* ATCC 25922, oysters were exposed to the relevant bacteria for 24 hours and placed in 20L flow through tanks. The flow rate of 120L/h was maintained over three days, with samples taken daily. Again, no significant

differences were observed between genotypes of *S. Newport*, though all were depurated less quickly than *E. coli*.

The conclusions of this research are: 1) *S. Newport* LAJ160311 does not have a growth advantage over other strains of *S. Newport* in the enrichment procedure used by Brands, et al. 2) *S. Newport* LAJ160311 does not possess any particular fitness in fresh or brackish water which would allow it to infect oysters at a higher rate than other *S. Newport* strains. 3) Vertical transmission of *S. Newport* LAJ160311 does not occur in oysters. 4) *S. Newport* LAJ160311 does not differ from other strains of *S. Newport* in rate of clearance from oysters in depuration conditions; however *S. Newport* appears to remain in oyster tissue under these conditions longer than indicator organisms such as a representative *E. coli*. Together this information suggests that *S. Newport* LAJ160311 does not have a special fitness in the aquatic environment or oysters, though the question of why this strain was so commonly isolated from these environments remains unanswered.

2.4 Summary of Appendix D

Appendix D presents the results of a two year surveillance of *Salmonella* in oysters and additional environmental samples from two previously positive bays identified in the 2003 Brands study (16), in Oregon, US.

Oyster (*Crassostrea gigas*) samples were obtained from two bays over a period of three years. Samples were obtained at least bi-monthly over the entire period from a bay in which sentinel oysters were placed, and as available from a second previously positive bay's already present oyster population. Clams (*Mya arenaria*) were used to investigate the presence of lower salinity waters upstream of oyster growing areas. Water samples were also obtained from oyster growing areas, and regions up and downstream of these areas. All samples were assayed for *Salmonella* within 48 hours of collection. *Salmonella* isolates were confirmed by PCR and characterized using Pulsed Field Gel Electrophoresis (PFGE) and antimicrobial resistance testing.

Only a single clam sample was positive and oyster samples were positive only sporadically. Three serovars of *Salmonella* were isolated from the various samples, Newport, I 4,[5],12:i:-, and O3A. The first two of these serovars of significant clinical importance as common causes of human salmonellosis (3). PFGE and antimicrobial resistance profiles were serovar specific. The Newport serovar was consistent with the serovar previously isolated in these bays and nationwide in the 2003 study.

These results indicate the persistence of a reservoir for the *S.* Newport strain so commonly isolated from oysters, which is responsible for sporadic contamination of

oyster growing waters. This reservoir remains to be identified, but is suspected to be cattle as they are both present near the oyster culture areas tested and have been found to be a reservoir for multidrug resistant *S. Newport* in previous surveys of dairy and feedlot cattle (5, 48). The origin of the sporadic occurrence of the other two serovars is not known and the relationship of these two serovars with oysters is less well characterized. Unlike previous studies which have found links between weather and rainfall events and sporadic *Salmonella* contamination, no major storm or rainfall events occurred immediately before or at time the positive samples were collected.

Importantly, *Salmonella* was not detected in water samples taken before or at the time of oyster collection. This supports the hypothesis that filter feeding animals like oysters can concentrate low levels of *Salmonella*, increasing their risk as a food source. This also suggests that in order to establish and maintain the safety of this food supply, oysters destined for raw consumption should periodically be tested for *Salmonella*.

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APPENDIX A: ORAL ADMINISTRATION OF A *SALMONELLA* VECTOR BASED VACCINE EXPRESSING A PUTATIVE PERIPLASMIC PROTEIN OR A PUTATIVE HEMOLYSIN FROM *CAMPYLOBACTER* REDUCES *C. JEJUNI* COLONIZATION IN BROILER CHICKENS

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Abstract

The most substantial risk factor for campylobacteriosis, a disease which sickens an estimated 2.4 million people annually in the US at a cost of more than \$3 billion, is the handling and consumption of raw and undercooked poultry. Based on mathematical modeling it is believed that even a modest reduction of *Campylobacter* in broilers will reduce human disease substantially. Current interventions and management strategies have proven themselves inadequate in the management of this pathogen. To this end, we examine the ability of two novel *C. jejuni* genes (*CjLAJ1* and *CjLAJ2*) to provide protection when administered via attenuated *Salmonella* Typhimurium vectors prior to challenge with *C. jejuni*. Cornish X rock chicks were vaccinated at 10 and 16 days post hatch, 10 days prior to challenge with the homologous *C. jejuni* strain NCTC 11168 or a heterologous clinical isolate M129. In all cases vaccination reduced cecal colonization at day 36.

Introduction

In addition to being a leading cause of diarrheal illness globally, *Campylobacter jejuni* causes an estimated 2.4 million illnesses annually in the US, at a cost of more than \$3 billion (5, 27). In addition to being the most common foodborne infection in the US, it is also responsible for more than 13,000 hospitalizations and 100 deaths annually (20). While the majority of these

illnesses resolve without treatment, serious and long term complications including bacteremia, meningitis, Guillain-Barre syndrome and reactive arthritis may occur (1, 21). The infectious dose for campylobacteriosis may be lower than 500 organisms, making cross contamination in food preparation and processing environments a serious risk to consumers (30). The primary risk factor for campylobacteriosis is the handling or consumption of uncooked or undercooked poultry products, in particular chicken.

Campylobacter colonization is of particular concern because once residence in the gut is established the organism may be shed in avian feces at levels higher than 10^9 CFU/ml (23). Chickens generally show no signs of colonization, and production traits such as growth are not affected (8). Up to 90% of flocks in both the US and Europe become positive, leading to cross contamination of carcasses from negative flocks during processing (7, 12, 23, 28). Currently, processing level interventions are said to be the most important in terms of impact on the final level of contamination on broiler meat (11). The prevalence of *Campylobacter* on retail poultry meats, however, is a strong argument that current control strategies at all levels are inadequate to protect the consumer. A 1999 study in Spain found a retail prevalence of 49.5%, while 70.2% of retail samples in the UK were found to be positive in 2011 and studies in the US have found levels as high as 70.7% and 77% (6, 9, 10, 33).

Mathematical modeling has suggested that with low levels of initial *Campylobacter* contamination on carcasses at the beginning of processing, post processing prevalence will not increase. However, high level, low prevalence initial contamination results in nearly 100% prevalence at post processing (11). Additionally, a two log reduction of *Campylobacter* on processed chicken would reduce the incidence of campylobacteriosis in consumers by a factor of 30 (25). This underscores the importance of pre-processing preventative measures; however recent studies indicate that biosecurity remains inadequate despite its continued use and prebiotic or probiotic interventions which have been proposed show little promise (22, 24, 29).

Vaccination has been proposed on several occasions as a means of reducing both flock colonization and the level of shedding in individual broilers. A number of approaches have been reviewed elsewhere, and it has been found that killed whole cell, flagellin subunit and mutated non-colonizing strain vaccines, among others, have provided limited protection against colonization by wild type strains (7, 17). Arguably the most promising results have come from expression of *Campylobacter* proteins by recombinant attenuated *Salmonella* vectors (RASVs). These vaccines have the benefit of low production cost, animal and human safety through attenuation, uncomplicated storage and transport, and potentially high efficacy (14). A greatly successful example of this approach was the use of a putative ABC transporter protein, CjaA, to reduce cecal

colonization of broilers by six logs in nearly all tested birds (31). More recently, the use of CjaD/Omp18 expressed on the surface of a *Salmonella* vector reduced colonization of the ileum to undetectable levels in broilers (15). Other attempts to use *Salmonella* vectored *Campylobacter* antigens have also had some success (4), though issues remain which stand in the way of commercialization of these vaccines, such as the presence of antibiotic markers and the use of genes that are not novel, a critical step in the actual reduction of *Campylobacter* in market chicken.

In this study, broiler chicks were vaccinated with an attenuated *Salmonella* Typhimurium vaccine into which one of two novel *Campylobacter jejuni* genes thought to be important to virulence were cloned. Chicks were challenged with a homologous or heterologous strain of *Campylobacter* to assess protection from colonization.

Materials and Methods

Cultivation of bacterial strains. A list of bacterial strains and plasmids can be found in Table A1. *C. jejuni* strains NCTC 11168 and M129 were routinely cultured on Mueller Hinton (MH) agar (BD, Sparks, MD) supplemented with 5% citrated bovine blood under microaerophilic conditions at 42°C. *Escherichia coli* χ 6212 pYA3493 was maintained on Luria Bertani (LB) agar (BD) supplemented with 0.1% glucose (LBG). *E. coli* χ 6212 without plasmids was grown on LBG agar

supplemented with 50µg/ml diaminopimelic acid (DAP) at 37°C. *S. enterica* strain χ 9992 without plasmids was routinely cultivated on LBG-DAP agar supplemented with 0.2% mannose and 0.05% arabinose (LBGMA) at 37°C. Strains containing pYA3493 were grown on LBGMA at 37°C. All strains were sub cultured at 48 h intervals.

Construction of the *Salmonella enterica* serovar Typhimurium vaccines.

LAJ1 was amplified using primers CjLAJ1F and CjLAJ1R. *LAJ2* was amplified primers CjLAJ2F and CjLAJ2R. These inserts were then cloned separately into the expression plasmid pYA3493 (13) in the proper reading frames, and introduced into *E. coli* χ 6212 via electroporation. PCR and sequencing were performed to ensure proper construction of all plasmids.

Salmonella enterica serovar Typhimurium strain χ 9992 was used as the vector strain and is a further modified version of strain χ 9088 described by Li, 2009 (16), a list of features is available in Table A1. Plasmids expressing the relevant genes were amplified in *E. coli* χ 6212, extracted and then introduced into χ 9992 by electroporation. Nucleotide sequencing was used to ensure proper construction of all vaccine strains.

Chickens. Day-old straight run Cornish X Rock broiler chicks were obtained from a commercial supplier (Murray McMurray, Webster City, Iowa). On arrival, chicks were divided into treatment groups and assayed for the presence of *Campylobacter* and *Salmonella*. Briefly, fecal samples were plated on

modified Campy-Cefex agar or Xylose Lysine Deoxycholate (XLD) (BD), respectively. Animals were housed and cared for in accordance with protocols approved by the University of Arizona IACUC.

Vaccination using *Salmonella enterica*. Chickens were vaccinated on days 10 and 16 post hatching. To prepare the vaccine, *Salmonella* strain χ^{9992} vectors were grown in LBGMA broth O/N at 37°C with aeration (200 RPM) until OD₆₀₀ = 1.0. Once OD 1.0 was obtained, cultures were centrifuged (5000Xg, 10 min) and the cells resuspended in PBS to a final OD = 10.0. Serial dilutions were performed to determine the exact titer of the vaccine. On the day of vaccination, feed was removed from the chicks 8 hours prior to vaccination. Each chick was administered 1 ml of the appropriate vaccine via oral gavage. Feed was returned 30 minutes post vaccination. Vaccination studies were performed in triplicate, with the exception of a single trial for χ^{9992} pYA3493-LAJ2 with heterologous challenge.

Screening of *Salmonella* shedding. Fecal samples were obtained from a group of broilers vaccinated with χ^{9992} pLAJ1 at 1, 3, and 7 days after the initial vaccination to determine duration of shedding. Birds were placed individually into sterilized pet carriers until defecation. Feces was collected from the sterile box floor, serially diluted and plated on XLD supplemented with 0.05% arabinose. After incubation, plates were scored positive or negative for *Salmonella*.

Challenge of chickens with *C. jejuni*. Chickens were challenged with the specified *C. jejuni* strains 10 days after the final vaccination. Briefly, *C. jejuni* was grown for 18 h as described above. Cells were harvested in PBS and diluted to a final titer of $\approx 1 \times 10^5$ CFU/ml for strain NCTC 11168 and $\approx 1 \times 10^7$ CFU/ml for strain M129. Serial dilutions were performed to determine the exact titer. Each chicken was challenged with 1.0 ml of the *C. jejuni* suspension via oral gavage.

Quantification of *Campylobacter jejuni*. Ten days post challenge, chickens were sacrificed, blood collected for serological analysis, and cecal contents serially diluted and plated for enumeration of *C. jejuni* on modified Campy-Cefex agar (BD).

Results

Screening of *Salmonella* shedding. Broilers were found to be positive for the χ^{9992} strain of *Salmonella* at days one and three. Shedding of the organism was absent from all birds at day seven.

Vaccination with χ^{9992} pLAJ1 against homologous challenge. Enumeration of *Campylobacter jejuni* NCTC in cecal contents following sacrifice revealed a greater than three log reduction in the vaccinated group as compared to controls which received either no vaccination or χ^{9992} pYA3493 (Empty Vector). No difference was found between unvaccinated and Empty Vector

controls (Table A2). Negative controls remained free of *Salmonella* and *Campylobacter* for the duration of the study.

Vaccination with χ^{9992} pLAJ2 against homologous challenge.

Vaccination with χ^{9992} pLAJ2 reduced cecal colonization by *C. jejuni* NCTC in the vaccinated group by approximately four logs as compared to unvaccinated controls (Table A3). Unvaccinated controls were not available for this study. Negative controls remained free of *Salmonella* and *Campylobacter* for the duration of the study.

Vaccination with χ^{9992} pLAJ2 against heterologous challenge.

Vaccination with χ^{9992} pLAJ2 reduced cecal colonization by *C. jejuni* M129, a heterologous strain, in the vaccinated group by more than one log as compared to unvaccinated controls and more than 1.5 logs as compared to Empty Vector vaccinates (Table A4). Negative controls remained free of *Salmonella* and *Campylobacter* for the duration of the study.

Discussion

Previous work in our laboratory has indicated that the *Campylobacter jejuni* genes *CjLAJ1*, a putative periplasmic protein, and *CjLAJ2*, a protein of unknown function with partial homology to a hemolysin of *Brachyspira hyodysenteriae*, are potential virulence factors important to the colonization of poultry. Studies using a *CjLAJ2* defective mutant which was unable to colonize broilers support the role

of this gene in broiler colonization (Shaner, et al., manuscript in preparation). Based on this information these proteins were chosen for expression in a recombinant attenuated *Salmonella* Typhimurium vector for the vaccination of broiler chickens against *Campylobacter* challenge.

Previous studies have demonstrated some success with the use of *Campylobacter* outer membrane proteins expressed by RASVs to protect or partially protect broilers from colonization, however in many cases the results of these studies have not been fully reproducible, the genes used have not been novel, the site of reduced colonization was not the ceca, or the *Salmonella* vectors required antibiotic selection (4, 15, 31). The use of *Asd* on pYA3493 to compliment the mutation in χ 9992 without requiring antibiotic selection makes this vaccine potentially useful in commercial broiler raising facilities. The attenuation of this strain allowed it to be reduced to undetectable levels in broiler feces by 7 days post vaccination.

A truly successful vaccine needs to exhibit a number of difficult to attain features simultaneously; including safety in both humans and animals, early protection against colonization which will take over where maternal immunity leaves off, protection against multiple strains and ideally species of *Campylobacter*, and finally a cost low enough to be attractive to large scale production industry (7). RASVs in general offer the benefits of safety through attenuation and low production cost (7, 14).

Vaccination of broilers with χ^{9992} pLAJ1 or χ^{9992} pLAJ2 significantly reduced colonization with the homologous strain, *C. jejuni* NCTC as compared to unvaccinated groups. No difference was found between unvaccinated groups and those vaccinated with the Empty Vector strain, indicating that any protection was the effect of the genes expressed rather than colonization by the vector or priming of cross protective responses. Vaccination with χ^{9992} pLAJ2 shows early promise in the reduction of *Campylobacter* colonization following heterologous challenge indicating the potential of this vaccine to provide broad protection which should be confirmed with additional trials and strains. The greater variation in this study is likely attributable to the relatively small number of animals in this single replicate. In order to further reduce or prevent colonization of broilers the possibility of expressing multiple proteins from a single RASV, as has been done recently with for *Helicobacter pylori*, should be considered (18).

Chicks often have high levels of *Campylobacter* specific IgY which is likely to reduce if not entirely prevent colonization for the first few weeks of life (7). That said, commercial broilers generally enter the food chain at less than two months of age necessitating protection by the vaccine as early as is feasible. This must be accomplished without establishing immune tolerance, as is possible when initial exposure occurs during the development of the avian immune system (2, 4). In order to assess the optimal dosage schedule for vaccination with

χ^{9992} pLAJ2 chicks were vaccinated on three schedules; 1 and 3 days, 3, 10 and 16 days, and 10 and 16 days. No significant differences in colonization were seen following challenge with *C. jejuni* NCTC 11168 at day 26 and sacrifice at day 36 (data not shown). Days 10 and 16 days post hatch were chosen for further trials due to evidence that the immaturity of the chicken immune system may reduce efficacy of vaccination earlier than this (3, 7, 19). Additionally, maternal antibodies may offer chickens younger than 10 days protection from *Campylobacter* colonization prior to immunization (7, 26).

Previous studies have indicated that expression of *Campylobacter* proteins by RASV stimulate the production of IgY and IgA which may be responsible for the reduction of colonization seen in those studies (4, 15, 31). This has not been conclusively established, and it is additionally possible that for proteins which may be localized to the inner membrane, such as the previously used *CjaA* or *CjLAJ1*, a cell mediated immune response reduces colonization by targeting intracellular *Campylobacter*. Broiler immune responses to *CjLAJ1* and *CjLAJ2* responsible for reduction of colonization, as well as immune responses to χ^{9992} remain to be assessed. Additional clarification of the role of cellular invasion in the persistence of *Campylobacter* in broilers, which is currently incompletely understood, may be required to fully elucidate the function of the vaccine (32).

Campylobacter jejuni is a significant human pathogen for which current management strategies are insufficient. Vaccination represents an increasingly

attractive option for the control of *Campylobacter* in poultry, a primary reservoir for its entry into the food chain. The present study illustrates the potential of two novel *Campylobacter* proteins expressed by attenuated *S. Typhimurium* to reduce *Campylobacter* colonization in broilers, though additional research is necessary to elucidate the protective immune response and the breadth of efficacy against additional strains and species of *Campylobacter* in additional broiler lines.

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Table A1: Strains and plasmids used in this study.

Strain or Plasmid	Relevant Characteristics	Source
Strains		
<i>C. jejuni</i>		
NCTC	Sequenced clinical isolate	NCTC
M129	Clinical isolate	L.A. Joens
<i>E. coli</i>		
X6212	ϕ 80d <i>lacZ</i> Δ M15 <i>deoR</i> Δ (<i>lacZYA-argF</i>)U169 <i>supE44</i> λ - <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> Δ <i>asd</i> Δ <i>zhf-2::Tn10</i> <i>hsdR17</i>	R. Curtiss 3 rd
<i>S. enterica</i>		
X9992	Δ <i>pmi-2426</i> Δ (<i>gmd-fcl</i>)-26 Δ P _{fur77} ::TT <i>araC</i> P _{BADfur} Δ P _{crp527} ::TT <i>araC</i> P _{BAD} <i>crp</i> Δ <i>asdA27</i> ::TT <i>araC</i> P _{BADC2} Δ <i>araE25</i> Δ <i>araBAD23</i> Δ <i>relA198</i> :: <i>araC</i> P _{BADlacI} TT	R. Curtiss 3 rd
Plasmids		
pYA3493	<i>Asd</i> ⁺ , β -lactamase signal sequence	R. Curtiss 3 rd
pLAJ1	<i>Asd</i> ⁺ , β -lactamase signal sequence, LAJ1	This work
pLAJ2	<i>Asd</i> ⁺ , β -lactamase signal sequence, LAJ2	This work

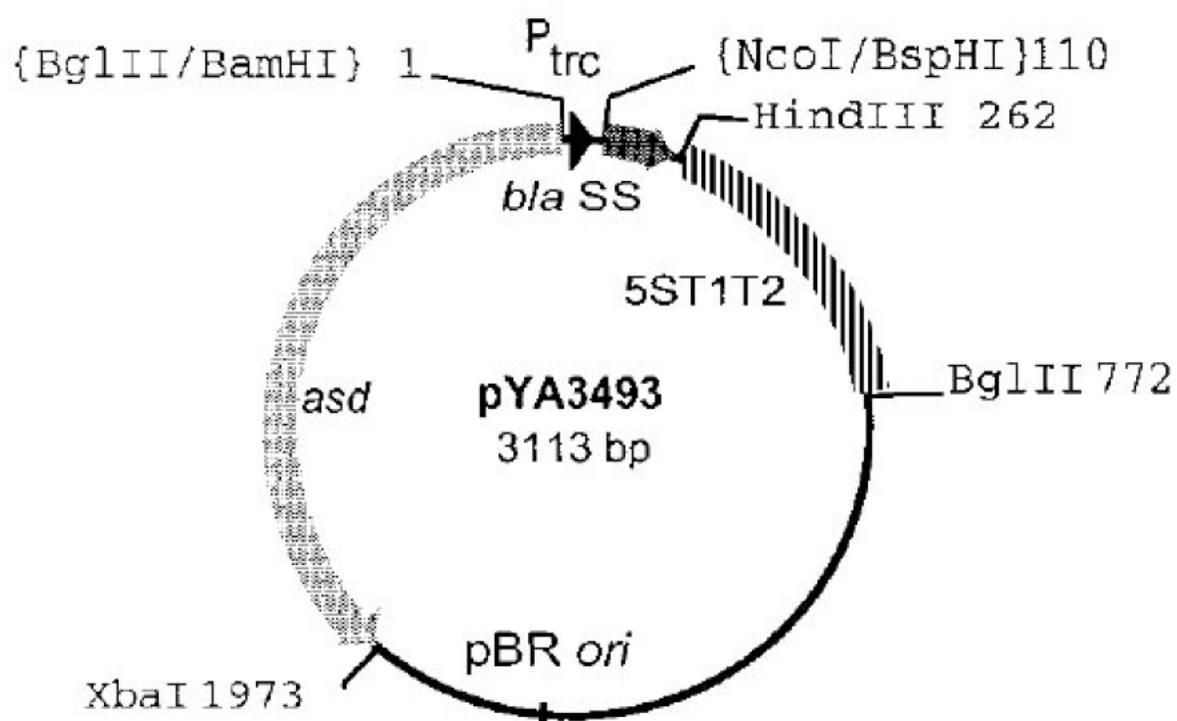


Figure A1: pYA3493

Table A2: Oral vaccination of broilers with χ^{9992} pLAJ1 challenged with the homologous strain, *Campylobacter jejuni* NCTC 11168.

<i>CjLAJ1</i> NCTC 11168 - Trial 1				<i>CjLAJ1</i> NCTC 11168 - Trial 2		
	<i>CjLAJ1</i>	EV	Control	<i>CjLAJ1</i>	<i>CjLAJ1</i>	EV
	1.0E+03	1.0E+07	3.0E+08	1.40E+04	2.00E+05	5.00E+07
	7.0E+04	1.2E+07	6.0E+07	3.20E+03	1.70E+05	3.00E+07
	7.4E+04	1.0E+08	1.0E+07	7.00E+04	3.00E+04	2.00E+07
	8.2E+04	2.0E+08	1.0E+07	1.00E+02	2.70E+05	1.70E+08
	1.0E+04	7.0E+07	2.0E+08	7.00E+04	3.00E+05	3.00E+07
	2.0E+04	9.0E+07	2.0E+07	9.00E+03	2.40E+04	
	3.0E+04	1.0E+08	4.0E+08	1.05E+04	1.50E+04	
	2.0E+03	1.0E+08	1.2E+08	1.00E+00	5.00E+06	
	6.0E+04	2.0E+07	1.0E+07	6.00E+03	2.30E+06	
	3.0E+04	1.0E+07	2.0E+07	3.00E+03	1.00E+07	
				6.00E+04		
Geometric mean	2.0E+04	4.4E+07	4.8E+07	3.78E+03	2.84E+05	4.33E+07
Combined data - Trials 1 and 2						
	<i>CjLAJ1</i>	EV	Control			
Geometric mean	2.60E+04	4.41E+07	4.83E+07			

Table A3: Oral vaccination of broilers with χ^{9992} pLAJ2 challenged with the homologous strain, *Campylobacter jejuni* NCTC 11168.

<i>CjLAJ2</i> NCTC11168				
	<i>CjLAJ2</i>	<i>CjLAJ2</i>	<i>CjLAJ2</i>	EV
	1.10E+03	5.00E+03	1.20E+05	5.00E+07
	1.50E+03	9.00E+04	1.00E+00	3.00E+07
	1.00E+02	3.00E+03	1.20E+04	2.00E+07
	1.00E+02	1.00E+00	6.00E+04	1.70E+08
	5.00E+03	4.00E+01	1.00E+02	3.00E+07
	1.00E+03	3.00E+07	4.00E+07	
	2.00E+03	5.00E+01	2.70E+04	
	7.00E+02	1.70E+04	6.00E+02	
	2.00E+01	5.00E+07	7.00E+04	
	5.00E+04	2.00E+02	1.20E+05	
	7.00E+01	1.00E+00	2.00E+04	
	5.60E+03	1.00E+00	2.00E+04	
Geometric mean	7.73E+02	1.24E+03	1.28E+04	4.33E+07
Combined Data			2.31E+03	4.33E+07

Table A4: Oral vaccination of broilers with χ^{9992} pLAJ1 challenged with the heterologous strain, *Campylobacter jejuni* M129.

	<i>CjLAJ2</i>	EV	Control
	6.0E+03	1.0E+04	2.0E+04
	7.0E+02	2.0E+04	7.0E+04
	7.0E+03	1.0E+04	1.0E+05
	3.3E+03	1.0E+05	3.0E+04
	2.0E+02	3.0E+04	3.0E+04
	7.0E+02	3.0E+04	7.0E+04
	8.0E+03	1.0E+04	1.0E+04
	3.0E+03	3.0E+04	1.0E+06
	3.0E+03	3.0E+04	1.0E+04
	3.2E+03	5.0E+04	1.0E+04
	1.0E+03	2.3E+04	3.5E+05
	4.0E+03	1.0E+04	7.0E+04
	6.8E+03	1.0E+04	2.1E+06
	2.3E+03	3.0E+08	7.0E+04
	1.4E+03	1.0E+04	3.0E+05
	7.0E+03		6.0E+05
Geometric mean	2.5E+03	3.8E+04	8.4E+04

APPENDIX B: A LONGITUDINAL STUDY OF *SALMONELLA* IN BEEF CATTLE IN A SMALL FEEDLOT ENVIRONMENT FROM RANGE THROUGH PROCESSING

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Abstract

Salmonella is an important pathogen of both humans and animals, causing 1.4 million cases of human diarrheal illness annually in the US (27) and major economic losses to the cattle industry in treatment, morbidity and mortality. In an effort to better understand transmission of *Salmonella* in the understudied small feedlot environment, bovine fecal, environmental, avian fecal, fly and processing samples were taken over a period of one year from the range through dry aging to ground beef. *Salmonella* was isolated from bovine associated samples only following transport, indicating that stress is a major contributing factor in *Salmonella* shedding. This is further supported by differences in shedding prevalence (17% vs. 100%) in animals transported 250 miles and 800 miles respectively at feedlot arrival. 92% of isolates collected were resistant to at least one antimicrobial agent. The *Salmonella* serotypes most commonly isolated were *S. Newport* and *S. Alachua*, which has not previously been isolated from cattle.

Introduction

Salmonella is an important pathogen of humans and animals (8, 27, 28). In 2010, the CDC's Foodborne Diseases Active Surveillance Network (FoodNet) reported 2,290 hospitalizations and 29 deaths attributable to *Salmonella*. They additionally reported that rates of infection caused by *Salmonella* have not

declined significantly in more than ten years, leading to direct medical expenditures of \$365 million each year (2). Because of the self-limiting nature of most cases of salmonellosis, it is estimated that for every laboratory confirmed case, 29 to 35 people have actually become ill (9, 33) with 1.4 million cases of human diarrheal illness being thought to occur annually in the US (27). *Salmonella* is responsible for lethal to subclinical disease in cattle and is thought to be one of the few diseases of cattle currently increasing in prevalence, making it a major economic and welfare concern to the industry (3).

Cattle are a major reservoir for *Salmonella*, potentially responsible for the introduction of the organism into the food chain at several points (16, 17). Both beef and dairy products have previously been recognized as important vehicles in outbreaks of human salmonellosis. One recent study found a *Salmonella* prevalence of 72.6% in pre-harvest feedlot cattle (30) and *Salmonella* in the feces or on the hides or carcasses of feedlot cattle has been routinely noted (18, 20). Once a cow becomes infected shedding of the organism may continue beyond the resolution of symptoms; a median duration of shedding was found to be 50 days with a maximum shedding period of 391 days (14).

Infection of cattle with *Salmonella* is all the more troubling because these animals have been identified as a reservoir for, and possibly a source of, multidrug resistant strains of nontyphoidal *Salmonella* species (22, 36). Use of antimicrobials in livestock production selects for and maintains these strains in

dairy and beef cattle (37). A recent survey found that multidrug resistant strains of *Salmonella* were significantly more likely to be of bovine than human origin (23). Multidrug resistant strains of *Salmonella* are associated with increased human morbidity and mortality and are inherently less treatable with commonly used antibiotics prescribed for serious infections which do not resolve on their own or are complicated by additional factors. (22, 37).

Bovine feces is used as fertilizer and deliberately spread on produce to increase its growth. Fecal material from cows and other species is treated, generally composted, to reduce pathogen load. This method is considered very good, and reduces pathogen levels more than 4 logs, with some difference in bacterial species reductions (11, 13). This process, then, does not completely eliminate high levels of bacteria in manure which is then introduced directly to foods which are likely to be consumed raw (35). Additionally, run off from beef and dairy production areas remains completely untreated, and may enter irrigation or aquaculture areas necessitating a greater understanding of the interactions of *Salmonella* in these production environments.

Pre-harvest interventions are critical to prevention of cattle morbidity and mortality, reduction of *Salmonella* amplification and reintroduction to the environment through the cattle host, and contamination of hides and ultimately beef products responsible for illness in the consumer (30). A complete understanding of the sources of *Salmonella* in the feedlot environment is

important to maximize the effectiveness of these strategies, and currently little is known about the sources and transmission of *Salmonella* in feedlots (3, 30). What little research has been done on transmission dynamics of *Salmonella* within feedlots has concentrated on large operations. The purpose of this study is to explore the timeline and patterns of infection in a small cohort of feedlot cattle, as well as to investigate possible sources of infection. We additionally investigate the role of the feed additives tylosin and monensin in this environment, the antimicrobial resistance of isolated strains and the effect of dry aging as a processing control strategy.

Materials and Methods

Cattle. All protocols followed the standards and policies of the University of Arizona Institutional Animal Care and Use Committee (IACUC). Hereford and crossbred steers were housed at the V-Bar-V range (Rimrock, AZ) until weaning. At approximately six months of age, cattle (n=36) were trucked to the University of Arizona, College of Agriculture Feedlot (Tucson, AZ). This cohort was examined at the range, arrival at the feedlot, months 3 and 6, and pre-harvest for *Salmonella* presence. A secondary group of nine steers were sampled at the range and following trucking to a large feedlot in Kansas, US. Fecal samples were obtained from each animal rectally at all sampling points. Cattle at the primary feedlot were randomly assigned to six pens (n=6 per pen). Pens 1-3 were fed an 85% concentrate diet free of antibiotics and growth promotants. Pens

5-7 were fed a traditional grain diet supplemented with tylosin and Rumensin® (monensin sodium, Elanco, Greenfield, IN), feed additives. Pen 4 remained empty throughout the study and separated the two treatment groups physically. Cattle were kept at the feedlot for 8-10 months depending on harvest group, with harvest occurring locally at the University of Arizona Meat Science Laboratory # 966, federally inspected (Tucson, AZ).

Wild birds. Birds were humanely collected by cannon net. Captured birds were manually removed from the net and individually placed into disposable cardboard carriers. Upon defecation on the sterile box floor, fecal samples were collected by sterile swabs and birds were released. Birds were collected at the feedlot before cattle arrival, and at months three and six post-arrival (n=60 per sampling). (Federal Fish and Wildlife Permit # MB209713-1; Arizona Game and Fish Department license # SP749148)

Environmental sampling. Samples were collected from pens 1-7 before the arrival of the cattle, and monthly until the first harvest (seven months). Drag swabs (n=4) (Solar Biologicals, Ogdensburg, NY), feed bunk swipes (n=1) (VWR, Westchester, PA), and water unit samples (n=1) were collected from each pen. Flies were trapped in bait bags (Rescue® Fly Bag, Spokane, WA) and samples pooled from all seven pens. Feed samples (1g) were collected prior to distribution at pre-arrival and at months three and six post-arrival. See Table B1 for an overview of samples taken.

Cattle Harvest. Cattle were harvested in three groups of 12 from May through July 2009. Sterile gauze squares (2x2 inches) pre-moistened in sterile phosphate buffered saline (PBS) were used to swab the carcasses at the ventral midline and USDA-FSIS locations after dehidng and evisceration. Carcasses were dry aged two weeks and re-sampled at the ventral midline and USDA-FSIS areas. Both of these locations were sampled as both have been variably used in the detection of *Salmonella* on beef hides and carcasses. These areas were also processed into ground beef samples (Figure B1).

Isolation of *Salmonella* spp. All samples were kept chilled and transported to the laboratory for immediate processing. Flies were weighed (0.5 g) and macerated by pestle and mortar in sterile PBS, then suspended in 4.5ml Buffered Peptone Water (BPW). Water unit samples were centrifuged at 1500xg for 15 minutes, and the pellet resuspended in 1ml BPW. Ground beef samples (25g) were diluted in 225ml BPW and stomached for 5 min at normal speed. All samples were serially diluted ten-fold in triplicate in BPW. After 12 hours of growth at 37° C, all samples were diluted 10 fold into Tetrathionate broth (TT). Samples were incubated for 24 hours at 37° C and plated on Xylose-Lysine-Tergitol 4 (XLT4) agar media. Following an additional 24 and 48 hours of growth at 37° C plates were inspected for growth of potential salmonellae. Suspect colonies were isolated and screened by PCR, primers used were: forward 5'-

GTGAATTATCGCCACGTTTCGG-3'; reverse: 5'- TCATCGCACCGTCAAAA GGAAC-3' for *Salmonella* virulence gene *invA* (3) and agglutination using commercial antisera (BD, Sparks, MD). The Animal Public Health Inspection Services (APHIS) in Ames, IA performed all serotyping.

Antimicrobial resistance tests. *Salmonella* isolates were tested for antimicrobial resistance using the Kirby-Bauer disc technique (5) on Mueller-Hinton agar (BD, Sparks, MD). Antimicrobial disks (BBL Sensi-Discs, BD, Sparks, MD) used in testing were: amikacin (30 µg), ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10µg), nalidixic acid (30 mg) streptomycin (10 µg), sulfisoxazole (250 µg), tetracycline (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75µg). Diameters of the zones of inhibition were measured and resistance was determined based on the manufacturer's specifications. Quality control of the antimicrobials was performed per the manufacturer's instructions using *Echerichia coli* ATCC 25922.

Pulsed-field gel electrophoresis. PFGE was performed on isolates using a PulseNet protocol and XbaI for restriction digestion (31).

Results

Cattle. At the range all cattle were negative for *Salmonella*. Upon arrival at the Tucson, AZ feedlot, 6 of 36 animals (17%) were positive for *Salmonella*. Two

serovars were detected, *S. Alachua* and *S. Albany* with a mean MPN of 2.59×10^4 CFU/gram of feces (Table B2). All nine (100%) animals transported to Kansas were positive for *S. Newport* at arrival.

Birds. The only time point at which avian fecal samples were positive for *Salmonella* was immediately before the arrival of the cattle (October). 12% (7/60) of birds were positive for *Salmonella* with a mean MPN of 1.39×10^7 CFU/gram of feces. Three serovars were isolated from the wild birds, *S. Typhimurium* (3/7), *S. Typhimurium* var. Copenhagen (3/7) and *S. Alachua* (1/7).

Flies. Flies were positive for *S. Alachua* only prior to the arrival of the cattle.

Environmental samples. Environmental samples were positive in three of the sampled months; immediately prior to the arrival of the cattle, and for the following two months. With the exception of a single feed bunk swab, all positive samples were drag swabs. All water unit and feed samples were negative for *Salmonella*.

Cattle processing. While fecal samples taken at processing were negative, three carcass swipes from two animals (5%) were found to be positive for *Salmonella*. One animal was positive for *S. Alachua* at the midline swipe following dehidng and the USDA swipe following evisceration (mean MPN 9.3×10^3 CFU). This serovar was also isolated from this animal's feces at arrival. A second animal was positive for *S. Newport* at the midline swipe following

dehiding (MPN 4.3×10^4 CFU). Both animals had been housed in pens from which the serovars isolated by carcass swipe had previously been isolated by drag swab. The two animals were from separate pens, however both were in groups receiving growth promotants.

Aging and ground beef. No carcass swipes taken following the dry aging period or ground beef samples were found to contain *Salmonella*.

PFGE and antimicrobial resistance. PFGE profiles were serovar specific and serovars Typhimurium and Typhimurium var. Copenhagen were indistinguishable from one another. *S. Newport* isolates from Arizona and Kansas were indistinguishable. Antimicrobial resistance profiles varied substantially within serovars. Among *S. Alachua* isolates the most common pattern was intermediate resistance to nalidixic acid and tetracycline (6 isolates), however four isolates were additionally intermediately resistant to ceftriaxone and one isolate was additionally intermediately resistant to sulfisoxazole. Single *S. Alachua* isolates were completely susceptible to all tested agents, intermediately resistant to tetracycline or ceftriaxone only and resistant to chloramphenicol and ceftriaxone only. The *S. Albany* isolate was resistant to tetracycline but susceptible to all other tested agents. *S. Newport* isolates shared the greatest level of resistance, however isolates from Arizona and Kansas differed. The two isolates from Arizona were entirely resistant to ampicillin, amoxicillin/clavulanic acid, chloramphenicol, streptomycin, and tetracycline as

well as intermediately resistant to nalidixic acid. Nine isolates from Kansas were only intermediately resistant to amoxicillin/clavulanic acid but were additionally resistant to gentamycin. Four *S. Typhimurium* isolates were intermediately resistant to nalidixic acid and tetracycline, one was intermediately resistant to tetracycline only and one was entirely susceptible to the agents tested. Each *S. Typhimurium* var. Copenhagen isolate differed, with one entirely susceptible, one each intermediately resistant to tetracycline and nalidixic acid, and one intermediately resistant to these two agents with additional total resistance to ceftriaxone. Antimicrobial resistance levels did not rise over time, were not associated with the use of rumensin and tylosin, and particular profiles were not isolated from single sampling periods.

Discussion

Antibiotics and growth promotants are frequently used in cattle to improve feed conversion and prevent illness, though increasingly there exists a market for beef cattle raised without these agents (12). Two commonly used agents, tylosin and monensin, were included in the feed of three of the six pens of cattle in this study. The addition of these agents was not anticipated to have an effect on *Salmonella* presence, levels, or shedding in cattle because the majority of salmonellae are resistant to tylosin (28) and monensin has been shown to have no in vitro effect on *Salmonella* (16). That said, one study found that monensin increased levels of *Salmonella* in the ceca and livers of chickens experimentally

infected indicating the potential for this effect in other animals (24). At arrival cattle had not yet been exposed to growth promotants of any kind. Following the arrival of cattle and the introduction of these agents, *Salmonella* was isolated more frequently from pens in which the agents were used. While the use of these particular additives did not appear to influence resistance, some worrying trends were seen. Resistance to nalidixic acid (the most common resistance found in this study) is predictive of fluoroquinolone resistance not detectable by disc diffusion in *S. Typhi* (29). Multidrug resistance as seen in *S. Newport* isolates are associated with increased human morbidity and mortality (22, 36, 37). Only three isolates were susceptible to all tested agents.

While no animals were found to be positive for *Salmonella* at the range, *Salmonella* was isolated from the feces of six animals at arrival in Arizona. The serovar isolated from five of the six cattle was present in the feedlot environment prior to their arrival, which likely indicates that most were initially infected in the feedlot environment. Alternatively, the short duration of their stay prior to sampling and the additional serovar not previously detected in the feedlot environment may indicate that they were previously infected with these serovars at the range and began shedding the organisms due to the stress of trucking and acclimation to a new environment. Calves are frequently infected with *Salmonella* between 4 and 28 days of age and may be infected within hours of birth; (28) up to 50% of calves from calf ranch operations and dairies have been found to be

infected (7). This indicates the necessity of further study of *Salmonella* in the range environment, for which to date there is essentially no information.

S. Alachua, the most commonly isolated serovar in this study, is rare in the US and uncommon in the literature. To the best of our knowledge it has not previously been isolated from cattle, though it was on one occasion isolated from non-fat dry milk (15). This serovar was first isolated from swine in Florida in 1952, and since then has been primarily associated with neonatal meningitis in India, illustrating its potential as a human health hazard (1, 10, 26). The persistence of *S. Alachua* from arrival to processing is consistent with a recent study (30). It is not known why animals from the same original location were shedding two different serovars upon arrival at their destinations. Vehicles used to transport cattle may become contaminated with *Salmonella* (6), making it possible that these trucks were the initial site of exposure. The presence of *S. Newport* at both locations supports the idea that cattle were potentially infected with this serovar at the range, though the varying antimicrobial resistance patterns and delayed appearance at the Tucson feedlot may indicate that the two strains of *S. Newport* have different origins. *S. Newport* is commonly isolated from cattle (19, 35).

Fecal samples taken at the feedlot, prior to transportation of the animals to the processing facility, were negative for *Salmonella* for all 36 animals; however *Salmonella* was isolated from the carcass swipes of two animals during

processing. The antimicrobial resistance pattern of *S. Alachua* isolated from carcass swipes was identical to the isolate from the fecal sample of that animal at arrival, and different from *S. Alachua* isolated from the pen floor; however contamination could have occurred through direct fecal or hide contamination. Transportation to processing facilities has been shown to increase hide contamination with *Salmonella* from roughly 20% to more than 50% in one study (6) and 6% to 89% in another (4). Transportation stress immediately prior to processing may increase shedding of *Salmonella* by cattle, thus increasing the potential for carcass contamination during processing (4). Various stresses have long been suspected of increasing the shedding of some pathogens in feces, and this has been demonstrated in select cases (32). The group of 36 cattle transported to Tucson, AZ traveled approximately 250 miles, while the Kansas group were transported more than 800 miles in close quarters with many additional animals. The additional stress associated with longer transit time may explain the greater prevalence of *Salmonella* at arrival in the cattle transported to Kansas. It is likely that *Salmonella* was not isolated from the cohort of 36 cattle following arrival because they were able to acclimate to their environment and were held in relatively comfortable conditions as compared to those in large feedlot operations. This acclimation effect has been seen previously in Australian cattle held for 80 days prior to slaughter, as compared to 2 and 18 days (21).

While frequency of carcass contamination was low, our results support previous studies which indicate dry aging reduces the survival of *Salmonella* on beef carcasses. *S. Newport* has previously been found to survive more poorly than other serovars in dry aging, but all *Salmonella* serovars in artificial inoculation studies were found to be significantly reduced by dry aging (25, 34).

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Table B1: Sample types and schedule.

Sample Type	Time Point
Drag Swab	Pre-arrival, monthly n=28 per sampling (4 per pen), 3x3" square
Feed Bunk	Pre-arrival, monthly n=7 per sampling (1 per pen), 1.5 x3x 5/8" sponge
Water Unit	Pre-arrival, monthly n=7 per sampling (1 per pen), 50ml
Fly Sample	Pre-arrival, monthly n=1 per sampling, if flies available (pool pens 1-7), 0.5g
Avian Fecal Sample	Pre-arrival, 3 and 6 months n=60 per sampling , 0.1g
Calf Fecal Sample	Range, arrival, 3 and 6 months, at processing n=36 per sampling (1 per calf), 1g
Feed Samples	Arrival, 3 and 6 months n=6 per sampling , 1 per pen (control pen has no feed), 1g
Carcass Swipes	After dehiding, evisceration, and aging at USDA and ventral midline areas, n=6 per calf, 2x2" square
Ground Beef	After aging carcass swipes, swiped areas are processed into ground beef n=2 per calf, USDA and ventral midline areas, 25g

TableB2: Arizona feedlot samples positive for *Salmonella*, with mean MPN per sample in CFU/g or CFU/ml and serovars.

S. Typhimurium var. Copenhagen is referred to here as Copenhagen. (N/A) Sample not taken for this time period.

Sample Type	Pre-Arrival (Oct 2008)		Arrival (Oct 2008)		Month 1 (Nov 2008)		Month 2 (Dec 2008)		Harvest (Summer 2009)	
	Serovars		Serovars		Serovars		Serovars		Serovars	
Wild Birds Dove	(1/4) 2.1 x 10	Copenhagen	N/A		N/A		N/A		N/A	
Wild Birds Pigeon	(6/56) 9.2 x 10 ⁵	Typhimurium Alachua Copenhagen	N/A		N/A		N/A		N/A	
Environmental Feed Bunk	(1/7) 4.3 x 10 ²	Alachua	N/A						N/A	
Environmental Drag Swab	(2/28) 2.0 x 10 ⁴	Alachua	N/A		(3/28) 1.1 x 10 ⁵	Alachua Typhimurium	(5/28) 4 x 10 ⁴	Typhimurium Alachua Copenhagen Newport	N/A	
Flies	(1/1) 1.5 x 10 ³	Alachua	N/A						N/A	
Calf Fecal	(0/36)	Negative	(6/36) 2.6 x 10 ⁴	Alachua Albany	N/A		N/A		N/A	
Cattle Processing	N/A		N/A		N/A		N/A		(2/36) 1.6 x 10 ⁴	Alachua Newport

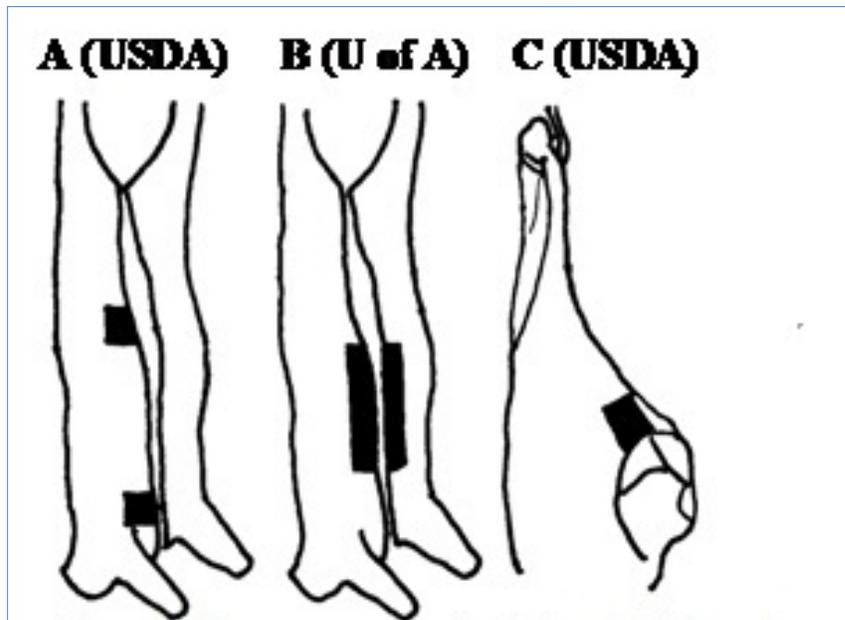


Figure B1: Sample sites for processing and aging carcass swipes.

APPENDIX C: SURVIVAL OF *SALMONELLA* NEWPORT IN OYSTERS

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ABSTRACT

Salmonella enterica is the leading cause of laboratory-confirmed foodborne illness in the United States and raw shellfish consumption is a commonly implicated source of gastrointestinal pathogens. A 2005 epidemiological study done in our laboratory by Brands et al., showed that oysters in the United States are contaminated with *Salmonella*, and in particular, a specific strain of the Newport serovar. This work sought to further investigate the host–microbe interactions between *Salmonella* Newport and oysters. A procedure was developed to reliably and repeatedly expose oysters to enteric bacteria and quantify the subsequent levels of bacterial survival. The results show that 10 days after an exposure to *Salmonella* Newport, an average concentration of 3.7×10^3 CFU/g remains within the oyster meat, and even after 60 days there still can be more than 10^2 CFU/g remaining. However, the strain of Newport that predominated in the market survey done by Brands et al. does not survive within oysters or the estuarine environment better than any other strains of *Salmonella* we tested. Using this same methodology, we compared *Salmonella* Newport's ability to survive within oysters to a non-pathogenic strain of *E. coli* and found that after 10 days the concentration of *Salmonella* was 200-times greater than that of *E. coli*. We also compared those same strains of *Salmonella* and *E. coli* in a depuration process to determine if a constant 120 L/h flux of clean seawater could significantly reduce the concentration of bacteria within oysters and found that after 3 days the oysters retained over 10^4 CFU/g of *Salmonella* while the oysters exposed to the non-pathogenic strain of *E. coli* contained 100-times less bacteria. Overall, the results of this study demonstrate that any of the clinically relevant serovars of *Salmonella* can survive within oysters for significant periods of time after just one exposure event. Based on the drastic differences in survivability between *Salmonella* and a non-pathogenic relative, the results of this study also suggest that unidentified virulence factors may play a role in *Salmonella*'s interactions with oysters.

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1. Introduction

Foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5000 deaths in the United States each year (Mead et al., 1999) and estimates attribute 10–19% of these illnesses to seafood consumption (Butt et al., 2004). A study over a four year period in Great Britain calculated that shellfish were the riskiest food to eat in terms of number of cases per serving, over six times the risk of poultry and nearly 27 times the risk of red meat (Adak et al., 2005). In the decade between 1999 and 2008, the United States' supply of oyster meat, both imported and domestically produced, averaged over 69 million pounds (NOAA, 2008), and while exact data are not kept on the amount of oysters consumed raw, estimates place the percentage at roughly 50% (FDA, 2005b). Oysters are filter feeders that concentrate and digest planktonic food from their surroundings, deriving nutrition from algae, phytoplankton, bacteria, and detritus. Oysters also concen-

trate potentially harmful contaminants such as toxins and heavy metals, as well as bacterial and viral pathogens (Butt et al., 2004) which can have negative consequences for human health. One study showed that fecal contaminants are 3 to 62 times greater in an oyster as compared to the surrounding water (Burkhardt and Calci, 2000). Based on these findings, the United States Food and Drug Administration (FDA) established the National Shellfish Sanitation Program (NSSP) to provide state governing bodies with guidelines for improving the safety of the U.S. supply of shellfish. Current regulations require a body of water used for shellfish production to be tested for fecal coliform bacteria (FDA, 2005a). Along with testing water and oyster samples for coliforms, a commonly used process known as depuration is used to improve the safety of bivalves destined to be consumed raw by maintaining the oysters in clean, rapidly circulating seawater in an attempt to purge oysters of bacteria. However, the effectiveness of this technique for certain microbes is still in question (Chae et al., 2009; Rowse and Flett, 1984; Savini et al., 2009; Son and Fleet, 1980; Sunnotel et al., 2007).

The United States Centers for Disease Control (CDC) estimated that there are approximately 1.4 million cases of *Salmonella* in the U.S. each year, resulting in 15,000 hospitalizations, and 400 deaths

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(Voetsch et al., 2004), and an estimated cost to society between 500 million dollars and 2.3 billion dollars (Frenzen et al., 1999). Despite numerous studies, the role of *Salmonella* in mollusk-associated acute gastrointestinal disease is difficult to estimate and the results of direct surveillance research have varied considerably. A mid-1990's study in Ireland found the overall prevalence of *Salmonella* was 8% amongst the 433 mollusks they tested (Wilson and Moore, 1996), while a 2004 study in Italy showed only a 1% prevalence in their samples of mollusks (Macaluso et al., 2004). An extensive study in Spain found the overall prevalence of *Salmonella* in oysters to be 2.5% with a slight upward trend during the course of the four-year study which concluded in 2001 (Martinez-Urtaza et al., 2003). A Brazilian study surveyed both water samples and oysters and found 30% of water samples and 10% of oysters from an estuary near Sao Paulo to be positive for *Salmonella* (Ristori et al., 2007).

In 2007, the FDA conducted a comprehensive year-long nationwide survey of market oysters and found *Salmonella* in 8.6% of their pooled market oyster samples by PCR and 1.5% via culture methods (DePaola et al., 2010). Previous work in our laboratory by Brands et al. (2005b) found an overall prevalence of *Salmonella* in 7.4% of individual market oysters in the United States in 2002. Seventy-seven percent of the isolates found in 2002 (Brands et al., 2005a) were the Newport serotype and of those isolates, 98% of them shared one Pulsed Field Gel Electrophoresis (PFGE) genotype. This PFGE profile, designated JJPX01.0014 by the CDC, matched the first multi-drug resistant strain of Newport submitted to the CDC's PulseNet program and the strain isolated from oysters was confirmed to be resistant to at least 7 antimicrobials (Brands et al., 2005a).

Based on the current state of our understanding of *Salmonella*'s interactions with oysters, this study sought to further examine the strain of *Salmonella* isolated from oysters in 2002 by Brands et al. (2005a) by quantifying its ability to live within oysters and comparing this survivability to other strains of *Salmonella* as well as a non-pathogenic strain of *E. coli*.

2. Materials and methods

2.1. Bacterial cultivation and enumeration

Strains used in these experiments are summarized in Table 1. Due to logistical constraints during preliminary studies that were continued with the current studies for the sake of consistency, inoculums for both the water and oyster survival studies were prepared from 48 h cultures, with 24 h spent stationary at room temperature and 24 h spent shaking at 37 °C. Other experiments in our laboratory have used a more typically-prepared late-stationary phase culture with the same results (data not shown). Enumeration of viable bacteria, from both the water samples and oyster homogenates described below, was done by serial dilutions in phosphate-buffered saline (PBS) and direct

plating on the appropriate detection media for the species being tested; Xylose–Lysine–Deoxycholate (XLD) agar incubated at 37 °C for *Salmonella* (Andrews and Hammack, 2006), and 4-methylumbelliferyl-D-glucuronide (MUG) agar at 45.5 °C, made by adding agar to EC MUG broth media, for *E. coli* (Venkateswaran et al., 1996). Both XLD and MUG agar were compared to LB agar in preliminary studies (data not shown) to insure that the enumeration process was not biased by the type of media used. Preliminary studies were also done to show that endogenous glucuronidase activity from oyster tissues was easily distinguishable on EC-MUG Agar from the same reactions done by the colonies of *E. coli* (data not shown). All growth media and agar additives were obtained from Difco Laboratories (Becton, Dickson and Company, Sparks, MD, USA), except for the EC MUG broth (Sigma, St. Louis, MO, USA).

2.2. Survival in water

Samples of fresh water from the Yaquina River and brackish water from Yaquina Bay were obtained near Newport, OR, USA (no relation to the origin of the serovar's name) and shipped overnight using standard commercial parcel carriers to Tucson, AZ, USA. The river water samples were split into 5 sterile 1 L Nalgene containers and maintained at an ambient temperature between 18 and 20 °C. Aeration of the water samples was provided by standard aquarium air pumps (Tetra, Blacksburg, VA, USA) and stones (Penn-Plax, Hauppauge, NY, USA). Each container was then inoculated with cultures of human, bovine, or oyster isolates of *Salmonella* Newport or *E. coli* ATCC 25922, grown as previously described, with all strains having an initial concentration between 1.0×10^7 and 5.1×10^7 CFU/mL for the river water experiments and between 1.8×10^6 and 2.5×10^6 CFU/mL for the bay water experiments. One container of each type of water was left uninoculated to serve as a negative control. Every three days for 12 days, 10 mL samples of each container were taken and vacuum filtered using a Microfil filtration system and sterile mixed cellulose ester filters with a pore size of 0.22 µm (Millipore, Billerica, MA, USA). After a sample had passed through the flexible filters, they were placed in 1 mL of sterile PBS and vortexed thoroughly to resuspend any bacteria, thereby concentrating the sample 10-fold. The surviving bacteria were then enumerated by serial dilutions and direct plating as described previously.

2.3. Survival in oysters

Pacific oysters, *Crassostrea gigas*, were acquired from a commercial source in Alaska (Kachemak Shellfish Growers Cooperative, Homer, AK, USA). Alaskan oysters were chosen because they have always been free of *Salmonella* (Brands et al., 2005b). Prior to any experiments, 10–15% of every incoming batch of oysters were homogenized by aseptically shucking, weighing, and blending the oyster tissues into a 1:5

Table C1

Table 1
Bacterial strains.

Strain	Characteristics and source
<i>Salmonella</i> Newport LAJ160311	Oyster Isolate from Brands et al. (2005b) with the JJPX01.0014 PulseNet PFGE Profile
<i>Salmonella</i> Newport TEB9356	Human Isolate from T.E. Besser, Washington State University
<i>Salmonella</i> Newport 3082	Bovine Isolate from T.E. Besser, Washington State University
<i>Salmonella</i> Newport SL254	Sequenced multi-drug resistant strain (GenBank: CP001113) obtained from the Salmonella Genetic Stock Centre (SGSC), University of Calgary
<i>Salmonella</i> Newport SL317	Sequenced pan-susceptible strain (GenBank: ABEW00000000) obtained from the SGSC, University of Calgary
<i>Salmonella</i> Enteritidis PT4 NCTC 13349	Sequenced strain (GenBank: AM933172) obtained from SGSC, University of Calgary (Thomson et al., 2008)
<i>Salmonella</i> Javiana CVM35943	Sequenced strain (GenBank: ABEH00000000) obtained from SGSC, University of Calgary
<i>Salmonella</i> Typhimurium LT2 ATCC 700720	Sequenced strain (GenBank: AE006468) obtained from SGSC, University of Calgary (McClelland et al., 2001; Zinder and Lederberg, 1952)
<i>Escherichia coli</i> ATCC 25922	Common coliform control strain obtained from C.P. Gerba, University of Arizona (Gerba and McLeod, 1976; Hood and Ness, 1982)

dilution of sterile phosphate-buffered saline (PBS) using a T18 Basic Ultra-Turrax Tissue Homogenizer (IKA, Wilmington, NC, USA). The resulting oyster homogenates were then confirmed to be free of *Salmonella*, and *E. coli* if relevant to the experiment, by the same direct plating method described previously.

The oysters were maintained in aquaria containing approximately 57 L (15 gal) of constantly aerated artificial seawater (ASW) (Instant Ocean, Marineland, Moor Park, CA, USA). A commercial saltwater mixer/dispenser (Marineland, Moor Park, CA, USA) was used to mix and store the ASW at a salinity of 25 ppt, as measured by a refractometer. Upon arrival at our lab in Tucson, AZ, USA, the oysters were allowed 5–10 days to acclimate to the tanks, during which time they were given two feedings of an algae-based invertebrate food according to the manufacturer's directions (Micro-Vert, Kent Marine, Franklin, WI, USA). One third of the water in each tank was changed three times per week, which amounts to an influx of new ASW at a rate of 0.34 L/h, to remove nitrogenous wastes and maintain water quality throughout the study. To minimize the effect residual food might have on the uptake of the bacteria, the food mixture was withheld from the oysters for the 5 days prior to inoculation. In order to expose the oysters to the bacteria, the oysters were removed from their tanks and placed in plastic buckets containing 10 L of ASW. Cultures of bacteria were diluted in sterile ASW and added to the buckets so that the estimated final concentration in each bucket was 10^6 CFU per mL of ASW, an amount that was determined in preliminary studies to be a reliable and consistent exposure concentration (data not shown). Just prior to the addition of the oysters to the buckets, samples of the ASW containing the bacteria were taken and concentrations of bacteria were measured by serial dilutions and plating on LB agar. One group of oysters in each experiment was unexposed to bacteria to serve as a negative control. The oysters were left in buckets for approximately 24 h before being returned to their larger aquaria, where the twice-weekly feedings resumed.

On post-exposure sample days, one oyster per tank (3 tanks per group) was aseptically shucked and homogenized and the surviving bacteria quantified to determine the number of colony forming units per gram of oyster meat. Water samples, including any expelled feces and other detritus that may have accumulated in the bottom of the tanks, were taken at the same time oysters were removed from the tanks and serially diluted, plated, and enumerated on the same media used for oyster homogenates to determine if there was any viable non-oyster-bound bacteria within any of the tanks.

2.4. Depuration

Oysters were acquired, exposed to bacteria in buckets for 24 h, and tested as described previously, however, for the depuration experiments they were maintained using the facilities of the Molluscan Broodstock Program (MBP) at Oregon State University's Hatfield Marine Science Center (HMSC). During acclimation and following exposure to bacteria, each group of oysters was housed in 20 L flow-through tanks where they were continuously exposed to an influx of treated Yaquina Bay water, supplemented with an algal diet of diatoms (*Chaetoceros* sp.) and flagellates (*Isochrysis galbana* Tahitian strain), at a flow rate of 120 L/h using the standard facilities in place to support the MBP. The incoming water was pumped from the Yaquina Bay into a reservoir at the HMSC and then passed through a variety of filters down to approximately 1 μ m in diameter. The filtered water was then irradiated with ultraviolet light before being pumped into the tanks housing the oysters. Effluent from the tanks was treated by the standard chlorination/dechlorination protocols used by the HMSC (Langdon et al., 2003). Every 24 h for three days, 10 oysters per group were removed from the tanks, aseptically shucked and homogenized, and their surviving bacterial contents enumerated as described previously. Samples of the influent water were also tested daily for the presence of *Salmonella* and *E. coli*.

2.5. Vertical transmission

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Oysters were exposed once to an average of 2.3×10^6 CFU/mL of *Salmonella* Newport LAJ160311 in buckets for 24 h as previously described and maintained at the HMSC facilities under the same conditions as the depuration study but with a reduced influent flow-rate of 60 L/h. Three times per week, the oysters were removed from their tanks into dry mesh holding bags and their tanks were cleaned. To account for the possibility that either a chronic infection or an acute infection would be required for vertical transmission, the oysters were re-exposed to *Salmonella*, as described previously, to an average of 2.8×10^7 CFU/mL, twenty-eight days after the initial exposure. Three days after this second exposure, the oysters were induced to spawn by following the standard conditioning procedures of the MBP (Langdon et al., 2003). Briefly, groups of 12 oysters were placed in 5 L containers where the water temperature was alternated every 30 min between 20 °C and 28 °C. After 7 h, the released gametes were filtered through an 80 μ m screen, rinsed in bay water that was filtered and irradiated as previously described, and then retained on a 25 μ m screen. The collected gametes were then resuspended in 50 mL of the treated bay water and allowed to settle for 15 min. Samples of the settled gametes were then ground by sterile mortar and pestle sets and then plated in triplicate on XLD agar. The adult oysters were tested for the amount of *Salmonella* remaining in their tissues as previously described.

2.6. Statistics

Prior to any analysis of CFU data, the values were transformed to a normal distribution by taking the base-10 logarithm of all data to account for the skew inherent in the quantification of exponential growth (Limpert et al., 2001).

When comparisons were made between just two groups, such as the comparison between *Salmonella* and *E. coli* in oysters, a Student's *T*-test was used with an α of 0.05 at each time point. In the rest of the experiments, multiple groups were simultaneously compared to each other, and a one-way analysis of variance (ANOVA) for independent samples was conducted followed by a Tukey HSD test to determine significance at a 0.05 level at each time point. All tests were performed using the open-source statistical software, R.

All studies were done at least in triplicate and repeated at least three independent times. The composite means of all three replicate trials $\pm 95\%$ confidence intervals of all data points are presented in the results.

3. Results

3.1. Survival in fresh river water

The survivability of three isolates of *Salmonella* Newport: TEB9356 a human clinical isolate, TEB3082 a bovine isolate, and LAJ160311 a strain isolated from oysters, in a fresh water environment was tested and compared to that of a representative fecal coliform bacterium. Each strain of bacteria had consistent results, which are summarized in Fig. 1A. The *Salmonella* strains dropped from an average starting concentration of 2.2×10^7 CFU/mL to an average of 8.0×10^5 CFU/mL, while *E. coli* dropped from an average starting concentration of 3.2×10^7 CFU/mL to an average of 2.2×10^3 CFU/mL. This approximately 370-fold difference between the average concentration of all three strains of *Salmonella* and *E. coli* ATCC 25922 after three days of incubation is statistically significant ($p < 0.01$). After 6 days of incubation, an approximately 32-fold, statistically significant ($p < 0.05$) difference remained with *Salmonella* dropping to an average of 2.5×10^2 CFU/mL and the *E. coli* strain falling to 7.8 CFU/mL. By day 9, the *E. coli* strain was no longer detectable and the *Salmonella* strains had dropped to an average of 4.8 CFU/mL, and by day 12 none of the bacterial strains could

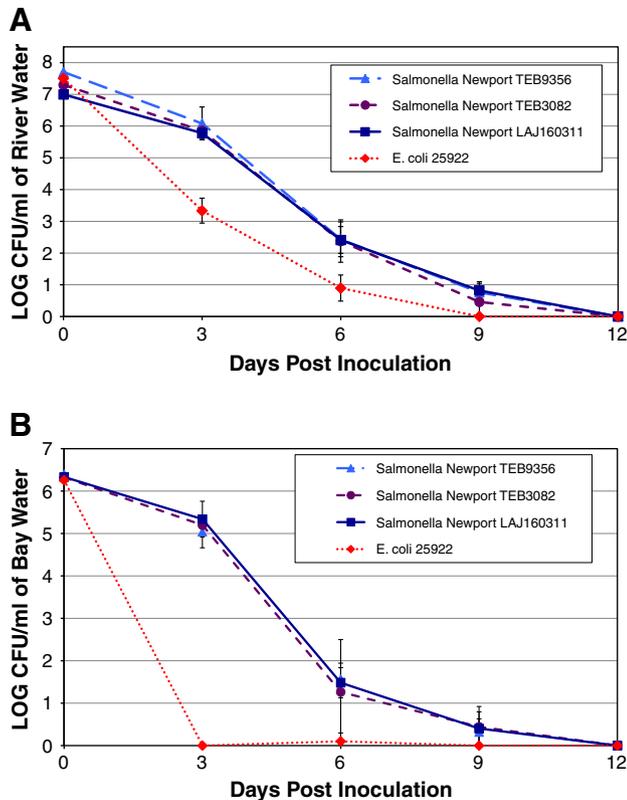


Figure C1

Fig. 1. Survival of *Salmonella* Newport and *E. coli* in water. Water samples from the Yaquina River (A) Yaquina Bay (B) in western Oregon were inoculated with three strains of *Salmonella* Newport, one of human origin TEB9356, one from bovine origin TEB3082, and one isolated from oysters LAJ160311, and *E. coli* ATCC 25922, used to represent a typical fecal coliform. Every 3 days for 12 days, the numbers of remaining viable bacteria were enumerated. The mean of three independent replicates \pm 95% confidence intervals are displayed.

be detected in the fresh water samples. At no point were there any significant differences between the three strains of *Salmonella*.

3.2. Survival in brackish bay water

The results summarized in Fig. 1B show that, like the fresh water samples, all of the strains tested in brackish water samples showed a consistent decline in viability. After three days of incubation, *E. coli* was not detected in the bay water, while the three strains of *Salmonella* had an average reduction from an initial concentration of 2.2×10^6 down to 1.6×10^5 . The difference between *E. coli* and the three *Salmonella* strains was statistically significant ($p < 0.01$). Six days after inoculation, *E. coli* was detected at an average of 1.3 CFU/mL, while the *Salmonella* was found at an average of 27.3 CFU/mL. By day 9, the *E. coli* strain was once again undetectable and the *Salmonella* strains had dropped to an average of 2.4 CFU/mL. Like the fresh water samples, none of the bacterial strains could be detected in the brackish water samples by day 12 and there were no significant differences between the three strains of *Salmonella* at any of the time points tested.

3.3. Survival of *Salmonella* in oysters

Fig. 2 shows that 10 days after exposure to the various strains of *Salmonella*, the amount of culturable *Salmonella* remaining within the oysters ranged between a low of 3.7×10^2 CFU/g for Newport strain LAJ160311 and a high of 3.7×10^3 CFU/g for Newport strain SL254. By 30 days post-exposure, the range of surviving concentrations remained similar to the initial time point with a low of 4.8×10^2 CFU/g for *Salmonella* Enteritidis and a high of 2.0×10^3 CFU/g for *Salmonella*

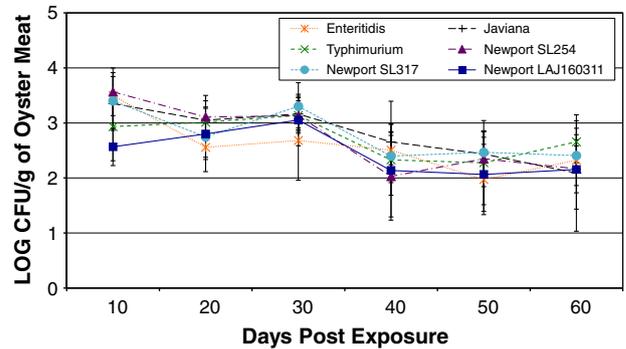


Figure C2

Fig. 2. Survival of *Salmonella* serovars in oysters. Pacific oysters, *Crassostrea gigas*, were exposed to six different strains of *Salmonella*. Every 10 days for 60 days, the numbers of viable bacteria remaining within the oysters were enumerated. The mean of three independent replicates \pm 95% confidence intervals are displayed.

Newport SL317. By the final time point of 60 days post-exposure, the range narrowed and fell to between 1.2×10^2 CFU/g for *Salmonella* Javiana and 4.5×10^2 CFU/g for *Salmonella* Typhimurium. There were no consistent patterns of statistically significant differences between any of the strains of *Salmonella* studied. There was never any culturable *Salmonella* detected in any of the tank water samples or any of the unexposed negative-control oysters.

3.4. Survivability of *Salmonella* Newport versus that of *E. coli*

As shown in Fig. 3, 15 days after being exposed to Newport LAJ160311, an average of 10^3 CFU of *Salmonella* remained within each gram of oyster meat. During that same time period, an average of only 5.0 CFU/g of *E. coli* ATCC 25922 survived, 200-times less than the Newport strain, despite similar average exposure concentrations of 1.6×10^6 CFU/mL of *Salmonella* and 2.0×10^6 of *E. coli*. By day 30, the average amount of *Salmonella* had fallen to 3.7×10^2 CFU/g while the *E. coli* dropped to an average of 2.2 CFU/g. At this point, only two of the nine oysters tested had any detectable amounts of *E. coli* within them. After 45 days, the oysters exposed to *Salmonella* still had an average of 67.9 CFU/g surviving within their tissues, whereas only 2.64 CFU/g of the *E. coli* inoculum had survived, with six of the nine oysters testing negative for *E. coli*. By day 60, the *E. coli* was no longer detected in any of the oysters, while the *Salmonella*-exposed oysters retained an average of 71.9 CFU/g within their meat. At each time point, the difference between *Salmonella* and *E. coli* was statistically significant ($p < 0.01$). At no point during the studies were culturable *Salmonella* or *E. coli* detected in any of the tank water samples collected or in any of the unexposed negative control oysters.

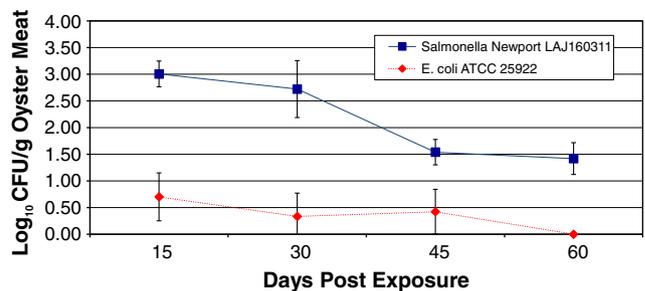


Figure C3

Fig. 3. Survivability comparison of *Salmonella* Newport and *E. coli* in oysters. Pacific oysters, *Crassostrea gigas*, were exposed to *Salmonella* Newport LAJ160311, previously isolated from oysters, and *E. coli* ATCC 25922, used to represent a typical fecal coliform. Every 15 days for 60 days, the numbers of viable bacteria remaining within the oysters were enumerated. The mean of three independent replicates \pm 95% confidence intervals are displayed.

3.5. Survival in depurated oysters

Fig. 4 shows that oysters exposed to an average of 3.0×10^6 CFU/mL of *Salmonella* retained an average of 2.3×10^4 CFU/g of oyster meat after 24 h of depuration. At the same time point, oysters exposed to 3.2×10^6 CFU/mL of *E. coli* retained an average of 2.0×10^3 CFU/g, which is more than a 10-fold reduction when compared to the *Salmonella* strains. Following 48 h of depuration, the *Salmonella*-exposed oysters contained an average of 1.6×10^4 CFU/g, while *E. coli* had an average of 2.2×10^2 CFU/g still within the oysters, a nearly 73-fold difference. After 72 h of depuration, the oysters exposed to *Salmonella* retained an average of 1.2×10^4 CFU/g while *E. coli* retained an average of 1.2×10^2 CFU/g, a 100-fold difference. At all three time points, the *Salmonella* strains survived at statistically significantly higher levels than the *E. coli* strain ($p < 0.01$) but none of the *Salmonella* strains were different from each other.

After taking the \log_{10} of each concentration of bacteria, and subtracting the concentrations of bacteria in the oysters at 72 h from the concentrations at 24 h and dividing by the time elapsed, a rate of clearance can be derived from this depuration data. For the *Salmonella* strain, a clearance rate of 0.006 log-CFU/g per hour was observed, while *E. coli* was cleared over 4 times faster with a rate of 0.025 log-CFU/g per hour.

3.6. Vertical transmission

The results of our experiments showed that there was no *Salmonella* detected in any of the egg samples that were collected after the oysters were induced to spawn, despite the fact that the parental oysters carried an average of 5.6×10^4 CFU/g of *Salmonella* Newport within their tissues.

4. Discussion

In designing the experiments of this study it was assumed that oysters infected with *Salmonella* are exposed to this pathogen via contaminated estuary water, which was itself polluted by either agricultural, industrial, or urban runoff. It would be impossible to conduct experiments in the actual environment these contamination events occur, so in order to further examine the relationship between oysters and *Salmonella*, we established laboratory models. The first such experimental setup involved water samples that included all of the natural microbiota, flora, and fauna that could help or hinder the survival of enteric bacteria, from a representative watershed that was previously positive for *Salmonella* (unpublished details from Brands et al., 2005a). The second experimental model developed was a method in which laboratory-maintained oysters could be reliably and consis-

tently exposed to enteric bacteria and the relative survivability of each strain of bacteria within the oysters could be quantified.

When examining the survivability of these enteric bacteria in aquatic environments relevant to oysters, all three strains of *Salmonella* Newport could survive at higher concentrations than *E. coli* in these sample aquatic environments, but none of the bacteria survived for 12 days. The results in the various water samples establish a baseline for the bacteria's ability to survive within water alone, and when compared to the survival data within oysters, help to illustrate that the oysters themselves play a significant role in *Salmonella*'s ability to survive in this model environment.

In order to quantify *Salmonella*'s ability to survive within oysters, a simple, yet effective method of exposure and controlled maintenance of the oysters was refined in preliminary experiments and used to compare the survivability of various strains of enteric bacteria within oysters. Because the oysters were maintained in closed tanks with a slow flux of water for all but the depuration experiments, water samples were tested for bacteria alongside the oyster homogenates. Because no viable bacteria were ever detected in the water samples, the results show that the numbers of viable bacteria enumerated in these experiments originated only from within the oyster tissues and were not due to any residual survival within the oysters' expelled excrement or the tank water alone.

One of the key conclusions from these oyster survival studies was that the genotype of Newport found so predominantly in the 2002 survey by Brands et al. (2005a) was no better at surviving within oysters or the related water samples than any of the other tested *Salmonella* isolates. When we compared our oyster isolate to bovine and human isolates of Newport with different PFGE profiles in water samples and in depurated oysters there was no significant difference found. When we compared two other strains of Newport along with three other commonly isolated serovars of *Salmonella* to our oyster isolate in their long-term survival in oysters, there also was no difference found between any of the strains. Unfortunately, the reasons behind the predominance of that one particular genotype in the 2002 study (Brands et al., 2005b) still remains elusive. We did perform studies that verified that the enrichment process used in the original study (Brands et al., 2005b) did not artificially select for that particular genotype (data not shown).

The depuration experiment is of particular interest because the results appear, on the surface, to contrast a study that found complete elimination of a *Salmonella* Typhimurium strain after just 12 h of depuration (de Abreu Corría et al., 2007). However, if you calculate a clearance rate for their results, based on their maximum observed starting concentration of 2×10^4 CFU/g, you find that their clearance rate was approximately 0.09 log(CFU)/g per hour, a rate that is significantly higher than our observed rate of 0.006 log(CFU)/g per hour, but reasonable when you take into consideration that their flow rate was nearly 20-times higher than ours. It seems reasonable to assume that clearance rates would be proportional to the flow rate used within the depuration system, but it is clear from the comparisons between *Salmonella* and *E. coli* in this study, that not all bacteria are cleared at the same rate, presumably due to differing mechanisms of persistence. Besides showing the relative difference in depuration dynamics between our strains of *Salmonella* and *E. coli*, the depuration results also confirm the repeatability of our exposure method. The depuration experiments were performed at a different facility in Oregon under flow-through maintenance conditions using a natural source of seawater, but the oysters were found to have levels of contamination consistent with the static tank studies conducted in Arizona with artificial seawater.

Perhaps the most important conclusion of this study, and the one that raises the most questions, is that there is a significant difference between the abilities of the pathogenic *Salmonellae* to survive in oysters and a non-pathogenic strain of *E. coli*. In both the static and depuration systems, the concentration of *E. coli* was at least 100-times lower than that of *Salmonella* Newport after the oysters were exposed

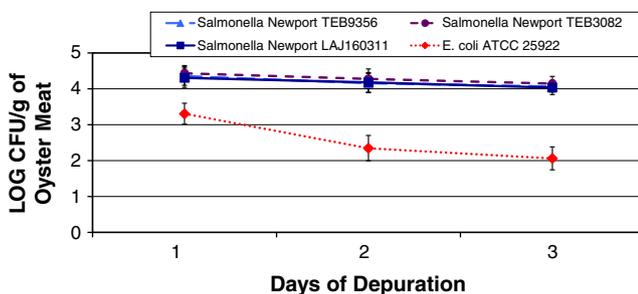


Figure C4

Fig. 4. Survival of *Salmonella* and *E. coli* in depurated oysters. Pacific oysters, *Crassostrea gigas*, were exposed to three strains of *Salmonella* Newport, one of human origin TEB9356, one from bovine origin TEB3082, and one isolated from oysters LAJ160311, and *E. coli* ATCC 25922 and then placed in depuration tanks with an influent flow rate of 120 L/h. Every 24 h days for 3 days, the numbers of viable bacteria remaining within the oysters were enumerated. The mean of three independent replicates \pm 95% confidence intervals are displayed.

to similar amounts of bacteria. These results imply that genetic differences between *Salmonella* and this strain of *E. coli* are important to *Salmonella*'s survival within oysters, and suggest an explanation for the inability of coliform testing to reliably predict the presence of *Salmonella* in oysters, a phenomenon that was observed in the study by Brands et al. (2005b) and by others (Catalao Dionisio et al., 2000; Wilson and Moore, 1996).

From an evolutionary biology perspective, this study also raises interesting questions about the nature of the interactions between *Salmonellae* and oysters, because it appears that *Salmonella* is capable of long-term persistence within or even a colonization of an invertebrate host, a type of interaction that has typically been associated only with higher order animals. There are many potential factors that are found in *Salmonellae* but not in *E. coli* that could provide the pathogen greater fitness within oysters, but the most notable, and the ones that warrant immediate further study, are the well-characterized type three secretion systems that *Salmonellae* use to infect host cells. Further study into the mechanisms behind this interesting and important host-microbe interaction could provide useful insights into a possibly overlooked aspect of *Salmonella*'s biology.

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**APPENDIX D: PREVALENCE AND CHARACTERIZATION OF
SALMONELLA SPP. IN CLAMS, OYSTERS AND ENVIRONMENTAL
SAMPLES FROM TWO PREVIOUSLY POSITIVE LOCATIONS**

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Abstract

Salmonella is an important foodborne pathogen responsible for 1.4 million cases of illness in the US annually. Oysters, many of which are consumed raw, pose a significant health risk because of their ability to concentrate pathogens such as *Salmonella* from the waters in which they are cultured. In this study we monitored oysters, clams, and water samples from upstream areas and two bays from which *Salmonella* positive oysters had previously been collected. *Salmonella* serovars Newport, I 4,[5],12:i:- and Typhimurium were sporadically isolated from oysters and clams. The multidrug resistant Newport serovar was consistent with the strain previously isolated from these bays, indicating a persistent reservoir for this strain. No seasonality or significant weather events were associated with the isolation of *Salmonella*. No water samples were found to be positive for *Salmonella*, this indicates the importance of testing oyster meat which may be consumed raw prior to distribution.

Introduction

The CDC estimates that 48 million illnesses, 128,000 hospitalizations, and 3,000 deaths annually are attributable to foodborne illnesses. In 2010, their Foodborne Diseases Active Surveillance Network (FoodNet) reported 2,290 hospitalizations and 29 deaths attributable to *Salmonella* (3). Because illnesses are not always laboratory confirmed, it is suspected that there are actually 1.4

million cases of salmonellosis annually in the US (21). Rates of infection caused by *Salmonella* are currently higher than any other bacterial foodborne pathogen in the US and have not declined significantly in more than ten years, leading to direct medical expenditures of \$365 million annually (3). *Salmonella* infection does not uniquely affect the US, and salmonellosis is recognized as a common cause of gastrointestinal illness worldwide.

While the most common identified sources of *Salmonella* infection in humans are birds and poultry, or various forms of fresh produce, shellfish may be an underreported and underappreciated source of the bacterium in the food supply. Shellfish in general have come to be known as a high risk food, due to their carriage of a variety of viral and bacterial pathogens. Oysters in particular pose a significant risk because of the likelihood that they will be consumed both raw and whole (4, 11, 15, 23). It is estimated that 50% of the more than 69 million pounds of oyster meat eaten annually in the US is consumed raw (1, 15, 18).

Because oysters are filter feeders known to concentrate not only food particles, but associated toxins and pathogens (11), regulations have been introduced to increase the safety of this food product. The cooperative state and federal National Shellfish Sanitation Program recognized by the Food and Drug Administration (FDA) provides guidelines for state and international regulatory agencies, including a model ordinance which may be adopted directly (as it has been in Oregon) to regulate shellfish cultivation and distribution in the interest of

food safety (2, 14). These guidelines include routine testing for coliforms, which are considered indicator organisms for other bacterial pathogens, but not specific routine monitoring for *Salmonella* spp.

Despite these regulations, *Salmonella* has been found on multiple occasions in oysters. A Spanish study found that 2.4% of 269 oysters tested were positive for *Salmonella*, but below European Union guideline levels for coliforms (20). A previous study in our lab found a *Salmonella* prevalence of 7.4% in oysters taken from 36 US bays. No correlation was found in that study between *Salmonella* and fecal coliform levels (9). In a year-long survey of nine US states, the FDA found 1.5% of samples to be culture positive, while 8.6% of samples were positive for *Salmonella* by real time PCR.

Several studies have noted seasonal variations in the prevalence of *Salmonella* in oysters, though this variation has been inconsistent, with increased prevalence being reported more commonly in the winter months (6, 9, 20, 32). Increased prevalence and diversity of serovars has also been associated with storm and other weather related events (6, 19, 34). However, an original source for predominant serovars has not often been identified.

A 2003 study found an overall *Salmonella* prevalence of 7.4% in tested market oysters from multiple bays in the US. The two bays from this study were included in that survey. That study additionally found that the majority of the isolates were *S. Newport* (77%), and the overwhelming majority of those (98%)

were of a single PFGE type (LAJ160311), indicating multiple reservoirs for this strain (9). The purpose of this study is to examine variations in *Salmonella* presence and serovar type within this economically important Pacific oyster production area, in order to better understand the complications posed by this foodborne pathogen for oyster production, and variables which may contribute to the contamination of growing waters.

Materials and Methods

Oyster and clam processing and *Salmonella* isolation. Pacific oysters (*Crassostrea gigas*) and soft-shell clams (*Mya arenaria*) were obtained from two bay areas in Oregon, USA over a three year period from 2008 to 2010. Oysters from bay area 1 were sentinel animals taken from the Hatfield Marine Science Center in Newport, Oregon; these were placed at their collection locations six months prior to the beginning of sampling. Oysters from bay area 2 were obtained from commercial suppliers. Native clam samples were sent monthly, when available, and were included to allow sampling of areas with lower salinity than is tolerated by oysters. All oysters and clams were packed with sealed ice packs, and were delivered and processed within 48 hours of removal from the aquatic environment. Upon delivery, the oysters and clams were rinsed individually in DI water confirmed to be negative for *Salmonella*, and any dead shellfish were discarded. The shellfish were processed using a modified version of the FDA's

Bacteriological Analytical Manual (BAM) protocols. These modifications have been used in previous studies (10), and allow processing of shellfish samples with less incubator space at a lower material cost. *Salmonella* detection within oyster meat did not differ when standard and modified BAM protocols were compared (unpublished data).

Oysters and clams were shucked using sterile shucking knives. Meat from individual oysters and clams was transferred into 25 ml of lactose broth (BD, Sparks, MD). The tissue was homogenized using an Ultra-turrax tissue homogenizer (IKA, Werke Stauffen, Germany) at ~15,000 rpm until a liquid consistency was obtained. Homogenates were incubated aerobically at 37°C for 24 h. Following primary enrichment, one milliliter of the lactose broth homogenate was transferred to 10 ml of Tetrathionate Broth (TT) (BD) containing potassium iodide and incubated aerobically at 37°C for 24 h. 100µl of the second enrichment was streaked onto xylose lysine deoxycholate (XLD) agar (BD) plates, which were incubated aerobically at 37°C for 24 h.

Water sample processing. Water samples were obtained at the time of oyster collection from the same location and from additional locations upstream. One liter samples were filtered through .45µm filters (Millipore, Billerica, MA). Filters were placed in 10 ml of lactose broth and incubated at 37°C for 24 h. Further enrichment and plating was performed as with oyster and clam samples.

PCR and serotyping. Black colonies on XLD, indicative of *Salmonella*, were confirmed by PCR using the primers SHIMA-L (5'-CGTGCTCTGGAAAACGGTGAG) and SHIMA-R (5'-CGTGCTGTAATAGGAATATCTTCA), which target the *Salmonella*-specific *himA* gene (8). *Salmonella* Newport isolates were serotyped using commercial antisera (BD, Sparks, MD) specific for the O and H antigens of *S. Newport*. The Animal Public Health Inspection Services in Ames, IA, USA performed all additional serotyping.

Antimicrobial resistance tests. *Salmonella* isolates were tested for antimicrobial resistance using the Kirby-Bauer disc technique (7) on Mueller-Hinton agar (BD, Sparks, MD). Antimicrobial disks (BBL Sensi-Discs, BD) used in testing were: amikacin (30 µg), ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10µg), nalidixic acid (30 mg) streptomycin (10 µg), tetracycline (30 µg), and trimethoprim/sulfamethoxazole (1.25/23.75µg). Diameters of the zones of inhibition were measured and resistance was determined based on the manufacturer's specifications. Quality control for the antimicrobial tests was performed per the manufacturer's instructions, using *Escherichia coli* ATCC 25922.

Pulsed-field gel electrophoresis. PFGE was performed on isolates using a PulseNet protocol and XbaI for restriction digestion (25).

Results

Oyster and clam samples. Oyster samples from the bay 1 area were positive for the presence of *Salmonella* only from April to August of 2009 (samples were not collected in July). For this four month sampling period, 26/80 (32.5%) individual oysters were found to contain *Salmonella*. In the first month only serovar I 4,[5],12:i:- was present, however, in subsequent months serovar Newport was also present in a total of 9/26 isolates.

Oysters from bay area 2 were positive on two occasions, each in February of two separate years. In 2008, 9/20 oysters were positive, and a single serovar, Newport, was isolated. In 2010, 2/6 oysters were positive, and a different serovar, Typhimurium, was isolated.

Only a single clam, from the bay 2 area, was found to contain *Salmonella*. This clam was collected in June of 2008, the first year *Salmonella* was isolated from oysters in bay 2, however, the serovar was consistent with that isolated in 2009, I 4,[5],12:i:- (Table D1).

Water samples. None of the tested water samples were positive for the presence of *Salmonella*.

PFGE and antimicrobial resistance. All I 4,[5],12:i:- isolates were found to be of a single PFGE type, and all shared an identical antibiotic susceptibility profile. I 4,[5],12:i:- isolates were susceptible to all agents with the exception of tetracycline and nalidixic acid, to which they had intermediate resistance. All Newport isolates were also found to be of a single PFGE type, which was consistent with *Salmonella* previously isolated from oysters at these locations (LAJ160311). All *S.* Newport isolates were resistant to ampicillin, amoxicillin-clavulanic acid, chloramphenicol, ceftriaxone, naladixic acid, streptomycin, and tetracycline but were susceptible to all other agents tested. These results are consistent with the LAJ160311 strain. Typhimurium isolates were of a single PFGE type, and were resistant to streptomycin and intermediately resistant to amikacin and nalidixic acid. Typhimurium isolates were susceptible to all other tested antimicrobials.

Discussion

Salmonella serovars isolated in this study are of significant clinical importance. In 2010 the CDC serotyped a total of 7,564 human isolates. Of these, the second most common serovar was *S.* Newport (1,079 isolates) and the fifth most common was *S.* I 4,[5],12:i:- (256 isolates) (12). *S.* I 4,[5],12:i:- is a monophasic variant of *S.* Typhimurium characterized by the deletion of the flagellar genes *fliA* and *fliB* (26). *S.* Newport was previously detected in a

nationwide study of oysters and is of particular concern as it is often, and in this case, multidrug resistant. Multidrug resistant strains of *Salmonella*, including *S. Newport*, have been associated with increased human morbidity and mortality (17, 31, 33).

The presence of *Salmonella* in environmental samples is highly variable and is influenced heavily by contamination events and other variables such as weather (24, 29). Thus, the continued presence of multidrug resistant *S. Newport* in oysters from this geographic region points to a reservoir of the bacteria, which is periodically released into the aquatic environment. On two occasions sampling of restaurant oysters in Tucson, Arizona also isolated *S. Newport* consistent with LAJ160311 from oysters. One of these restaurants served exclusively Eastern oysters (*Crassostrea virginica*) which are not grown in the Pacific Northwest, indicating the persistence or recurrence of this organism in other estuarine environments as well (10).

The fact that *Salmonella* was not detected in water samples from the same area as the contaminated shellfish either before or during the months in which the *Salmonella* isolates were collected indicates that the level of contamination in the water was probably very low, and it is only the concentration of *Salmonella* by filter feeding animals that allows their detection in this case. This is of critical importance, as oysters are not routinely tested for this pathogen before they are distributed for raw consumption. This evidence suggests that in order to

establish and maintain the safety of this food supply, oysters destined for raw consumption should periodically be tested for *Salmonella*.

Unlike some previous studies, no major weather events were associated with any of the time periods during which *Salmonella* positive samples were collected. Because two serovars of *Salmonella* were present in both bays, common environmental sources are suspected. *Salmonella* has previously been isolated from vertebrate species which live in or have contact with the aquatic environment. Pinnipeds (seals, sea lions, otters) are of particular interest here as they have previously been found to carry *Salmonella* and frequently consume mollusks such as oysters. These animals may therefore be significant in both the amplification and distribution of *Salmonella* (22, 27, 28). Human fecal contamination, for example from leaking septic tanks, has also been suspected in previous studies in which serovars of clinical importance have been isolated (24). Given the consistency and geographic diversity of *S. Newport* isolation from oysters this seems an unlikely source of this particular serovar, but it cannot be ruled out entirely as a source of *S. I 4,[5],12:i:-*, as this strain has been increasingly reported from human infection (26).

S. Newport has frequently been associated with cattle (5, 16). Cattle and calves are listed as the top livestock inventory items in the counties in which both bay 1 and bay 2 are located, with one county housing tens of thousands of head of cattle (30). Fecal shedding of *Salmonella* by cattle can persist up to 391 days

(13), making dairy and beef production facilities a potential year round source of *Salmonella*. These facilities may contaminate growing waters by way of untreated bovine feces in runoff. These results may indicate that monitoring of cattle production facilities should be included in future efforts to determine the source of *Salmonella* contamination of oysters.

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Table D1: Overview of *Salmonella* isolated from oyster and clam samples.

Date	Location	Sample Type	Number Positive	Serovar
February, 2008	Bay 2	Oyster	9/20	Newport
June, 2008	Bay 2	Clam	1/20	I 4,[5],12:i:-
April, 2009	Bay 1	Oyster	11/20	I 4,[5],12:i:-
May, 2009	Bay 1	Oyster	3/20	I 4,[5],12:i:- / Newport
June, 2009	Bay 1	Oyster	7/20	I 4,[5],12:i:- / Newport
August, 2009	Bay 1	Oyster	5/20	I 4,[5],12:i:- / Newport
February, 2010	Bay 2	Oyster	2/6	Typhimurium