COMPETITIVE INHIBITION BACTERIA OF BOVINE ORIGIN AGAINST *SALMONELLA* SPP.

by

MICHELLE DAWN DANYLUK

(Under the direction of Michael P. Doyle)

ABSTRACT

108 fecal samples were obtained from calves, dairy and beef cows. *Salmonella* spp. was isolated from 9.25% of samples. Of 1097 bacterial isolates from cattle feces screened, 30 demonstrated anti- *S*. Typhimurium DT104 activity in vitro. Isolates included 22 *Escherichia coli*, 6 *Bacillus circulans*, 1 *Serratia fonticola*, and 1 *Enterobacter cloacae*. Identification by pulsed-field gel electrophoresis revealed 19 distinguishable profiles among the 24 gram-negative isolates. Competitive inhibition isolates did not significantly reduce *S*. Typhimurium DT104 during 21 days of storage at 37°C in cattle feces. *B. circulans* (10^5 CFU/g inoculum) significantly reduced *S*. Newport on days 3 and 5 and at day 21 with a 10^8 CFU/g inoculum at 37°C. At 21°C, significant reductions of *S*. Typhimurium DT104 occurred due to 10^8 CFU/g of gram-negative competitive inhibition bacteria and 10^5 CFU/g of *B. circulans* at day five only. No significant reductions were seen with *S*. Newport at 21°C.

INDEX WORDS: *Salmonella*, *Salmonella* Typhimurium DT104, *Salmonella* Newport, Competitive Inhibition, Competitive Exclusion, Cattle, Feces

COMPETITIVE INHIBITION BACTERIA OF BOVINE ORIGIN AGAINST *SALMONELLA* SPP.

by

MICHELLE DAWN DANYLUK

BSc with Specialization, University of Alberta, Canada, 2000

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2002

COMPETITIVE INHIBITION BACTERIA OF BOVINE ORIGIN AGAINST *SALMONELLA* SPP.

by

MICHELLE DAWN DANYLUK

Approved:

Major Professor: Michael P. Doyle

Committee: Larry R. Beuchat

Jinru Chen

Electronic Version Approved:

Maureen Grasso

Dean of the Graduate School

The University of Georgia

December 2002

DEDICATION

I would like to dedicate this thesis to the Bank of Mom and Dad. Thanks for not drying up just yet.

ACKNOWLEDGMENTS

I have been influenced by so many people, this page does little credit to recognize and thank all those who have made a mark in my life thus far…

Dr. W. Page, my first exposure to microbiology was through you. It was your class, and perhaps your cow drawing, that convinced me I didn't want to be a biochemist, and was influential in my switch in to Microbiology.

Dr. M.E. Stiles, who taught my first course in Food Microbiology, foodborne pathogens, that opened my eyes to a whole new way of looking at things, and introduced me to an area that I wanted to be a part of.

Drs. M.B. and B.R. Yacyshyn, for the two summers they welcomed me into their lab and their hearts. Bruce especially for showing me the world of a MD. Mary-Beth, for all her advice, and most importantly showing me that it is possible to have both a family and a career, and to be successful at both.

Dr. M.P. Doyle, for allowing me to be one of the few students to enter your lab. Your guidance, support, insight and encouragement have been in key in getting me through the last year.

Dr. L.R. Beuchat and Dr. J. Chen for being on my advisory committee, and all your help through this learning process.

Dr. G. Siragusa, for all the advice and learning experiences you provided me with during my short time in your lab.

Dr. R. Shewfelt, for being someone who just understood, and an awesome college bowl coach. I learnt so much about food science because of you.

For all the professors at the University of Georgia who taught me how to think like an engineer, a processor, a packager, and all the other disciplines that encompass this field we call food science.

The faculty and staff at the griffin campus, especially: Larry Goodridge, Barb Adler, Lynne Thurber, Rhonda Howell, Dr. Rob Blodgett, Ping and Tong Zhao, Allen Scouten, Kim Hortz, Sonya Lambert, Joy Adams, Dr. Lin, Dr. Yan, Kristen Bray, Bobby Goss, and The Crap Collectors: David Wilkerson, Brent Crawley, and Dawn Wilkerson

The students who became my family away from home: Wendy Wade, Jamie Rudlof, Christine Chu, Michelle Kazmerski, Janice Hunter, Joy and Brad Wright, Hannah Osborn, Jared Henderson, Manan Sharma, Megan Lang, John Allen, Steve Kenney, Mei Yang, and Josh Gurtler.

My surrogate mothers, Auntie Rene in Edmonton and Auntie Gail in Florida, for offering an escape when things got crazy, and for always making me feel at home.

My friends, Diana Snell, Rachel Parker, and Tirian Eynon, for putting up with me through the good times and the bad, and letting be know that no mater what, no matter where we are in the world we will be there for one another.

My brothers, Mark and Robert, for the jokes and laughs, putting up with my phone calls, bring me back to reality, and never letting me take my self too seriously.

And finally my parents, whose love and support is unfailing and has allowed me to become the person that I am today. They taught me the value of hard work and the necessity of a good education, as well as always allowing me to follow my dreams towards making myself happy. And of course how would I have ever survived without the Bank of Mom and Dad.

TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION

In the 1980s, surveillance data of cattle and human isolates indicate that *Salmonella enterica* serovar Typhimurium DT104 emerged worldwide. *S*. Typhimurium DT104 typically is resistant to the antibiotics ampicillin, chloramphenicol, streptomycin, sulphonamides and teracycline (Threlfall et al. 1994). Currently, data suggest that a multi-resistant *Salmonella enterica* serovar Newport is emerging in the United States (MMWR Weekly 2002). *S*. Newport typically is resistant to at least nine antibiotics. Recent studies by Zhao et al. (2002) revealed that 3.5% of retail ground beef was positive for *Salmonella* spp. of which 35.6% was *S*. Typhimurium DT104. Between January and April 2002, a five-state outbreak of *S*. Newport occurred. Exposure to raw or undercooked ground beef was implicated as the vehicle of transmission (MMWR Weekly 2002). Cattle are thought to be a primary reservoir through which both these multi-resistant pathogens can enter the food supply.

Surveys of feedlot cattle in the United States done in 1998 revealed that 38% of feedlots were *Salmonella* spp. positive, and 5.5% of all fecal samples collected were positive for *Salmonella* spp. *S*. Typhimurium DT104 was detected in 2.6% of the feedlots, and 2.9% of the positive fecal samples (Fedorka-Cray et al. 1998). A similar study conducted on beef cattle in 2000 revealed that 11.2% of all operations tested positive for *Salmonella* spp., and 1.4% of all fecal samples were positive (Dargatz et al. 2000). There are clear associations between *S*. Typhimurium DT104 infection in food production animals and humans (Fone and Barker 1994, Wall et al. 1994, Wall et al 1995, Davis et al. 1996).

The administration of probiotic bacteria is an approach to reduce carriage of enteric pathogens through competitive exclusion. Successful studies with competitive

exclusion bacteria have prevented growth of certain *Salmonella* spp. in chickens (Rantala and Nurimi 1973, Pascia; et al. 1999. Audisio et al. 1999, Seo et al 2000, van der Wielen et al. 2002). Competitive exclusion bacteria have also successfully reduced the duration of ruminal carriage and shedding in feces of *Escherichia coli* O157:H7 in cattle (Zhao et al 1998). The objective of this study was to isolate and identify potential competitive exclusion bacteria from the feces of cattle that are not shedding *Salmonella* spp. and evaluate them for anti-*S*. Typhimurium DT104 activity in vitro and in cattle feces artificially contaminated with *S*. Typhimurium DT104 and *S*. Newport. *Salmonella* spp. isolated from cattle were also serotyped.

CHAPTER 2

LITTERATURE REVIEW

Introduction

Typhoid fever, the first salmonellosis identified, was defined by clinical symptoms before the bacteria was identified. Once the bacteria were clearly defined, their transmission, pathogenesis, control and treatment could be studied. Eberth first observed the typhoid bacillus in 1880, while examining the spleen and mesenteric lymph nodes of a patient who died of typhoid fever (Le Minor 1981). Koch verified the finding that same year. However, it was not until 1884 that the bacteria were successfully cultured by Gaffy (Le Minor 1981), and 1886 that Widal developed agglutination tests to be used as a serodiagnosis of typhoid fever (Le Minor 1981). It was found that some patients having signs of typhoid fever were serodiagosed as negative. Bacteria cultured from these patients were distinct from the typhoid bacillus, both culturally and serologically, leading to two independent findings of paratyphus bacillus A and B, in 1896 and 1898, respectively (Le Minor 1981).

Bacteria resembling the paratyphus bacillus were isolated from diseased animals. In 1885 Salmon and Smith isolated a microbe from pigs with hog cholera and named it *Bacillus cholerae-suis*, as they assumed it was the causative agent of hog cholera (Le Minor 1981). Similar bacteria were isolated from patients with foodborne intoxication by Gaffky and Paak in 1885, from meat in 1888 by Gärtner, and other animal diseases in 1892 by Loeffler. In 1900, Lignieres created the bacterial genus *Salmonella* to include all of these organisms. It was named after Dr. Salmon, the USDA bacteriologist who first described *Salmonella cholerae-suis* (Le Minor 1981).

Members of the family Enterobacteriaceae, *Salmonella* spp. are facultatively anaerobic, gram-negative rods. Most members are motile due by peritrichous flagella;

however, some nonflagellated strains and strains with dysfunctional flagella exist, and are not motile. Salmonellae are chemoorganic, with the ability to metabolize nutrients by both respiration and fermentation. Several biochemical characteristics enable the identification of *Salmonella* spp. They catabolize D-glucose into acid and gas, are oxidase-negative and catalase-positive, capable of growth on citrates as the sole carbon source, cannot utilize the sugars lactose or sucrose, and are unable to hydrolyze urea. As well, hydrogen sulfide can be produced and lysine and ornithine are decarboxylated into alkaline by-products (D'Aoust 1997).

The optimum growth temperature for *Salmonella* spp. is between 35ºC to 37°C, but they are capable of growth between 4ºC and 54ºC (D'Aoust 1991). Psychrotrophic growth can also occur between 2°C and 4°C in various food matrices (D'Aoust 1991). Growth can occur within the pH range of 4.5 to 9.5, with the optimum being between pH 6.5 and 7.5. Currently, it is accepted that *Salmonella* will not grow in foods that maintain a water activity below 0.93. Three to four percent sodium chloride (NaCl) also inhibits the growth of *Salmonella*; however, an increase in growth temperature moderates this effect (D'Aoust 1997). The time and temperature required to kill 90% of viable organisms, or the D-value, varies depending upon the food medium (Jay 2000).

Classification

Simple classification of *Salmonella* spp. done for strictly epidemiological purposes divides the species into three groups (Jay 2000). The first group includes salmonellae that infect humans only and are the agents of typhoid and paratyphoid fevers. *S.* Typhi, *S*. Paratyphi A, and *S* .Paratyphi B fall into this group. The second group is

composed of host-adapted serovars, of which some are human pathogens that can be contracted through foods. Examples of this group include *S*. Gallinarum from poultry and *S*. Dublin from cattle. Serovars in the third group have no host preference (unadapted), and are pathogenic to both humans and animals. The majority of foodborne serovars are in this group.

Antigenic analysis of *Salmonella* began as early as 1902, when Castellania described a method for absorbing antisera (Le Minor 1981), and continued in 1903 when somatic and flagellar antigens were differentiated by Smith and Reagh (Le Minor 1981). In 1918, these somatic and flagellar antigens were renamed O and H, antigens, respectively by Weil and Felix (Le Minor 1981). White developed the first antigenic scheme for *Salmonella* in 1926, which was further developed by Kauffmann (Le Minor 1981). In 1941, using the Kauffman-White scheme, there were 100 serotypes of *Salmonella* spp. (Le Minor 1981), whereas of 1998, this number increased to 2435 (Brenner and McWhorter-Murlin 1998).

The nomenclature for *Salmonella* spp. is complex. Five subgenera, I through V, are defined, and serovars are given species status. Subspecies I (*S. enterica* subsp. Enterica) is comprised of the majority of human isolates, found in the first nine O groups, with *Salmonella* ser. Typhimurium and *S*. ser. Enteritidis being the most common. Also included are *S*. ser. Typhi, *S*. ser. Paratyphi, and *S*. ser. Choleraesuis. Subspecies *II (S. enterica* subsp. Salamae) is comprised of serotypes in O groups F11 through 67. Subspecies III includes two groups, IIIa (*S. enterica* subsp. Arizonae) for monophasic strains and IIIb (*S. enterica* subsp. Diarizonae) for diphasic strains. These strains were formerly classified in the genus *Arizona*. Subspecies IV (*S. enterica* subsp. Houtenae) are

usually isolated from cold-blooded animals and the environment, and are in O groups F11 through 67. Subspecies VI (*S. enterica* subspecies Indica) have been isolated from warm-blooded and cold-blooded animals and the environment, but rarely from humans. Most of the serotypes of subspecies VI previously belonged to subspecies I or II. *S. bongor* was described as subspecies V, until 1989 when Reeves et al. (1989) proposed a second species. The designation of V is still used for this species to avoid confusion (Brenner and McWhorter-Murlin 1998).

Serotypes of *Salmonella* spp. are based on their O (somatic), Vi (capsular), and H (flagellar) if present, antigens. Names of new serotypes belonging to subspecies I are given based on the geographic location where the serotype was first isolated. Unnamed serotypes are designated by their antigenic formula. Following 1966, new serotypes in subspecies II, IV, V, and VI are not named but designated by their antigenic formula. All pre-1966 names for these subspecies have also been eliminated, and are now also designated by their antigenic formula (Brenner and McWhorter-Murlin 1998).

Phage typing is the method for subtyping *Salmonella* spp. that has the longest history. Initially developed by Felix and Callow in 1943 and designated Scheme 1, it was extended to Scheme 2 in 1959 by Callow. The main identification system used worldwide today is a refined form of Scheme 2, known as the Anderson phage-typing scheme. It was developed by Anderson et al. in 1977. The basis of this typing system is a collection of *Salmonella* phages, which were propagated on particular hosts of the species *S.* Typhimurium. All phage typing done today is done using aliquots of the stock phages obtained decades ago (Schmieger 1999).

Modern subtyping methods, based on the characterization of genotype of the organism, are currently being developed. There is currently no "gold standard" for molecular typing of *Salmonella.* Studies have recently been completed evaluating the efficiency of plasmid profiling, ribotyping, pulsed-field gel electrophoresis (PFGE) (Liebana et al. 2001), random amplified polymorphic DNA analysis (RAPD) (De Cesare et al. 2001), and multiplex polymerase chain reaction (Khan et al. 2000) to detect and identify different serotypes of *Salmonella* spp. No consensus has been reached on the most discriminatory method for differentiating strains.

Reservoirs

Salmonella spp. are widespread with in the environment. Their primary habitat is the intestinal tracts of birds, reptiles, animals (especially those on the farm), humans, and occasionally insects. They may also be found in other parts of the body from time to time, for example in the spleen, liver, bile, mesenteric and portal lymph nodes, diaphragm, and pillar in slaughterhouse pigs (Jay 2000).

Serovars that cause human salmonellosis are most often found in foods of animal origin, such as pork and poultry meats, and dairy products (Oosterom 1991). The persistence of salmonellae in slaughterhouses and meat processing facilities continues due to the exposure of livestock to environmental sources of contamination, contaminated feeds, and parental transmission of infection (D'Aoust 1997). The feces of infected humans and animals contaminate water sources, which subsequently infect farm animals, that contaminate meat during slaughter, and subsequently infect humans, beginning the cycle anew (Jay 2000). This cycle is augmented by the practice of international shipping

of animal products and feed, which has lead to the worldwide distribution of salmonellosis (Jay 2000).

Different parts of the world are predominated by different serovars of *Salmonella* (D'Aoust 1989). Serovars such as *S.* Typhimurium, and *S*. Enteritidis are found consistently throughout the world, whereas other serovars can be found with high frequency for short periods of time, then all but disappear (D'Aoust 1989). Certain serovars are associated at a high frequency with specific foods, such as *S.* Enteritidis in eggs.

Antibiotic Resistance and *Salmonella* spp.

Development of antibiotic resistance in bacteria is based on two main factors, selective pressure by the use of antibiotics and the presence of resistance genes. Resistance genes may arise from mutations within the chromosome, or by acquisition of genes coding for a resistance mechanism. Resistance is disseminated by either clonal spread of the resistant strain, or by spreading of the resistance genes through plasmid transfer, transposition or integrons (Witte 2000).

Concern about antibiotic resistance in human pathogens has been on the rise over the last three decades, and has increased in intensity in the last five years (Barton 2000). Increased applications of antimicrobial agents over the last fifty years have created an ecological imbalance. The application of about half of the world's antibiotics is in agriculture (Teuber 1999).

Antibiotics are used for three main purposes in animals: therapeutic use to treat sick animals, prophylactic use to prevent infection, and as growth promoters to improve

feed utilization and production. Therapeutic treatment generally involves treatment of individual animals over short periods with doses exceeding the minimal inhibitory concentration (MIC) of the known or expected pathogen. The antibiotic is often delivered through feed or drinking water, which leads to doubtful efficacy as sick animals often do not eat or drink. Prophylactic treatment involves moderate to high doses of antibiotic, usually given to a group of animals in feed or water for a defined period. Antibiotics used as growth promoters are given at subtherapeutic levels in feed over extended periods to entire herds and flocks. Many of these antibiotics are available for purchase by feed manufactures over the counter (Teuber 1999, Barton 2000).

The development of antibiotic resistance in farm animals can be evaluated in two ways. The first is to analyze the resistance of bacteria isolated in food hygiene and animal health investigations. This approach enables collection of a large number of bacterial strains unbiased by the origin from which the animal was produced and the processor who processed food from the animal. These investigations often result in significant data regarding the type of bacteria and their resistances, but lack critical information such as the amounts of antibiotic applied, due to lack of control at the farm. Results from this approach reflect the overall effects of antibiotic uses for different purposes in a specific agricultural environment. The second approach involves analysis of resistance development under experimental conditions controlling all factors involved. This method had been applied in surveillance experiments with control of both antibiotics and animals in both prospective and retrospective studies. These experiments can only be done on a limited quantitative basis, which may not reflect the actual situation (Teuber 1999).

Resistance to antibiotics associated with the use of antibiotics in animals leads to several concerns. The first of which is the transfer of antibiotic-resistant pathogens through the food chain and the associated risk of transferring these resistance genes from the enteric flora to strict human pathogens. The second is the reduced efficacy of antibiotic therapy in animals colonized with resistant bacteria (Barton 2000).

There is evidence for the spread of antibiotic resistance among *Salmonella* spp. since the application of antibiotic use in human and veterinary medicine in 1948. One hundred *S*. Typhimurium isolates obtained in the USA from farm animals (fowl) and humans between 1940 and 1948 were all sensitive to tetracycline. Hence, they provide baseline information for the development of resistance. Within 50 years of the application of tetracycline into human and veterinary medicine as well as its use for growth promotion, resistance to tetracycline has increased to 90% of isolates. Tetracycline has subsequently lost its usefulness to treat disease caused by *S*. Typhimurium (Teuber 1999).

A study from October 1, 1999 through September 30, 2000 of 502 *Salmonella* isolates from domestic and imported foods screened against 12 antimicrobial agents revealed that 49.2% were resistant to one or more antimicrobial agents (Kiessling et al. 2002). None of the isolates was resistant to norfloxacin, and only one to ciprofloxacin. This study provides a baseline for determining the evolution of antibiotic resistance in *Salmonella* spp. in the U.S. food supply in the future.

Antibiotic resistance of *S.* Typhimurium has become more serious with the spread of an epidemiologically predominant strain, identified as *S.* Typhimurium definitive type DT104.

Salmonella Typhimurium DT104

Salmonella Typhimurium DT104 was first identified in humans in England and Wales in 1984 (Threlfall et al. 1994). It was resistant to the antibiotics ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline (R-type ACSSuT) (Threlfall et al. 1994). Other isolates of *S.* Typhimurium DT104 were obtained from wild and exotic birds in 1984. These birds were imported from countries in the Far East, Africa, Germany, and the Netherlands (Hollinger et al. 1998). The PHLS Laboratory for Enteric Pathogens in the United Kingdom identified less than 50 isolates of this phage type/R-type annually in England and Wales before 1988. This number increased to 50-100 isolates per year between 1988 and 1990, over 200 isolates per year in 1991, more than 800 in 1992 and more than 1000 isolates from humans in only the first 10 months of 1993 (Threlfall et al. 1994). This increase throughout England, Wales, and Scotland made it the second most common phage type of *S.* Typhimurium DT104 in 1991, and the most common in 1992. Approximately half of 267 *S.* Typhimurium isolates from cattle in England, Wales, and Scotland in 1992 also were of this R-type. That same year, DT104 ACSSuT was also isolated from poultry, sheep, and pigs (Threlfall et al. 1994).

By 1996, 4006 isolates of DT104 were isolated in the United Kingdom and 95% of these had an R-type of ACSSuT. Concurrently that year in the United Kingdom, DT104 was isolated from cattle, sheep, goats, pigs, chickens, and turkeys (CDR Weekly 1997). Between August 1st and September 8th, 2000, 265 cases of DT104 infection were confirmed by the PHLS Laboratory of Enteric Pathogens. In the same time frame in 1999, only 97 isolates were confirmed (CDR Weekly 2000).

S. Typhimurium DT104 was first isolated from cattle in Ireland in 1995 (Murphy et al. 2001). During the summer of 1996, an outbreak amongst 127 persons attending a wedding reception in Dublin, Ireland, was investigated. Of those experiencing symptoms, guests older that 64 years had the highest attack rates (100%), whereas those between 45-65 years had the lowest rate (43%). Symptoms of watery diarrhea, general malaise, nausea, fever, and vomiting, began within 24 hours of the meal, and the median duration of illness was five days. Seven cases required admission to an acute care hospital. 85% of stool samples obtained were positive for *S*. Typhimurium, and all isolates were resistant to ampicillin, amoxycillin, chloroamphenicol, and sulphonamides. The cultures that were phage typed were DT104. 47% of the kitchen workers of the hotel where the reception was held carried *S*. Typhimurium in their stools, with an antibiotic pattern that matched the outbreak strain; however, no phage typing was performed. None of the turkey served at the reception remained to be sampled; however, a litter sample from the farm that had supplied the birds was positive for DT104. Further evidence of turkey being the vehicle of transmission was the occurrence of salmonellosis in five persons who ate at the hotel restaurant the same day as the wedding reception. Three of the persons ordered turkey sandwiches, and all developed gastroenteritis within 24 hours; the other two did not order turkey and remained healthy. A stool sample from one of the persons was positive for the same outbreak strain of *S*. Typhimurium DT104 (Grein et al. 1999). The prevalence of DT104 in Ireland increased to 94% of all *S.* Typhimurium isolates from cattle in 1998 (Murphy et al. 2001). Murphy et al. (2001) used PFGE and antimicrobial susceptibility techniques to differentiate *S*. Typhimurium DT104 isolates. Considerable genetic homology was observed among epidemiologically unrelated isolates, with the majority

having a common PFGE profile. This suggests DT104 may be endemic among cattle in Ireland.

A remarkable increase of DT104 infection was observed in Scotland between 1993 and 1996, when it accounted for 16% of human isolates, second only to *S.* Enteritidis phage type 4 (Calvert et al. 1998). DT104 was the most common phage type isolated from farm animals, of which cattle were thought to be the main reservoir of the pathogen. Unlike the trends observed in the UK, the proportion of isolates from cattle resistant to ciprofloxacin was lower than the proportion of resistant isolates from humans in Scotland. In 1997, 9% of human isolates were resistant to ciprofloxacin (0.125mg/l) as compared to only 1% of cattle isolates (Calvert et al. 1998). This is inconsistent with the hypothesis of direct transmission from cattle to people. Seasonality of DT104 infection of both cattle and humans followed a similar pattern, with peaks matching the pattern of calving in spring and autumn when clinical disease is likely to be highest. Also noted was that infection rates were higher in rural populations with less that 4000 people, than it was in urban populations. Similarly of adult cases of DT104 infection, when an occupation was known, 37.8% had jobs involving regular contact with animals, compared to only 9.4 % when other *Salmonella* spp. were involved. Fifty-seven percent of DT104 infected individuals were children under the age of six, who normally only account for 28.4 % of all *Salmonella* infections (Calvert et al. 1998). Sheep were also determined to be infected with DT104, as 19.4% of cases of salmonellosis in sheep reported between 1993 and 1996 were caused by DT104 (Calvert et al. 1998).

Resistance patterns of *S.* Typhimurium in bovine isolates from Belgium also changed significantly during the 1990's. In 1991, 37% of bovine isolates of *S.*

Typhimurium were resistant to at least ampicillin, chloramphenicol, tetracycline, and streptomycin. That same year, only one pig and one chicken isolate out of 67 and 154 strains examined, respectively, were multi-resistant. By 1997, 25% of *S.* Typhimurium isolates from pigs, 60% of isolates from poultry, and 70% of isolates from cattle were positive. DT104 was the predominant phage type in all three species of animals in Belgium since 1993 (Imbereshets et al. 1998).

Until 1997, DT104 infections accounted for less than one percent of the total number of human *Salmonella* infections in Denmark, and other than an outbreak of DT104 salmonellosis at a small hospital in 1996, only sporadic cases had been recorded (Molbak et al. 1999). In June of 1998, the first community outbreak occurred, with 25 culture-confirmed cases. Eleven patients were hospitalized, and two died. *S.* Typhimurium, with an unusual resistance pattern, was cultured from five patients suffering from gastroenteritis. At the same time, *S.* Typhimurium DT104 with the same resistance pattern was isolated from a sample of pork collected in May from a slaughterhouse. Two pork samples collected by food inspectors were also found to contain *S.* Typhimurium DT104 with this resistance profile. Further investigation revealed that the two wholesalers had received the pork from a slaughterhouse from which the same strain of *S.* Typhimurium DT104 was isolated. This resistance profile had not been detected previously in *Salmonella* isolated from Danish animals or animal-derived foods, and only twice in *Salmonella* from humans. It is likely that the source of the outbreak was the two swine herds identified as being slaughtered on the day when the slaughterhouse sample was obtained in May (Molbak et al. 1999).

From September until the end of December, 2000, there was an explosive increase in the number of *S.* Typhimurium DT104 isolated from humans in the Netherlands (Eurosurveillance Weekly, 2001). The number of which has since plateaued to double the level of previous years, and is now among the highest in Europe. Outbreaks linked to the same strain occurred in September 2000 and July-August 2001 in Sweden, Australia, Germany, the United Kingdom and Norway linked to contaminated batches of a sesame seed product (Eurosurveillance Weekly, 2001). The observed clinical course of infection was severe. The number of hospital admissions was as high as that reported in England and Denmark, over 40%, but twice the number of patients had blood in their stools and there was one death as a result of infection. An important cause for the explosive increase of DT104 in humans in 2001 was its sustained increase in cattle and pigs. Dispersal of contaminated manure may play a role in this increase. A case-control study determined that the risk of infection with *S.* Typhimurium on farms that had bought pig manure was over 20 times higher than those that had not. Also, the practice of keeping animals inside modern dairies was associated with contamination and this practice has been increasing. In addition, the rise in national dispersal of manure in the Netherlands is an important, fairly new, risk factor for the spread of DT104 and possibly other pathogens (Eurosurveillance Weekly, 2001).

Between 1993 and 1996, 41% of 544 cattle in Canada infected with *Salmonella* and one sheep were found to carry DT104 (Poppe et al. 1998). In 1995, there were 59 documented cases of DT104 infections in humans, which increased to 148 human cases in 1996. Of the human isolates obtained between 1993-1996, 41% were DT104. As well, the antibiotic resistance profile ACSSuT of *S.* Typhimurium isolates increased from 46%

in 1995 to 57.5% in 1997. In addition, 32.4% of *S.* Typhimurium isolates collected from animals, foods and environmental sources were typed as DT104. During the first 10 months of 1997, 38.2% of *S.* Typhimurium isolated were typed as DT104, indicating that this phage type is still increasing (Poppe et al. 1998).

The first reports of DT104 in the United States were in 1996 (Besser et al. 1997). An increase in the occurrence of outbreaks of salmonellosis in dairy cattle in the Pacific Northwest was observed in 1994, with many of the isolates being *S*. Typhimurium R-type ACSSuT. A subsequent examination of preserved bovine isolates of *S.* Typhimurium from this region revealed this R-type accounted for 13% and 64% of isolates from 1986 to1991 and 1992 to 1996, respectively. *S.* Typhimurium DT104 was also isolated from sheep, pigs, horse, goat, emu, cat, dog, elk, mouse, coyote, ground squirrel, raccoon, chipmunk and birds, including pigeons, starlings, and pine siskins. The preserved cultures of human isolates in the Pacific Northwest were also examined and two of 46 *S.* Typhimurium isolates from 1989 were R-type ACSSuT, and 42.5% of 188 isolates from 1994 also were of this R-type. All human isolates with this R-type were typed as DT104 (Besser et al. 1997).

S. Typhimurium was the second most common human serotype of *Salmonella* isolated in the United States in 1995, accounting for 24% of isolates (MMWR, 1997). As phage typing is uncommon in the United States, it is not known if this increase as associated with the emergence of DT; however, 32% of the *S.* Typhimurium isolates were determined to have the typical DT104 R-type of ACSSuT. This was detected in only 7% of *S.* Typhimurium isolates in 1990 (MMWR, 1997). During October of 1996, an outbreak of salmonellosis occurred in an elementary school in Nebraska. The source was

never identified, although chocolate milk, and a turtle and a sick kitten brought to show and tell, were identified as potential sources. Stool samples obtained from seven children all yielded *S.* Typhimurium R-type ACSSuT, and phage typing confirmed the isolates as DT104 (MMWR, 1997).

In early 1997, a five-fold increase in salmonellosis was seen in the Hispanic population of Yakima County Washington. Fifty-four culture confirmed cases of *S.* Typhimurium were reported between January 1 and May 5, 1997, of which the median age was 4 years and 91% were Hispanic. Unpasteurized Mexican-style soft cheese was consumed by 77% of patients in the 7 days before onset of illness. All isolates from patients eating the cheese were phage typed as DT104, with the R-type ACSSuT. Cheese produced and eaten by two unrelated patients was made with raw milk traced to the same local farm and *S.* Typhimurium DT104 was isolated from milk samples from nearby dairies. The increase in human salmonellosis appears to reflect the emergence of DT104 among dairy cows in the region (Villar et al. 1999).

Also in 1997, two overlapping outbreaks of DT104 infection occurred in Hispanic communities in the San Francisco Bay area (Cody et al. 1999). These outbreaks also were associated with the consumption of fresh Mexican-style cheese. One hundred and ten cases were culture confirmed as *Salmonella* Typhimuriun var Copenhagen DT104. The first outbreak peaked in February on 1997, and 94% of cases reported eating unpasteurized Mexican-style cheese. Cheese samples could not be obtained to confirm the association. The second outbreak peaked in April 1997, and all pateints ate fresh Mexican-style cheese from street vendors. Pulsed-field gel electrophoresis (PFGE) revealed three different patterns, hence 3 subtypes. One of the subtypes was obtained

from raw cow's milk, street-vended cheese and patients. The other two subtypes were recovered from street-vended cheese and patients. The California dairy industry is the sixth largest in the world and the volume of commercially sold Mexican-style cheese continues to increase. The presence of DT104 in dairy cattle and the legal sale of unpasteurized raw milk within the state portend increasingly severe outbreaks of DT104 infections (Cody et al. 1999).

Following the report of the outbreaks in the Pacific Northwest and Nebraska, 4627 cultures of *Salmonella* obtained by the *Salmonella* Reference Center at the University of Pennsylvania from the Northeast United States between April 1994 and June 1997 were examined (Benson et al. 1997). Of these, 461 isolates were *S.* Typhimurium, and 13.4% of these were phage typed as DT104. The incidence of *S.* Typhimurium among bovine isolates was lower than expected, and may indicate that the acquisition of DT104 by cattle in this region was recent (Benson et al.1997).

A historical review by the Centers of Disease Control and Prevention of the emergence of DT104 infections in the United States revealed the reported number of cases of *S.* Typhimurium has remained relatively constant between 1981 and 1996, excluding one large outbreak in 1985 (Glynn et al. 1998). However, the proportion of isolates with the five-drug resistance pattern increased, from less than 1% in 1979-1980 to 34% in 1996, with 91% of the penta-resistant isolates being DT104. Estimates indicate between 68 000 and 340 000 cases of penta-resistant *S.* Typhimurium infection, most of which were DT104, occurred in the United States in 1996. As compared to the United Kingdom, there have been in the United States fewer outbreaks of DT104 infections. However, case-control studies of sporadic cases in the United States of DT104 infections

in humanshave identified consumption of unpasteurized dairy products and direct contact with livestock as risk factors (Glynn et al. 1998).

Studies by both the USDA-APHIS National Antimicrobial Resistance Monitoring System (NARMS) and the USDA- ARS National Veterinary Services Laboratories (NVSL) on the prevalence of DT104 in animals in the United States were carried out. The NARMS study revealed that 10.2%, 10.5% and 17.7% of *S.* Typhimurium isolates were R-Type ACSSuT in 1995, 1996, and 1997, respectively. However, of these, only 64%, 22.2% and 63.8% were DT104. NVSL studied *Salmonella* isolates from both clinically ill and healthy animals submitted between October 1996 and February 1997, and found that 26% were the penta-resistant R-type. The NVSL results were substantially larger than those of the NARMS study; this may be due to differences in the number of isolates from healthy animals and differences in the duration of sampling (Akkina et al. 1999).

Similarities between increased human and domestic animal DT104 infections in the Pacific Northwest of the United States were observed at the beginning of DT104's emergence. Besser et al. (2000) determined that humans infected with DT104 were more likely to live in areas with more cattle farms and were more likely to have had direct contact with livestock compared to humans infected with other strains of *S.* Typhimurium.

Outbreaks of *S.* Typhimurium DT104 infections associated with pet contact were first reported in 1994 in the United Kingdom (Wall et al. 1994). The first outbreaks of DT104 infection associated with pets in the United States were reported from three states in 1999 (Canadian Communicable Disease Report 2001). Cases reported in Idaho

occurred in patients caring for kittens in a small animal veterinary clinic in September and October (Anonymous 2001), and in Minnesota DT104 isolated from nine cats and seven humans were indistinguishable by PFGE. All cases were associated with the presence of DT104 in an animal shelter, or through contact with a person who owned a cat from the animal shelter. In Washington State, an outbreak of DT104 infection was associated with a small animal veterinary clinic in late 1999, with indistinguishable PFGE profiles from the sick cats and humans. Information on the occurrence of DT104 in human cases and animal carriage in continents other than Europe and North America is not readily available (Poppe et al. 1998).

Salmonella Typhimurium DT104 and Antibiotic Resistance

The R-type of ACSSuT was originally ascribed to a large plasmid of approximately 60 MDa, which was present in all strains of DT104 ACSSuT isolated. However, the 60 MDa plasmid was determined to be the *S*. Typhimurium serovar-specific plasmid pSLT, which is carried by most *S*. Typhimurium strains and does not encode antimicrobal resistance. Rather, the antimicrobial resistance genes were on the chromosome (Threlfall et al. 1994).

Isolation of *S*. Typhimurium of phage types with multiple drug resistance from cattle have been well documented (Threlfall et al. 1994). Prior to 1991, the most common phage types were 204c and 139, both of whose antibiotic resistance pattern was plasmid encoded (Threlfall et al. 1994). *Salmonella* Typhimurium DT104 was first identified in humans in England and Wales in 1984, and is thought to have originated in veal calves (Threlfall et al. 1994). In 1997, Threlfall et al.(1998) determined that 12% of

multiresistant DT104 were additionally resistant to ciprofloxacin MIC 0.5-1.0 mg/L, 17% were resistant to trimethoprim, and 2% were resistant to both ciprofloxacin and trimethoprim. Although treatment of humans infections caused by DT104 with antibiotics is uncommon, should antibiotic therapy be necessary, the choice of antimicrobials is very limited. The genetic basis for resistance to nalidixic acid and ciprofloxacin commonly involves a point mutation in the *gyrA* gene (Piddock et al. 1998). In 1998, a large community outbreak of ciprofloxacin-resistant *S.* Typhimurium DT104 infection occurred in England (Walker et al. 2000). Epidemiologic investigations revealed that 79% of cases had consumed milk from a local dairy. DT104 strains isolated from dairy cattle, a milk filter and humans was defined as the outbreak strain and contained a GAC to GGC mutation at codon 87, leading to an aspartic acid to glycine substitution within the *gyrA* gene. Marobofloxacin, a fluoroquinolone, was used on the farm in the months preceding the outbreak. Although it is common for subclinical infection with DT104 to persist for several months of years, in this outbreak it is possible that the therapeutic use of marbofloxacin in cattle may have enhanced dissemination and persistence of the pathogen and increased the potential for contamination of milk (Walker et al. 2000).

In 1994, only 1% of all DT104 isolates in the UK exhibited increased resistance to ciprofloxacin, whereas in 1998, this increased to 16% of DT104 isolates (Eurosurveillance Weekly 2001). Of concern is that the same penta-resistance property associated with DT104 has been identified in isolates of *Escherichia coli* from farm animals. Chromosomal exchange of genes which provide advantageous attributes for survival, such as antibiotic resistance genes, across the species barrier is possible and

may occur increasingly with the growing prevalence of resistant DT104 strains (Eurosurveillance Weekly 2001).

Davis et al. (1999) examined changes in antimicrobial resistance profiles in *S.* Typhimurium in the northwestern United States and observed marked resistances to chloramphenicol in isolates from both humans and cattle. Before 1991, fewer than 5% of cattle isolates and 3% of human isolates were resistant to chloroamphenicol, whereas by 1995 more than 70% of cattle isolates and almost 50% of human isolates were resistant. The ACSSuT R-type associated with DT104 increased from 1% of cattle isolates before 1990 to 55% of isolates in 1997. A similar change was observed for human isolates, increasing from 2% before 1991to 36% in 1997.

Schmieger and Schicklmaier (1999) determined that *Salmonella* isolates harbor prophage genomes as general genetic elements, with most of them belonging to the P22-like group of lambdoid phages which are capable of generalized tranductions. Transduction of resistance genes in DT104 was induced with many different phages, including PDT104, which resides as a prophage in all DT104 strains. This implies that all DT104 strains carry in their genome a potent vehicle suitable for horizontal transfer and further spread of resistance genes. DT104 originates in cattle and predominantly inhabits animal breeding stations, under these conditions the number of bacterial cells which may participate in gene exchange is extremely high and even though transduction appears low under experimental conditions. This study also revealed the chromosomal organization of the resistance genes, i.e. that all the resistance determinants must be clustered in a range of less than 46kb, as this is the packaging capacity of the phage used. One other

surprising observation from this study was the transduction of strains other than DT104 having at least one resistance gene was either unsuccessful or extremely low.

Carlson and Ferris (2000) determined that in some isolates of multi-resistant *S.* Typhimurium, exposure to certain beta-lactam antibiotics could endow resistance to a wider range of antibiotics and quantitative increases in MIC values for antibiotics that were part of their preexposure antibiograms. A plausible explanation for this is that ampicillin is mediating the activation of alternative resistance machinery, such as *marA* which is a global mechanism for the efflux of antibiotics that exists as part of an archaic pathway for the survival of enteric bacteria in the presence of bile salts. The augmentation due to ampicillin parallels that of *marA*-mediated induction as both are relatively rapid, reversible, and related to stationary phase growth. This suggests that resistant *Salmonella* not only have a selective advantage over non-resistant *Salmonella* but their resistance phenotypes can be potentiated when an inappropriate antibiotic is used therapeutically.

Chloramphenicol was considered the drug of choice to treat salmonellosis in animals, and in August 1994, it was prohibited in Europe for veterinary use in farm animals. In January of 1995, florfenicol, a drug from this same family was licensed in France for therapeutic use for bovine pasteurellosis. Soon after its introduction, isolates of *S.* Typhimurium were detected which appeared to be cross-resistant to both the phenicol drugs. However, the first florfenicol-resistant strain was isolated in 1989, and in 1994 florfenicol-resistant strains outnumbered florfenicol-susceptible chloramphenicol-resistant strains. Thus, it can be concluded that florfenicol resistance was associated with ACSSuT multi-resistance previously described in DT104 (Arcangioli et al. 2000).

Antimicrobial resistance profiles of isolates continue to be a concern as additonal loss of susceptibilities is increasingly prevalent in food animal isolates (Hollinger et al. 1998).

Salmonella Disease in Humans

In 1888 *Salmonella* was first recognized as a human foodborne pathogen following an outbreak of salmonellosis that occurred in Germany due to the consumption of raw ground beef from a dying cow (Tauxe 1991). Typhoid fever, caused by *Salmonella* Typhi was the predominant disease seen resulting from a *Salmonella* infection in humans prior to 1949. Since then, typhoid fever has all but been eliminated in industrialized countries (Tauxe 1991). In 2000, a total of 32 022 confirmed cases of human salmonellosis were reported in the United States. This represents a 24% decrease since 1990, and a 2% decrease since 1999. The two most common serotypes responsible for human salmonellosis are *S*. Typhimurium and *S*. Enteritidis, of which substantial decreases have occurred in frequency of human illness when compared with 1990, with an incidence of 20% and 29%, respectively (CDC 2001).

Salmonella serotypes, other than Typhi and Paratyphi, cause a foodborne infection that results in gastroenteritis. Symptoms appear within 8 to 72 hours following ingestion of contaminated food and remain for as long as one week. The initial symptoms of nausea and vomiting subside after a few hours and are followed by abdominal pain and diarrhea, with the possibility of fever. The severity of pain and diarrhea vary depending upon the host. Treatment usually involves supportive therapy and electrolyte replacement. Use of antibiotics is not common, as they can to prolong the carrier state

(D'Aoust 1989). The infected individual will continue to excrete *Salmonella* for up to three months after symptoms have subsided. One to three percent of infected individuals can become chronic carriers, and continue to excrete *Salmonella* spp. for more than one year. This persistence of *Salmonella* spp. in the intestinal tract likely results from an antibiotic-dependent repression of native gut microflora, which would normally compete with salmonellae for nutrients and binding sites. Most cases of gastroenteritis occur in children less then 10 years of age, in which symptoms tend to be severe. A systemic form of the infection may develop from the gastroenteritis. It is likely to be seen in infants and immunocompromised adults, where host defenses are unable to respond effectively to the invasive salmonellae (D'Aoust 1989). Although *Salmonella* infection usually causes a self-limiting localized infection, in any large outbreak, deaths will occur in those who develop the systemic form of the disease. *Salmonella* spp. may also induce chronic conditions such as aseptic reactive arthritis, Reiter's syndrome, and ankylosing spondylitis (D'Aoust 1997).

Infective doses of *Salmonella* vary, but may be as low as one to ten cells (D'Aoust et al. 1985; Kapperud et al. 1990). The infective dose varies depending on a number of factors, including virulence of the strain, composition of the ingested food, and age and health status of the patient (D'Aoust 1989; D'Aoust 1997). Non-typhoid serovars range in their ability to infect humans, with *S.* Gallinarium at the less virulent end of the spectrum and *S.* Choleraesuis at the virulent end (Jay 2000). Foods associated with low infective doses have high fat contents, such as cheddar cheese and chocolate, with observed infective doses of *S.* Typhimurium of 10^0- 10^1 and $\leq 10^1$, respectively (D'Aoust 1997). This lower infective dose may be due to bacteria becoming trapped in lipid

micelles, thus bypassing the stomach acids, and being released into the small intestine once the micelles are dispersed by bile (D'Aoust 1997). Mortality rates vary depending upon the age of the infected individual, for infants' age 0-12 months the rate is 5.8%, between 1-50 years the rate is 2%, and after the age of 50 years, this rate increases to 15% (Jay 2000). Young, elderly and immunocompromised populations are the most susceptible populations to infection (D'Aoust 1997).

Clinical symptoms of *S.* Typhimurium DT104 in humans include diarrhea, fever headache, nausea, vomiting and abdominal pain. One-fourth of patients infected in a case-control study had bloody diarrhea, 41% of patients required hospitalization, and 3% of patients died. This is much higher than the case-fatality rate associated for non-typhoid *Salmonella* infections, which other than for DT104, is approximately 0.1% (Akkina et al. 1999).

A study by Threlfall et al. (1998) revealed a 1.3% incidence of DT104 isolated from blood culture in human beings, after being isolated from feces, in England and Wales. This was not significantly greater than the incidence of other *S.* Typhimurium phage types (1.0%) and was equal to the incidence of *S.* Enteritidis. The incidence of invasive disease of both *S.* Virchow and *S.* Dublin, which are both known to have a propensity of invasive disease in humans, was 2.5% and 40%, respectively.

Salmonella Disease in Animals

Salmonellosis in farm animals is responsible for serious economic loss due to the numerous diseases it can cause. The presence of *Salmonella* spp. in all types of animals may be a transient change in the bowl flora with no apparent illness, or may be at the

other extreme, causing a persistent disease with high mortality (LeMinor 1981). *S.* Abortus-ovis and *S.* Dublin, cause abortions in sheep and cows, respectively, more frequently than *Brucella* spp. (LeMinor 1981).

S. Typhimurium has a murine reservoir in which the bacterium produces typhoid-like disease (Salyers and Whitt 1996). In other animals it typically causes a localized gastroenteritis. The virulence factors employed by *S.* Typhimurium in infection of calves differ from those involved in virulence in mice (Tsolis et al. 1999). *S.* Typhimurium is a major cause of mortality and morbidity in calves in the United States and in Europe (McDonough et al. 1994). The infection in calves closely resembles the disease caused in humans. Once infected, calves develop diarrhea within 48 hours (Rankin and Taylor 1966). It is primarily an enteric infection with mortality resulting from dehydration and intestinal lesions. Necropsy of terminally infected calves revealed acute fibrinopurulent necrotizing enteritis at the villous tops of the ileum and Peyer's patches. Destruction of the mucosal epithelium, massive neutrophil infiltration, and depletion of lymphocytes in the germinal centers of intestinal lymphoid follicles occurs (Wray and Sojka 1978). Septicemia and respiratory disease are also common in intensively managed calves (McDonough et al. 1994). Salmonellosis in calves frequently occurs as an epidemic, with 90% morbidity and up to 50% mortality (Hinton 1986).

S. Typhimurium is common in dairy cows and to a lesser extent in beef cattle. Sporadic disease occurs in adult cattle, but rarely occurs in more than five animals in the herd (Wray et al. 1987). Epidemics have, however, been reported (Tutt and Hoare 1974, Hunter and Peek 1977). The disease has been associated with stress factors, and is most common in late summer and early autumn (Wray et al. 1987). One of the stress factors

often overlooked is nutrition, which may play a larger role in beef cows, that, unlike dairy cows, are often grown on a nutritionally marginal diet (Davis and Renton 1992). Symptoms that have been observed in suckler cows include depression, loss of appetite and febrile, as well as scouring for 48 hours. If infection with *S*. Typhimurium occurs late in pregnancy, abortion may occur (Davis and Renton 1992).

The stress of marketing cattle affects the fecal shedding of *Salmonella* spp. in cattle. Corrier et al. (1990) determined that of calves negative for *Salmonella* on Tennessee farms following transfer to auction yards in Texas, 1.5% were culture positive, and following 30 days of feed yard confinement, 8% were positive.

Clinical signs of DT104 infection in cattle include pyrexia, lethargy and mental dullness, decreased milk production, anorexia, dehydration, increased salivation and diarrhea progressing to dysentery (Akkina et al. 1999). Symptoms can progress to serious colitis, sepsis and miscarriage (Eurosurveillance Weekly 2001). Cattle can be clinically inapparent carriers and shed large populations of *S.* Typhimurium DT104 for up to 18 months after an outbreak. Cattle also can maintain a persistent infection after illness characterized by pyprexia, diarrhea and decreased milk production. DT104 can be isolated from milk three months following clinical infection, and in some cases for up to seven months following clinical episodes, including after a non-lactating period and subsequent parturition. Cats may shed DT104 for up to 12 weeks after infection, and nonclinical carriers can shed large populations of DT104 from their mouths (Akkina et al. 1999).

Individual adult cattle can be symptomless carriers of DT104, hence there have been reports of clinical salmonellosis reoccurring on farms at more that 3-month

intervals. Low et al. (1996) determined that fecal carriage of DT104 in clinically normal cattle occurs frequently after outbreaks of DT104 salmonellosis, and that the organism can be recovered from fecal samples of clinically normal cattle for up to 6 months from these herds. They found that the addition of one of these symptomless carrier animals into another herd of uninfected cattle led to the development of clinical disease in these animals. In 1996, it was determined that dibility due to vitamin A deficiency was a predisposing factor for clinical salmonellosis due to *S.* Typhimurium DT104 in a beef suckler herd (Anonymous 1997).

The incidence of DT104 disease is higher in calves than in adult cattle. Calves are usually affected in the first month after birth, commonly within the first two weeks. The incidence of infection declines to low levels by the fourth week after birth (Poppe et al. 1998).

Risk factors associated with *S.* Typhimurium DT104 disease for cattle herds included the premises of cattle dealers, introduction of newly purchased cattle and the 4-week introduction of newly purchased cattle, purchase of new cattle from cattle dealers, housing of cattle, lack of isolation facilities on the farm, and access of wild birds or cats to feed stores (Evans et al. 1996). Fecal shedding of *S.* Typhimurium DT104 by cattle peaks during the summer months (Sorensen et al. 2002).

Salmonella Virulence

Salmonellae adhere to and invade a number of different mammalian cell lines, but do not have a marked tissue tropism. In vivo they attach to and invade both intestinal epithelial cells and M cells overlying Peyer's patches. *Salmonella* spp. must successfully compete with natural gut microflora for attachment surfaces on the intestinal lumen and

evade capture by immunoglobin A. Colonization of *Salmonella* spp. occurs as a result of many factors, including mannose-sensitive or mannose-resistant fimbriae, surface adhesins, nonfimbriate hemagglutins, host glycoprotein receptors on microvilli or glycocalyx of the intestinal surface (D'Aoust 1997).

The invasion mechanism of *Salmonella* spp. into cells differs from that of many other enteric pathogens. *Salmonella* spp. trigger actin rearrangements that result in pseudopod formation, which subsequently engulf the bacteria. The *inv* gene group mediates this mechanism of actin rearrangement that leads to ruffling of the host cell, resembling a splash of a drop of liquid hitting a solid surface. The result is internalization of the bacteria inside an endocytic vesicle, with the host cell surface returning to normal (Salyers and Whitt 1994).

Unlike other invasive bacterial pathogens that replicate in the cytoplasm of host cells, salmonellae remain within endocytotic vacuoles. *Salmonella* spp. multiply within the phagosome within hours of internalization. Within 24 hours, the host cell is filled with bacteria, and release of these organisms can occur (Finlay et al. 1992). Infected vesicles may also be transported to the basal region of the host cells and release salmonellae into the lamina propoa (D'Aoust 1989). *Salmonella* spp. infections trigger immune responses such as increased blood flow, convergence of neutrophils, eosinophils, monocytes and macrophages to the infected tissue, and host cell release of prostaglandins (D'Aoust 1991). Intracellular survival and multiplication of *Salmonella* spp. within macrophages is in part host dependent, but is also dependent upon bacterial virulence. Their ultimate fate may not be related to oxygen-dependent mechanisms (Vladoianu et al. 1990).

The presence of virulence plasmids in *Salmonella* is limited, but has been observed in *S.* Typhimurium, *S.* Dublin, *S.* Gallinarum-pullorum, *S.* Enteritidis, *S.* Choleraesuis, and *S.* Abortus-ovis (Guiney et al. 1994). These plasmids have no effect on adhesion or invasion. Rather, it is thought that the plasmids enable salmonellae to multiply more rapidly within host cells and overwhelm host defense mechanisms (Kowarz et al. 1994).

Iron scavenging by sidophores is another contributor to of *Salmonella's* virulence. Host iron is sequestered to drive the electron transport chain and enzymes associated with iron co-factors. Fe^{+3} is sequestered from intercellular storage by a high affinity enterobactin, which then complexes with an outer membrane pump receptor. The iron is brought into the bacterial cytoplasm and released in the Fe^{+2} state, for use in the key metabolic functions of the cell (Crosa 1989). The content of enterobactin produced by the infecting *Salmonella* strain is directly related to its degree of virulence (D'Aoust 1989).

The overt clinical symptoms of *Salmonella* spp. are caused by an enterotoxin, which is released into the cytoplasm of the infected host cell (Salyers and Whitt 1994). Once released, the enterotoxin activates adenyl cyclase in the host cell membrane, thus increasing the cytoplasmic concentration of cAMP. This results in secretion of Cl^- ions from the crypt regions and depressed absorption of Na^+ by villi. This subsequently results in fluid release into the intestinal lumen, and causes diarrhea (D'Aoust 1997). The 90-to 110- kDa enterotoxin is chromosomally encoded and is thermolabile. It has identical gel mobility patterns to the cholera A and B toxin subunits suggesting a similar structural configuration (Chopra et al. 1987). However, there is weak DNA homology and lack of antitoxin cross-reactivity between the two toxins (Rahman et al. 1994).

In addition, a thermolabile 56-to 78-kDa cytotoxic protein is localized on the bacterial outer membrane. Acidic pH and elevated temperatures precipitate the release of the toxin, possibly as a result of induced bacterial lysis. The toxin appears to play a role in inhibiting protein synthesis of host cells leading to lysis, thus promoting the dissemination of salmonellae into surrounding host tissue (D'Aoust 1991).

The capsular Vi antigen polysaccharide increases the virulence of the carrier strain by inhibiting opsonization to surface lipopolysaccharide (LPS). Ospinization is a critical event in the induction of macrophage phagocytosis of invasive *Salmonella* (D'Aoust 1991). This occurs for most *S.* Typhi strains, occasionally for *S.* Paratyphi C, and rarely for *S.* Dublin. LPS plays a critical role in repelling the complement system that is potentially lytic to the salmonellae. Those strains with short LPS are more susceptible to lysis, and thus less virulent. As well, varying LPS carbohydrate composition affects the level of serum activation, thus affecting virulence (D'Aoust 1991).

Infection associated with *S.* Typhimurium DT104 caused a doubling in the rates observed for hospital admissions and a ten-fold increase in mortality over the rates seen in other salmonellosis cases (Wall et al. 1994). However, the incidence of DT104-mediated bacteremia was not significantly greater than that of other *S.* Typhimurium phage types and was equal to that of *S.* Enteritidis (Threlfall et al. 1998). Carlson et al. (2000A) determined that multi-resistant *S.* Typhimurium isolates were not more invasive but rather less invasive than related resistant and non-resistant *S.* Typhimurium controls. Not only were strains of *S.* Typhimurium showing the R-type ACSSuT essentially as invasive as the controls, but those having kanamycin resistance (R-type ACSSuTK) also were hypoinvasive. Carlson et al. (2000B) determined that the increased virulence of

DT104 was not due to increased invasiveness. Allen et al. (2001) determined that *S.* Typhimurium DT104 isolates were able to survive and replicate in activated murine macrophages at similar levels to *S*. Typhimurium 14028s. As well, DT104 isolates were not unusually susceptible to hydrogen peroxide, were similar in resistance to acidified nitrite after 3 hours of incubation, and were able to grow to the same extent in pH 5 Luria-Bertani (LB) broth lacking sodium nitrite. This study also verified that the invasive properties of DT104 isolates were not significantly different than that other *S.* Typhimurium strains. In vitro assays revealed that DT104 strains did not possess an increased ability to invade tissue culture cells, survive within murine macrophages, or withstand reactive oxygen or nitrogen species. In vivo virulence of DT104 compared to *S.* Typhimurium 14028s in a murine model revealed that all but one of the DT104 strains had similar lethality, and the remaining strain, a clinical isolate from Washington State, was unable to cause lethal infection. When a mixed infection assay was done with DT104 and strain ATCC 14028s, none of the DT104 isolates was able to compete with the 14028s strain as determined by the inability of DT104 to colonize either the spleen of liver of the infected mice. Hence, it was concluded that the apparent increase in hospital rates and mortality could not be attributed to enhanced virulence-associated phenotypes detectable by conventional assays.

Salmonella Typhimurium DT104 and Cattle

The first isolates of DT104 from food animals were obtained from two cattle farms in England in 1988 (Hollinger et al. 1998). The first was from a closed dairy herd that was located adjacent to a river, an abattoir, a dealer of cull animals and a cattle

dealer. The second farm from which the isolate was obtained was a calf-rearing facility with a large and constant throughput of calves on the premises. The number of reported isolates of DT104 from cattle in the United Kingdom has steadily increased since 1988 (Hollinger et al. 1998).

Threlfall et al. (1994) determined that in the United Kingdom *S.* Typhimurium DT104 accounted for most cases on salmonellosis in cattle since 1993. Between 1994 and 1995, 295 of 450 of all *Salmonella* spp. isolated were multi-antibiotic resistant DT104. DT104 infections continued to increase through 1996 in Great Britain, including the first appearance of *S.* Typhimurium DT104b. Strains of this phage type are resistant to more antibiotics *in vitro* than typical isolates of DT104. Interestingly, DT104 also was isolated in the foot well of a tractor that had moved between three herds under the same management (CDR 1997).

A survey of *Salmonella* spp. serotypes isolated from feedlot cattle in the United States was done in 1998. *Salmonella* spp. was isolated from cattle in 38% of feedlots, and 5.5 % of all 4977 samples were *Salmonella*-positive. *S.* Typhimurium was detected in 2.6% of the positive feedlots, and in 2.9% of the positive samples (Fedorka-Cray et al. 1998). A similar study conducted on beef cattle in 2000 (Dargatz et al. 2000) revealed that 11.2% of all operations tested were positive for *Salmonella* spp., and 1.4% of all fecal samples obtained were positive. The percentage of positive samples among states, ranged from 52.8% in Texas to 0% in Georgia and South Dakota. Three of the *Salmonella* spp. isolates were serotyped as Typhimurium; however, none of their antibiotic resistance patterns was of the typical penta-resistance pattern, so were not phage typed. Results of this study indicate DT104 was not common.

Fecal shedding of *Salmonella* spp. by dairy cattle on the farm and at cull cow markets was studied in the United States in 2001 (Wells et al. 2001). The serotype distribution in dairy cattle differed from the 1991 data of which 26.5% of dairy calf isolates were *S*. Typhimurium, and the 1992 results of which *S.* Typhimurium was the most common isolate in clinically ill cattle, at 27.3%. A low fecal shedding prevalence of *S.* Typhimurium by adult dairy cattle was observed in the 2001 study with only 1% of *Salmonella* spp. isolates identified as *S*. Typhimurium DT104.

A study of beef cattle at processing in Western Canada revealed only one *Salmonella* spp. isolate, which was typed as *S.* Typhimurium DT104 (0.08%), isolated from feces sampled every 2 to 3 weeks between September 1995 to August 1996 (Van Donkersgode et al. 1999). These results indicate that either *Salmonella* spp. is an uncommon fecal bacteria in these cattle or that insensitive methods were used for isolating *Salmonella* spp. A more recent study of fecal shedding of *Salmonella* spp. by beef cattle in Alberta feedlots (Sorensen et al. 2001) revealed that 10.5% of feedlots were *Salmonella*-positive, and only 1.1% of animals tested were positive. DT104 was detected in only 2 of the 2000 samples assayed, and only one of these was of the typical penta-resistant R-type.

Transmission of *Salmonella* DT104 from cattle to humans

There are clear associations between *S.* Typhimurium DT104 infections of farm animals used for food production and humans, indicating that DT104 readily infects people in contact with infected animals or their products (Fone and Barker 1994, Wall et al. 1994, Wall et al. 1995, Davis et al. 1996). Salmonellosis began in recently purchased

calves and heifers in Western Canada, and then moved into two veterinary clinics, and one animal health technician. DT104 was isolated from the calf-rearing facilities from where the calves were purchased, veal operations and dairies, and from horses and humans, showing the transmission of the pathogen through cattle production (Poppe et al. 1998).

A large increase in the number of human cases of DT104 infection throughout England and Wales in 1993 prompted an investigation into the source of the pathogen (Wall et al. 1995). Contact with sick calves was identified as a risk factor contributing to both direct and possible indirect transmission of DT104. Because all infected persons did not had contact with sick calves, it is likely that the outbreak of infection was associated with several different kinds of food (Wall et al. 1995).

Humans predominantly acquire *Salmonella* infection through the consumption of contaminated food of animal origin. Although *Salmonella* spp. are often associated with poultry and poultry products, DT104 is associated with a wide range of animals used in food production, especially cattle. The widespread occurrence of DT104 in animal populations complicates the challenge of eliminating or controlling *S.* Typhimurium DT104 in the food continuum (Crerar et al. 1999).

Salmonella DT104 in Foods

A national sampling of retail ground beef for DT104a in the United States revealed that 3.5% of 404 total samples were *Salmonella* spp. positive, of which 35.6% (14 samples) were *S.* Typhimurium DT104a (Zhao et al. 2002). All DT104 positive samples were from retail stores in San Francisco CA.

Cases of DT104 infection have been linked to consumption of cooked and processed foods such as sausages, pate and chicken (Wall et al. 1994). Humphrey et al. (1997) examined the impact that attachment of DT104 to pork muscle has on its heat tolerance. Eight separate experiments were performed, and in five, attachment increased heat tolerance; the D(58˚C) value increased from 2 minutes for free cells, to greater than 10 minutes for attached cells. They suggested that the attachment of DT104, which may occur naturally during the preparation of meat products, could permit greater survival during subsequent cooking, and may provide an alternate explanation to cross-contamination, for the involvement of cooked foods in DT104 infection.

The percentage of fat in meats contributes to the tolerance of bacteria. Goodfellow and Brown (1978) reported D-values ranging from 61 min at 51.6°C to 0.7 min at 62.7°C for a mixture of six *Salmonella* serotypes in ground beef. Juneja and Eblen (2000) determined that increasing the fat content of ground beef resulted in longer lag times and lower D values of DT104. They obtained D-values at 58°C for S. Typhimurium DT104 of 3.22, 2.46, 2.49, and 1.61 minutes in beef with 7,12,18, and 24% fat respectively. This suggests that the lag values must be taken into consideration and reported with the D-values for determining the time required at a specific temperature for achieving a specific lethality for DT104 in ground beef.

Dark, firm, dry (DFD) beef is a condition in beef carcasses that is known to cause increased susceptibility to microbial spoilage than normal beef due to either a high pH or reduced glucose (Newton and Gill 1981, Gill 1983). Studies by Hooper-Kinder et al. (2002) on the effects of DFD ground beef on growth of DT104 when packaged in

oxygen-permeable overwrap and stored at 10˚C revealed that DFD beef is no more susceptible to growth of DT104 than normal beef.

Wall et al. (1994) reported recovering *S.* Typhimurium DT104 from a stick of salami that displayed an identical plasmid profile to an isolate recovered from a sick patient who ate the salami and then became ill. Ihnot et al. (1998) reported that when DT104 was inoculated into pepperoni batter, a 1.3 log reduction occurred following fermentation at 36°C and 92% humidity to pH 4.8, and an additional 1.6 log reduction was observed following drying to 13°C and 65% RH. Following 56 days of storage under vacuum at 4 or 21°C, the pathogen population decreased by a total of 4.6 and 6.6 log units respectively.

Eggs are another vehicle that has been implicated in DT104 infection (Wall et al. 1994). Williams et al. (1998) determined that commercial laying hens are susceptible to systemic infection by DT104 following experimental oral inoculation. This resulted in a higher proportion of intact eggs being infected with DT104 than has been observed with S. Enteritidis PT4, which is the current concern regarding intact shell eggs. Leach et al. (1999) determined that experimental infection of DT104 by aerosol delivery resulted in an increase to as much as 25% in intact egg contamination, from 1.7% with oral infection. As well, the frequency of DT104 isolation from muscle increased from 0% following oral challenge to 27% following aerosol challenge.

D values and z values of acid-shocked and non-shocked cells of DT104 and non-DT104 cells in liquid whole egg, egg yolk, egg white, whole egg+10% salts and egg yolk +10% salts were determined (Jung and Beuchat 2000). Differences in the thermal sensitivity of the two cell types were few, and rates of thermal inactivation of DT104

cells indicate that the USDA pasteurization process would eliminate more than 8 log CFU/ml of liquid whole egg at 57°C and greater than 11 log CFU/ml of all the other egg products tested at 61°C. D values, however, of both cell types of DT104 cells were lower than those of non-shocked cells in liquid egg products. Jung and Beuchat (1999) also determined DT104 survival in egg powders, including whole egg powder, whole egg powder supplemented with corn syrup solids (38%) and salt (1.9%), egg yolk powder, and egg white powder at varying water activity. Rates of inactivation for DT104 cells and non-DT104 cells did not differ, with the exception of whole egg powder supplemented with corn syrup solids and salt whose survival was significantly higher. Death was enhanced at aW 0.29-0.37 when stored at 13°C. The opposite trends were observed at aW 0.51-0.61 and stored at 37°C. Both DT104 and non-DT104 cells survived in egg white powder containing 4.9% moisture, but not at 8.2% moisture at 54°C for 7 days. Heating to 82°C failed to eliminate 5 log of DT104 per gram of egg white powder, regardless of moisture content.

Although apple cider has not been associated with any foodborne infections of *S.* Typhimurium DT104, cider-associated infections have attributed to *S.* Typhimurium (CDC 1975). The presumptive route of contamination was the use of drop apples, which had contacted animal feces, and may contain DT104. Roering et al. (1999) determined that DT104 populations decrease by 4.5 log CFU/ml during 14 days of storage at 4 and 10°C in pasteurized cider and by 5.5 log CFU/ml during 14 days of storage in unpasteurized cider at these temperatures. However, after 7 days at 4°C, DT104 populations had only decreased by 2.5 log CFU/ml in both types of cider. Studies with combination treatments to achieve a 5 log reduction in DT104 in apple cider revealed that

at pH 3.3, a freeze-thaw treatment of cider was sufficient to decrease 5 $\log_{10}$ of organisms, as did storage of cider at 25°C for 12 hours or at 35°C for 2 hours, and when the pH was increased to 3.7 (Uljas and Ingham 1999).

Salmonella and the Rumen

Ruminant animals and ruminant microorganisms have developed a symbiotic relationship that facilitates fiber digestion. The host provides the microorganisms with a suitable habitat for growth and the microbes supply protein, vitamins and short-chain organic acids for the animal. Microbial protein accounts for as much as 90% of the amino acids reaching the small intestine, and energy from short-chain organic acids drives animal metabolism. Bacterial numbers in the rumen can reach in excess of 10^{10} cells per gram of contents, and will play a dominant role in all facets of ruminal fermentation. Some ruminal bacteria are capable of producing bacteriocins that form holes in the cell membranes of closely related bacteria, which compete for the same substrates. The impact of these bacteriocins on rumen ecology has yet to be fully determined. Mixed culture studies indicate that bacteriocins may affect cellulose digestion, amino acid degradation, and starch fermentation (Russell and Rychlik 2001).

Grain-rich rations used currently by the cattle industry lead to a low pH and a high concentration of volatile fatty acids, which act to limit the growth of enteric bacteria in the rumen (Brownlie and Grau 1967). As a result, enteric bacteria are thought to represent a minor and rather transient microflora in the rumen (Harmon et al. 1999). Although the growth conditions are adverse, the rumen is still considered an important

reservoir of enteric pathogens, and can provide a continuous flow of these bacteria into the intestinal tract (Harmon et al. 1999).

Many strategies are currently being examined to make the rumen environment more hostile to enteric pathogens, thereby reducing the potential for these pathogens entering the food chain. The use of prebiotic sugars to displace *Escherichia coli* O157:H7 from the rumen has been examined (de Vaux et al. 2002). The addition of the sugars sorbitol, L-arabinose, trehalose and rhamnose, all unfermentable by *E. coli* O157:H7, reduced the pathogen to undetectable numbers within 72 hours. However, when added to sterile rumen fluid, sorbitol-positive mutants of *E. coli* O157:H7 appeared. This suggests that the ruminal microflora must also control the pathogen by competitive exclusion. The bactericidal effect of sodium chlorate on both *E. coli* O157:H7 and DT104 has been studied by Anderson et al. (2000). When added at concentrations of 5mM to buffered rumen contents, pathogens were reduced from 10^6 CFU/ml to less than 10 CFU/ml in 24 hours, whereas the total number of culturable anaerobes remained constant.

Salmonella and Competitive Exclusion

The administration of probiotic bacteria has been proposed as an approach to reduce carriage of enteric pathogens through competitive exclusion. These competitive exclusion bacteria are introduced as boluses or into the feed of cattle, and produce antimicrobial substances, or otherwise outcompete the pathogen in the rumen or colon (de Vaux et al. 2002). This approach was first successfully applied in 1973 by Rantala and Nurimi (1973) who prevented the growth of *Salmonella* Infantis in chicks through the application of enteric bacteria from adult *Salmonella*-free chickens.

Development and identification of competitive exclusion cultures using a science-based approach involves: defining how the pathogen colonizes the site of interest, isolating other microbes that colonize the same site and produce metabolites that inhibit or kill the pathogen, and verifying that when introduced to pathogen-free animals, colonization of the pathogen is reduced or prevented (Zhao et al. 1995). The commercial implication of this technique is an essential part of the Hazard Analysis Critical Control Point (HACCP) plan of animal production, as it reduces the presence of the pathogen from the animals (Zhao et al. 1995).

The use of competitive exclusion bacteria to reduce the presence of *E. coli* O157:H7 in cattle has been examined. Zhao et al. (1998) determined that the administration of probiotic bacteria to calves prior to exposure to *E. coli* O157:H7 decreased the duration of ruminal carriage of *E. coli* O157:H7 from 26 days to 14 days. In addition, the presence of *E. coli* O157:H7 in feces of animals was reduced from 25-32 days to 14-19 days in five of the six calves treated with probiotic bacteria. The inability of the competitive exclusion bacteria to reduce *E. coli* O157:H7 in one animal may be due to its failure to colonize that animal, indicating that more than one administration of the competitive exclusion bacteria may be necessary to provide greater protection.

The administration of competitive exclusion bacteria to prevent *Salmonella* infection has been examined in chickens and mice. Pascual et al. (1999) determined that a rifampin-resistant *Lactobacillus salivarius* strain CTC2197 prevented *Salmonella* Enteritidis C-114 colonization in chickens. When administered by oral gavage into day old chickens, the pathogen was eliminated from the birds after 21 days. Similar results were observed when 10^5 CFU of the probiotic strain/g was administered through the feed

and drinking water. Colonization of the gastrointestinal tract by the probiotic strain occurred through one week; however, between 21 and 28 days, the probiotic bacteria could no longer be detected in some birds. This indicates that more than one dose may be necessary to ensure its maintained presence in the gastrointestinal tract.

Audisio et al. (1999) initially determined that *Enterococcus faecium* J96, isolated from the intestinal tract of free-range chickens, produced lactic acid and bacteriocins that inhibited *Salmonella* of different serotypes (Gallinarum, Pullorum, Enteritidis, and Typhimurium) when grown in mixed culture fermentations. Subsequently, they determined that when 10^9 CFU of *Enterococcus faecium* J96/ml were orally administered twice a day for three consecutive days as preventative treatments, the chickens survived a 10^5 CFU/ml challenge of *Salmonella* Pullorum (Audisio et al. 2000). However, the probiotic strain was not effective as a therapeutic agent.

Salmonella Enteritidis infection can be eliminated after treatment with competitive exclusion culture in chicks (Seo et al. 2000). Day-old chicks were treated with normal avian gut flora (NAGF) derived from a pathogen-free adult chicken and then were infected with 10^6 CFU of *Salmonella* Enteritidis by oral gavage. There was significantly less cecal colonization at 12 weeks of treated birds than untreated birds; however, no significant differences were observed in organ infection.

A mixed culture of *Lactobacillus crispatus* and *Clostridium lactatifermentans* has been used as a competitive exclusion culture for *Salmonella* Enteritidis in chickens (van der Wielen et al. 2002). The mixture, when placed in a sequencing fed-batch reactor that mimics the cecal ecophysiology of broiler chickens, inhibited *Salmonella* Enteritidis at pH 5.8, but was not effective at pH 7.0. *Lactobacillus crispatus* alone was unable to

prevent growth at either pH value. Inhibition was hypothesized to be due to the concentrations of the undissociated forms of acetic acid and propionic acid at pH 5.8 that result from *Clostridium lactaifermentans* fermentation.

The applications of probiotics against *Salmonella* spp. have also been studied in mice. Oral administration of *Lactobacllus acidophilus*, *Saccharomyces boulardii* and *Escherichia coli* to gnotobiotic mice ten days before challenge with *Salmonella* Typhimurium, did not affect the colonization of *S.* Typhimurium that continued to be present at 10^8 CFU/g of feces (Filho-Lima et al. 2000). Transgalactosylated oligosaccharides administered with bifidobacteria into mice during antibiotic treatments prevented antibiotic-induced disruption of colonization resistance to oral infection by *S.* Typhimurium. This represents a useful application for probiotics to provide protection against opportunistic enteric infections by antibiotic-resistant pathogens.

Research Needs

Cattle are a reservoir for *S*. Typhimurium DT104, which may move into the human food chain subsequently leading to increased risk of foodborne disease caused by this pathogen. One approach to reduce carriage of *S*. Typhimurium DT104 by cattle is the use of competitive exclusion bacteria that produce antimicrobials and compete with *S*. Typhimurium DT104 at sites of colonization. Potential competitive exclusion bacteria should be isolated from the fresh feces of cattle that are not shedding *Salmonella* spp. and evaluated for anti-*S*. Typhimurium DT104 activity in vitro. Isolates showing anti-*S*. Typhimurium DT104 activity in vitro should be evaluated for the ability to inhibit *S*. Typhimurium DT104 within a natural environment, such as feces. Results of these

studies would be useful as a first step to the application of competitive exclusion bacteria into cattle to reduce their carriage of *S.* Typhimurium DT104.

REFERENCES

Allen, C. A., P. J. Fedorka-Cray, A. Vazquez-Torres, M. Suyemoto, C. Altier, L. R. Ryder, F. C. Fang, S. J. Libby. 2001. In vitro and in vivo assessment of Salmonella enterica serovar tyohimurium DT104 virulence. Infect. Immun. 69:4673-4677.

Anderson, E. S., L. R. Ward, M. J. De Saxe, J. D. H. De Sa. 1977. Bacteriophage-typing designations of *Salmonella* Typhimurium. J. Hyg. 78:297-300.

Anderson, R. C., S. A. Buckley, L. F. Kubena, L. H. Stanker, R. B. Harvey, D. J. Nisbet. 2000. Bacteicidal effect of Sodium chlorate on *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT104 in rumen contents in vitro. J. Food Prot. 63:1038-1042.

Anonymous. 2001. Explosive increase of multiresistant *Salmonella* Typhimurium DT104 in 2001 in the Netherlands. Eurosurveillance Weekly. December 13, 2001 (http.//www.eurosurv.org/update/)

Anonymous. 2001. Outbreaks of multidrug-resistant *Salmonella* Typhimurium associated with veterinary facilities – Idaho, Minnesota, and Washington, 1999. Canadian Communicable Disease Report. Volume 27-21,1 November 1, 2001.

Anonymous. 2000. National increase in *Salmonella typhimurium* DT104—update. Communicable Disease Report CDR Weekly.10:311,314,323,326.

Anonymous. 1997. Investigating *Salmonella typhimurium* DT104 infections. Communicable Disease Report CDR Weekly. 7:137,170.

Anonymous. 1997. Increase in *S. typhimurium* DT104 infections in cattle. Vet. Rec. 139:5-7.

Akkina, J. E., A. T. Hogue, F. T. Angulo. 1999. Epidemiologic aspects, control, and importance of multiple drug resistant *Salmonella typhimurium* DT104 in the United States. J. Am. Vet. Med. Assoc. 214:790-798.

Arcangioli, M-A., S. Leroy-Setrin, J.-L. Martel, E. Chaslus-Dancla. 2000. Evolution of chloramphenicol resistance, with emergence of cross-resistance to florfenicol, in bovine *Salmonella typhimutium* strains implicates definitive phage type(DT) 104. J. Med. Microbiol. 49:103-110.

Asahara, T., K. Nomoto, K. Shimizu, M. Watanuki, R. Tanaka. 2001. Increased resistance of mice to *Salmonella enterica* serovar Typhimurium infection by synbiotic administration of bifidobacteria and transgalactosytated oligocaccharides. J. Appl. Microbiol. 91:985-996.

Audisio, M. C., G. Oliver, M. C. Appella. 2000. Protective effect of *Enterococcus faecium* J96, a potential probiotic strain, on chicks infected *with Salmonella pullorum*. J. Food Prot. 63:1333-1337.

Audisio, M. C., G. Oliver, M. C. Apella. 1999. Antagonistic effect *of Enterococcus faecium* J96 against human and poultry pathogenic *Salmonella* spp. J. Food Prot. 62:751-755

Baggesen, D. L., D. Sandvang, F. M. Aarestrup. 2000. Characterization *of Salmonella enterica* serovar Typhimurium DT104 isolated from Denmark and comparison with isolates from Europe and the United States. J. Clin. Microbiol. 38:1581-1586.

Barton, M. D. 2000. Antibiotic use in animal feed and its impact on human health. Nutr. Res. Rev. 13:279-299.

Benson, C. E., D. S. Munro, S. Rankin. 1997. *Salmonella typhimurium* DT104 in the northeast USA. Vet. Rec. 141:503-54.

Besser, T. E., M. Goldoft, L. C. Pritchett, R. Khakhria, D. D. Hancock, D. H, Rice, J. M. Gay, W. Johnson, C. C. Gay. 2000, Multiresistant *Salmonella* Typhimurium DT104 infections of humans and domestic animals in the Pacific Northwest of the United States. Epidemiol. Infection. 124:193-200.

Besser, T. E., C. C. Gay, J. M. Gay, D. D. Hancock, D. Rice, L. C. Pichett, E. D. Erickson. 1997. Salmonellosis associated with *Salmonella typhimurium* DT104 in the United States. Vet. Rec. 140:75.

Brenner, F. W. and A. C. McWhorter-Murlin. 1998. Identification and serotyping of *Salmonellae*. Centers for Disease control and Prevention, Atlanta, GA.

Brownlie, L. E. and F. H. Grau. 1967. Effect of food intake and growth on survival of *Salmonella* and *Eschericchia coli* in the bovine ruman. J. Gen. Microbiol. 46:125-134.

Callow, B. R. 1959. A new phage-typing scheme for *Salmonella typhimurium*. J. Hyg. 57:346-359.

Calvert, N., W. C. Stewart, W. J. Reilly. *Salmonella typhimurium* DT104 infection in people and animals in Scotland: a collaborative epidemiological study 1993-1996. Vet. Rec. 143:351-354.

Carlson, S. A. and K. E. Ferris. 2000. Augmentation of antibiotic resistance in *Salmonella typhimurium* DT104 following exposure to penicillin derivatives. Vet. Microbiol. 73:25-35.

Carlton, S. A., R. M. Willson, A. J. Crane, K. E. Ferris. 2000A. Evaluation of invasion-conferring genotypes and antibiotic induced hyper invasive phenotypes in multiple antibiotic resistant *Salmonella typhimurium* DT104. Microb. Pathogen. 28:373-378.

Carlson, S. A., M. Browning, K. E. Ferris, B. D. Jones. 2000B. Identification of diminished tissue culture invasiveness among multiple antibiotic resistant *Salmonella typhimurium* DT104. Microb. Pathogen. 28:37-44.

CDC. Salmonella Surveillance: Annual Summary, 2000. Atlanta, Georgia: US Department of Health and Human Services, CDC, 2001.

CDC. 1975. State outbreak traced to a commercial apple cider- New Jersey. Morbid. Mortal. Weekly Rep. 24:87-88.

Chopra, A.K., C. W. Houston, J. W. Peterson, R. Prasad, J. J. Mekalanos. 1987. Cloning and expression of the *Salmonella* enterotoxin gene. J. Bacteriol. 169:5095-5100.

Cody, S. H., S. L. Abbot, A. A. Marfin, B. Schulz, P. Wagner, K. Robbins, J. C. Mohle-Boetani, D. J. Vugia. 1999. Two outbreaks of multidrug-resistant *Salmonella* serotype Typhimurium DT104 infections linked to raw-milk cheeses in northern California. J. Am. Med. Assoc. 281:1805-1810.

Corrier, D. E., C. W. Purdy, J. R. DeLoach. 1990. Effects of marketing stress on fecal excretion of *Salmonella* spp. in feeder calves. Am. J. Vet. Res. 51:866-869.

Crerar, S. K., T. J. Nicholls, M. D. Barton. 1999. Multidrug-resistant *Salmonella typhimurium* DT104—implications for animal industries and the veterinary profession. Aust. Vet. J. 77:170-171.

Crosa, J. H. 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. 53:517-530.

D'Aoust, J.-Y., D. W. Warburton, A. M. Sewell. 1985. *Salmonella* Typhimurium phage-type 10 from cheddar cheese implicated in a major Canadian foodborne outbreak. J. Food Prot. 48:1062-1066.

D'Aoust, J.-Y. 1989. *Salmonella*. In Foodborne Bacterial Pathogens. M.P. Doyle (Ed.). p. 327-445. Marcel Dekker, New York.

D'Aoust, J.-Y. 1991. Pathogenicity of foodborne *Salmonella*. Int. J. Food Microbiol. 12:17-39.

D'Aoust, J.-Y. 1991. Psychrotrophy and foodborne *Salmonella*. Int. J. Food Microbiol. 13:207-216.

D'Aoust, J.-Y. 1994. Salmonella and the international food trade. Int. J. of Food Microbiol. 24:11-31.

D'Aoust, J-Y. 1997. Salmonella species. In Food Microbiology: fundamentals and frontiers. M. P. Doyle, L. R. Beauchat, and T. J. Montville (ed.). p. 129-158. ASM Press, Washington, D.C.

Dargatz, D. A., P. J. Fedorka-Cray, S. R. Ladely, K. K. Ferris. 2000. Survey of *Salmonella* serotypes shed in feces of beef cows and their antimicrobial susceptibility patterns. J. Food Prot. 63:1648-1653.

Davis, A., P. O'Neill, L. Towers, M. Cooke. 1996. An outbreak of *Salmonella* typhimurium DT104 food poisoning associated with eating beef. Communicable Disease Report CDR Rev. 6:159-162.

Davis, M. A., D. D. Hancock, T. E. Besser, S. H. Rice, J. M. Gay, C. Gay, L. Gearheart, R. DiGiacomo. 1999. Changes in antimicrobial resistance among *Salmonella enterica* Serovar Typhimurium isolates from humans and cattle in the Northwestern United States, 1982-1997. Emerg. Infect. Dis. 5:802-806.

Davis. T. G., and C. P. Renton. 1992. Some aspects of the epidemiology and control of *Salmonella typhimurium* infection in outwintered suckler cows. Vet. Rec. 131:528-531.

De Cesare, A., G. Manfreda, T .R. Dambaugh, M. E. Guerzoni, A. Franchini. 2001. Automated ribotyping and random amplified polymorphic DNA analysis for molecular typing of *Salmonella enteritidis* and *Salmonella typhimurium* strains isolated in Italy. J. Appl. Microbiol. 91:780-785.

De Vaux, A., M. Morrison, R. W. Hutkins. 2002. Displacement of *Escherichia coli* O157:H7 from rumen medium containing prebiotic sugars. Appl. Environ. Microbiol. 68:519-524.

Evans, S., and R. H. Davies. 1996. Case control study of multiple *resistant Salmonella typhimurium* DT104 infection of cattle in Great Britain. Vet. Rec. 139:557-558.

Farrington, L. A., R. B. Harvey, S. A. Buckley, R. E. Droleskey, D. J. Nisbet, P. D. Inskip. 2001. Prevalence of antimicrobial resistance in salmonellae isolated from market-age swine. J. Food Prot. 64:1496-1502.

Fedorka-Cray, P. J., S. R. Ladely, J. S. Bailey, N. J. Stern. 2001. Colonization of broiler chicks by *Salmonella* Typhimurium definitive phage type 104. J. Food Prot. 64:1698-1704.

Fedorka-Cray, P. J., D. A. Dargatz, L. A. Thomas, J. T. Gray. 1998. Survey of *Salmonella* serotypes in feedlot cattle. J. Food Prot. 61:525-530.

Ferris, K .E., and D. A. Millet. 1997. *Salmonella* serotypes from animals and related sources reported during July 1996-June1997. Proc. 101st Annu. Meet.United States Animal Health Assoc. Louisville, KY. pg.423-443.

Filho-Lima, J. V. M., E. C.Vieira, J. R. Nicoli. 2000. Antagonistic effect *of Lactobacillus acidophilus, Saccharomyces boulardii* and *Escherichia coli* combinations against experimental infections with *Shigella flexneri* and *Salmonella enterica* subsp. Typhimurium in gnotobiotic mice. J. Appl. Microbiol. 88:365-370.

Finlay, B. B., K. Y. Leung, I. Rosenshine, F. G. Portillo. 1992. *Salmonella* interactions with the epithelial cell. ASM News 58:486-489.

Fone, D. L., and R. M. Barker. 1994. Associations between human and farm animal infections with *Salmonella typhimurium* DT104 in Herefordshire. Communicable Disease Report CDR Rev. 4:R136-140.

Gill, C. O. 1983. Meat spoilage and evaluation of the potential spoilage life of fresh meat. J. Food Prot. 46:444-452.

Glynn, M. K., C. Bopp, W. Dewitt, P. Dabney, M. Mokhtar, F. J. Angulo. 1998. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. N. Engl. J. Med. 338:1333-1338.

Goodfellow, S. J., and W. L. Brown, 1978. Fate of *Salmonella* inoculated into beef for cooking. J. Food Prot. 41:598-605.

Grein, T., D. O'Flanagen, T. McCarthy, D. Bauer. 1999. An outbreak of multidrug-resistant *Salmonella typhimurium* food poisoning at a wedding reception. Irish Med. J. 92:238-241.

Guiney, D. G., F. C. Fang, M. Krause, S. Libby. 1994. Plasmid-mediated virulence genes in nontyphoidal *Salmonella* serovars. FEMS Microbiol. Lett. 124:1-9.

Harmon, B. G., C. A. Brown, S. Tkalcic, P. O. E. Mueller, A. Parks, A. V. Erin, T. Zhao, M. P. Doyle. 1999. Fecal shedding and rumen growth of *Escherichia coli* O157:H7 in fasted calves. J. Food Prot. 62:574-579.

Hinton, M. 1986. Veterinary Annual. 26th Issue. Bristol, Scientechnuca. p.81.

Hollinger, K., C. Wray, S. Evens, S. Pascoe, S. Chappell, Y. Jones. 1998. *Salmonella typhimurium* DT104 in cattle in Great Britain. J. Am. Vet. Med. Assoc. 213:1732-1733.

Hooper-Kinder, C. A., M. P. Davidson, S. K. Duckett. 2000. Growth of *Escherichia coli* O157:H7, *Salmonella* Typhimurium DT104, and *Listeria monocytogenes* in dark cutting beef at 10 or 22°C. J. Food Prot. 65:196-198.

Humphrey, T. J., S. J. Wilde, R. J. Rowbury. 1997. Heal tolerance of *Salmonella typhimurium* DT104 isolated attached to muscle tissue. Lett. Appl. Microbiol. 25:265-268.

Hunter, A. G. and I. S. Peek. 1977. Vaccination control of an outbreak of *Salmonella typhimurium* infection in suckler cows and calves. Br. Vet. J. 133:239-244.

Ihnot, A. M., A. M. Roering, R. K. Wierzba, N. G. Faith, J. B. Luchansky. 1998. Behavior of *Salmonella typhimurium* DT104 during the manufacture and storage of pepperoni. Int. J. Food Microbiol. 40:117-121.

Imberechts, H., M. DeFilette, C. Wray, Y. Jones, C. Godard, P. Pohl. 1998. *Salmonella typhimurium* phage type DT104 in Belgian livestock. Vet. Rec. 143:424-425.

Jay, J. M. 2000. Modern Food Microbiology, 6th ed. Aspen Publishers, Gaithersburg, MD.

Juneja, V. K., and B. S. Eblen. 2000. Heat inactivation of *Salmonella typhimurium* DT104 in beef as affected by fat content. Lett. Appl. Microbiol. 30:461-467.

Jung, Y. S. and L. R. Beuchat. 2000. Sensitivity of multidrug-resistant *Salmonella typhimurium* DT104 to organic acids and thermal inactivation in liquid egg products. Food Microbiol. 17:63-71.

Jung, Y. S. and L. R. Beuchat. 1999. Survival of mulitdrug-resistant *Salmonella typhimurium* DT104 in egg powders as affected by water activity and temperature. Int. J. Food Microbiol. 49:1-8.

Kapperud, G., S. Gustavsen, I. Hellesnes, A. H. Hansen, J. Lassen, J. Hirn, M. Jahkola, M. A. Montenegro, R. Helmuth. 1990. Outbreak of *Salmonella typhimurium* infection traced to contaminated chocholate and caused by a strain lacking the 60-megadalton virulance plasmid. J. Clin. Microbiol. 28:2597-2601.

Khan, A .A., M. S. Nawz, S. A. Khan, C. E. Cerniglia. 2000. Detection of multi-drug resistant *Salmonella typhimurium* DT104 by multiplex PCR. FEMS Microbiol. Lett. 182:355-360.

Kiessling, C. R., J. H. Cutting, M. Loftis, W.M. Kiessling, A. R. Datta, J. N. Sofos. 2002. Antimicrobial resistance of food related *Salmonella* isolates, 1999-2000. J. Food Prot. 65:603-608.

Kowarz, L., C. Coynault, V. Robbe-Saule, F. Norel. 1994. The *Salmonella typhimurium* katF (rpoS) gene: cloning, nucleotide sequence, and regulation of spvR and spvABCD virulance plasmid genes. J. Bacteriol. 176:6852-6860.

Leach, S. A., A. Williams, A. C. Davies, J. Wilson, P. D. Marsh, T. J. Humphrey. 1999. Aerosol route enhances the contamination of intact eggs and muscle of experimentally infected laying hens by *Salmonella typhimurium* DT104. FEMS Microbiol. Lett. 171:203-207.

Le Minor, L. 1981. The genus *Salmonella*. pp. 1148-1159. *In* Star, M. P., H. Stlop, G. H. Truper, A. Ballows, H. G. Schlegel (ed.), *The Prokaryotes*. Springer-Verlag, New York.

Liebana, E., D. Guns, L. Garcia-Migura, M. J. Woodward, F. A. Clifton-Hadley, R. H. Davies. 2001. Molecular typing of *Salmonella* serotypes prevalent in animals in England: assessment of methodology. J. Clin. Microbiol. 30:3609-3616.

Low, J. C., G. Hopkins, T. King, D. Munro. 1996. Antibiotic resistant *Salmonella typhimurium* DT104 in cattle. Vet. Rec. 138:650-651.

McDonough, S. P., C. L. Stull, B. I. Osburn. 1994. Enteric pathogens in intensively reared veal calves. Am. J. Vet. Res. 55:1516-1520.

Molbak, K., D. L. Baggesen, F. M. Aarestrup, J. M. Ebbesen, J. Engberg, K. Frydendahl, P. Gerner-Schmidt, A. M. Petersen, H. C. Wegener. 1999. An outbreak of

multidrug-resistant, quinolone-resistant *Salmonella enterica* serotype Typhimurium DT104. N. Eng. J. Med. 341:1420-1425.

Murphy, T. M., E. McNamara, M. Hill, N. Rooney, J. Barry, J. Egan, A. O'Connell, J. O'Loughlin, S. McFaddyen. 2001. Epidemiological studies of human and animal *Salmonella typhimurium* DT104 and DT104b isolates in Ireland. Epidemiol. Infect. 126:3-9.

Newton, K. G. and C. O. Gill. 1981. The microbiology of DFD fresh meats: a review. Meat Sci. 5:223-232.

Oosterom, J. 1991. Epidemiological studies and proposed preventive measures in the fight against human salmonellosis. Int. J. Food Microbiol. 12:41-52.

Pascual, M., M. Hugas, J. I. Badiola, J. M. Monfort, M. Garriga. 1999. *Lactobacillus salivarius* CTC2197 prevents *Salmonella enteritidis* colonization in chickens. Appl. Environ. Microbiol. 65:4981-4986.

Piddock, L. J. V., V. Ricci, I. McLaren, D. J. Griggs. 1998. Role of mutatuin in the gyrA abd parC genes of nalidixic acid resistant *Salmonella* serotypes isolated from food animals in the United Kingdom. J. Antimicrob. Chemother. 41:635-641.

Poppe, C., N. Smart, R. Khakhria, W. Johnson, J. Spika, J. Prescott. 1998. *Salmonella typhimurium* DT104: a virulent and drug resistant pathogen. Can. Vet. J. 39:559-565.

Rahman, H., V. B. Singh, V. D. Sharma. 1994. Purification and characterization of enterotoxic moiety present in cell-free culture supernatant of *Salmonella typhimurium*. Vet. Microbiol. 9:245-254.

Rankin, J. D. and R. J. Taylor. 1966. The estimation of doses of *Salmonella* Typhimurium suitable for the experimental production of disease in calves. Vet. Rec. 78:706-707.

Rantala, M., and E. Nurmi. 1973. Prevention of the growth of *Salmonella infantis* in chick by the flora of the alimentary tract of chicks. Br. Poultr. Sci. 14:627-630.

Roering, A. M., J. B. Luchansky, A. M. Ihnot, S. E. Ansay, C. W. Kaspar, S. C. Ingham. 1999. Comparative survival of *Salmonella typhimurium* DT104, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in preservative-free apple cider and simulated gastric fluid. Int. J. Food Microbiol. 46:263-269.

Russel, J. B., and J. C. Rychlik. 2001. Factors that alter rumen microbial ecology. Science 292:1119-1122.

Salyers, A. A., and D. D. Whitt. 1994. Bacterial Pathogenesis. ASM Press, Washington, D.C. pp. 229-243.

Schmieger, H. 1999. Molecular survey of the *Salmonella* phage typing system of Anderson. J. Bacteriol. 181: 1630-1635.

Schmieger, H.and P. Schicklmaier. 1998. Transduction of multiple drug resistance of *Salmonella enterica* serovar *typhimurium* DT104. FEMS Microbiol. Lett. 170:251-256.

Seo, K. H., P. S. Holt, R. K. Gast, C. L.Hofacre. 2000. Elimination of early *Salmonella enteritidis* infection after treatment with competitive-exclusion culture and enrofloxacin in experimentally infected chicks. Poult. Sci. 79:1408-1413.

Sorensen, O., J. van Donkersgoed, M. McFall, K. Manninen, G. Gensler, G. Ollis. 2002. *Salmonella* spp. shedding by Alberta beef cattle and the detection of *Salmonella* spp. in ground beef. J. Food Prot. 65:484-491.

Tauxe, R.V. 1990. *Salmonella*: a postmodern pathogen. J. Food Prot. 54:563-568.

Teuber, M. 1999. Spread of antibiotic resistance with food-borne pathogens. Cell. Mol. Life Sci. 56:755-763.

Threlfall, E. J., L. Ward, B. Rowe. 1998. Multiresistant *Salmonella typhimurium* DT104 and *Salmonella* bacteraemia. Lancet 352: 287-8.

Therlfall, E. J., J. A. Frost, L. R. Ward, B. Rowe. 1996. Increasing spectrum of resistance in multiresistant *Salmonella typhimurium*. Lancet 347:1052-1053.

Threlfall, E .J., M. D. Hampton, S. L. Schofield, L. R. Ward, J. A. Frost, B. Rowe. 1996. Epidemiological application of differentiating multiresistant *Salmonella* Typhimurium DT104 by plasmid profile. Communicable Disease Report 6:R155-R159.

Threlfall, E. J., J. A. Frost, L. R. Ward, B. Rowe. 1994. Epidemic in cattle and humans of *Salmonella typhimurium* DT104 with chromosomally integrated multiple durg resistance. Vet. Rec. 134:577.

Tsolis, R. M., L. G. Adams, T. A. Ficht, A. J. Baumler. 1999. Contribution of *Salmonella typhimurium* virulance factors to diarrheal disease in calves. Infect. Immun. 67: 4879-4885.

Tutt, J. B. and D. I. B. Hoare. 1974. Disease associated with *S. typhimurium* in cattle. Vet. Rec. 95:334.

Uljas, H. E. and S. C. Ingham. 1999. Combinations of intervention treatments resulting in 5 $\log_{10}$-unit reductions in numbers of *Escherichia coli* O157:H7 *and Salmonella typhimurium* DT104 organisms in apple cider. Appl. Environ. Microbiol. 65:1924-1929.

van der Wielen, P. W. J. J., L. J. A. Lipman, F. van Knapen, S. Biesterveld. 2002. Competitive exclusion of *Salmonella enterica* serovar Enteritidis *by Lactobacillus crispatu*s and *Clostridium lactatifermentans* in a sequencing fed-batch culture. Appl. Environ. Microbiol. 68:555-559.

Van Donkersgoed, J., T. Graham, V. Graham, V. Gannnon. 1999. The prevalence of verotoxins, *Escherichia coli* O157:H7, and *Salmonella* in the feces and rumen of cattle at processing. Can. Vet. J. 40:332-338.

Villar, R. G., M. D. Macek, S. Simons, P. S. Hayes, M. J. Goldofl, J. H. Lewis, L. L. Rowan, D. Hursh, M. Patnode, P. S. Mead. 1999. Investigation of multidrug-resistant *Salmonella* serotype *typhimurium* DT104 infections linked to raw-milk cheese in Washington State. J. Am. Med. Assoc. 281:1811-1816.

Vladoianu, I. R., H. R. Chang, J. C. Pechere. 1990. Expression of host resistance to *Salmonella typhi* and *Salmonella typhimurium*:bacterial survival within macrophages of murine and human origin. Microb. Pathogen. 8:83-90.

Walker, R. A., A. J. Lawson, E. A. Lindsay, L. R. Ward, P. A. Wright, F. J. Bolton, D. R. A. Wareing, J. D. Corkish, R. H. Davies, E. J. Threlfall. 2000. Decreased susceptability to ciprofloxacin in outbreak associated multi-resistant *S. typhimurium* DT104. Vet. Rec. 147:395-396.

Wall, P. G., D. Morgans, K. Lamden, M. Griffin, E.J. Threlfall, L. R. Ward, B. Rowe. 1995. Transmission of multi-resistant strains of *Salmonella typhimurium* from cattle to man. Vet. Rec. 136:591-592.

Wall, P. G., D. Morgan, K. Lamden, M. Ryan, M. Griffin, E. J. Threlfall, L. R. Ward, B. Rowe. 1994. A case control study of infection with an epidemic strain of multidrug resistant *Salmonella typhimurium* DT 104 in England and Wales. Communicable Disease Review. 4:R130-135.

Wells, S. J., P. J. Fedorka-Cray, D. A. Dargatz, K. Ferris, A. Green. 2001. Fecal shedding of *Salmonella* spp. by dairy cows on farm and at cull cow markets. J. Food Prot. 64:3-11.

Williams, A., A. C. Davies, J. Wilson, P. D. Marsh, S. Leach, T. J. Humphrey. 1998. Contamination of the contents of the intact eggs by *S.* Typhimurium DT104. Vet. Rec. 143:560-563.

Witte, W. 2000. Ecological impact of antibiotic use in animals on different complex microflora: environment. Int. J. Antimicrob. Agents 14:321-235.

Wray, C., J. N. Todd, M. Hinton. 1987. Epidemiology of *Salmonella typhimurium* infection in calves: Excretion of *S. typhimurium* in the faeces of calves in different management systems. Vet. Rec. 121:293-296.

Zhao, S., J. Meng, T. Zhao, M. P. Doyle. 1995. Use of vaccine and biological control techniques to control pathogens in animals used for foods. J. Food Safety 15:193-199.

Zhao, T., M. P. Doyle, P. J. Fedorka-Cray, P. Zhao, S. Ladely. 2002. Occurrence of *Salmonella enterica* Serotype Typhimurium DT104a in retail ground beef. J. Food Prot. 65:403-407.

Zhao, T., M. P. Doyle, B. G. Harmon, C. A. Brown, P. O. E. Mueller, A. H. Parks. 1998. Reduction of carriage of enterohemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. J. Clin. Microbiol. 36:641-647.

CHAPTER 3

COMPETITIVE INHIBITION BACTERIA OF BOVINE ORIGIN AGAINST *SALMONELLA* SPP.[1]

[1] Danyluk, M. D., T. Zhao, M. P. Doyle.
To be submitted to Journal of Food Protection

Surveillance data of cattle and human isolates of *Salmonella enterica* serovar Typhimurium DT104 indicate that this pathogen emerged world wide in the 1980s, particularly in cattle. Recent data suggest that multi-resistant *Salmonella enterica* serovar Newport is emerging in the United States and indicate that cattle are a reservoir through which *S*. Newport can enter the food supply. Studies were conducted to determine the prevlance of *Salmonella* spp. in cattle in Georgia, to isolate bacteria inhibitory to *S*. Typhimurium DT104 in vitro from cattle not carrying *Salmonella* spp., and to show the inhibitory activity of the isolated bacteria through competitive growth in cattle feces artificially contaminated with *S*. Typhimurium DT104 and *S*. Newport. A total of 108 fecal samples were obtained from dairy cows, beef cows, and calves. *Salmonella* spp. were isolated from 10 (9.25%) of samples. All samples positive for *Salmonella* spp. were from beef cows. Six different serotypes were identifed among the isolates, including *Salmonella* Newport (4), *Salmonella* Bareilly (1), *Salmonella* Mbandaka (1), *Salmonella* Montevideo (1), *Salmonella* Meleagris (1), and two monophasic *Salmonella* spp. All four *S*. Newports showed resistance to at least nine antibiotcs, including ampicillin, amoxicillin/clavulanic acid, defoxitin, ceftiofur, cephalothin, cholroamphenicol, streptomycin, sulphamethoxazole, and tetracycline. Thirty of 1097 bacterial isolates from cattle feces were screened and determined to inhibit the growth of *S*. Typhimurium DT104 in vitro. Twenty-two of the isolates were *Escherichia coli*, six were *Bacillus circulans*, one was *Serratia fonticola*, and one was *Enterobacter cloacae*. Genomic DNA identification by pulsed-field gel electrophoresis revealed 19 distinguishable profiles among the 24 gram-negative isolates. Competitive inhibition isolates were unable to significantly reduce *S*. Typhimurium DT104 during storage for 21 days at 37°C. *B.*

circulans was able to cause a significant reduction of *S.* Newport on days 3 and 5 with a low inocula of the competitive inhibition bacteria (10^5 CFU/g) and at day 21 with a high inocula of the competitive inhibition bacteria(10^8 CFU/g) at 37°C. When stored at 21°C, A high inocula of gram-negative competitive inhibition bacteria significantly reduced *S.* Typhimurium DT104 populations at day 5 only, the low inocula of *B. circulans* also significantly reduced *S.* Typhimurium DT104 at day 5 only. No significant reductions were seen with *S.* Newport at this temperature. These reductions indicate that the competitive inhibition bacterial isolates can competitively decrease the growth of *S.* Typhimurium DT104 and *S.* Newport in a natural environment.

Salmonella Typhimurium DT104 was first identified in humans in England and Wales in 1984 (Threlfall et al. 1994). It was resistant to the antibiotics ampicillin, chloramphenicol, streptomycin, sulphonanides and tetracycline (R-type ACSSuT) (Threlfall et al. 1994. Between August 1st and September 8th, 2000, 265 cases of DT104 infection were confirmed by the PHLS Laboratory of Enteric Pathogens in the United Kingdom. In the same time frame in 1999, only 97 isolates were confirmed (CDR Weekly 2000).

The first reports of DT104 in the United States were in 1996 (Besser et al. 1997). An increase in the occurrence of outbreaks of salmonellosis in dairy cattle in the Pacific Northwest was observed in 1994, with many of the isolates being *S.* Typhimurium R-type ACSSuT. A subsequent examination of preserved bovine isolates of *S.* Typhimurium from this region revealed this R-type accounted for 13% and 64% of isolates from 1986 to1991 and 1992 to 1996, respectively. *S.* Typhimurium DT104 was also isolated from sheep, pigs, horse, goat, emu, cat, dog, elk, mouse, coyote, ground squirrel, raccoon, chipmunk and birds including pigeons, starlings, and pine siskins (Besser et al. 1997).

A historical review by the Centers of Disease Control and Prevention of the emergence of DT104 infections in the United States revealed the reported number of cases of *S.* Typhimurium has remained relatively constant between 1981 and 1996, excluding one large outbreak in 1985 (Glynn et al. 1998). However, the proportion of isolates with the five-drug resistance pattern increased, from less than 1% in 1979-1980 to 34% in 1996, with 91% of the penta-resistant isolates being DT104. Estimates indicate between 68 000 and 340 000 cases of penta-resistant *S.* Typhimurium infection, most of which were DT104, occurred in the United States in 1996. As compared to the United

Kingdom, there have been in the United States fewer outbreaks of DT104 infections. However, case-control studies of sporadic cases in the United States of DT104 infections in humans have identified consumption of unpasteurized dairy products and direct contact with livestock as risk factors (Glynn et al. 1998).

Threlfall et al. (1994) determined that in the United Kingdom *S.* Typhimurium DT104 accounted for most cases on salmonellosis in cattle since 1993. Between 1994 and 1995, 295 of 450 of all *Salmonella* spp. isolated were multi-antibiotic resistant DT104. DT104 infections continued to increase through 1996 in Great Britain, including the first appearance of *S.* Typhimurium DT104b. Strains of this phage type are resistant to more antibiotics *in vitro* than typical isolates of DT104.

A survey of *Salmonella* spp. serotypes isolated from feedlot cattle in the United States was done in 1998. *Salmonella* spp. was isolated from cattle in 38% of feedlots, and 5.5 % of all 4977 samples were *Salmonella*-positive. *S.* Typhimurium was detected in 2.6% of the positive feedlots, and in 2.9% of the positive samples (Fedorka-Cray et al. 1998). A similar study conducted on beef cattle in 2000 (Dargatz et al. 2000) revealed that 11.2% of all operations tested were positive for *Salmonella* spp., and 1.4% of all fecal samples obtained were positive. Results of this study indicate DT104 was not common.

Fecal shedding of *Salmonella* spp. by dairy cattle on the farm and at cull cow markets was studied in the United States in 2001 (Wells et al. 2001). The serotype distribution in dairy cattle differed from the 1991 data of which 26.5% of dairy calf isolates were *S.* Typhimurium, and the 1992 results of which *S.* Typhimurium was the most common isolate in clinically ill cattle, at 27.3%. A low fecal shedding prevalence of

S. Typhimurium by adult dairy cattle was observed in the 2001 study with only 1% of *Salmonella* spp. isolates identified as *S.* Typhimurium DT104.

Humans predominantly acquire *Salmonella* infection through the consumption of contaminated food of animal origin. Although *Salmonella* spp. are often associated with poultry and poultry products, DT104 is associated with a wide range of animals used in food production, especially cattle. The widespread occurrence of DT104 in animal populations complicates the challenge of eliminating or controlling *S.* Typhimurium DT104 in the food continuum (Crerar et al. 1999).

A national sampling of retail ground beef for DT104 in the United States revealed that 3.5% of 404 total samples were *Salmonella* spp. positive, of which 35.6% (14 samples) were *S.* Typhimurium DT104a (Zhao et al. 2002). All DT104a positive samples were from retail stores in San Francisco CA.

The administration of probiotic bacteria has been proposed as an approach to reduce carriage of enteric pathogens through competitive exclusion. These competitive exclusion bacteria are introduced as boluses or into the feed of cattle, and produce antimicrobial substances, or otherwise outcompete the pathogen in the rumen or colon (de Vaux et al. 2002). This approach was first successfully applied in 1973 by Rantala and Nurimi (1973) who prevented the growth of *Salmonella* Infantis in chicks through the application of enteric bacteria from adult *Salmonella*-free chickens.

Development and identification of competitive exclusion cultures using a science-based approach involves: defining how the pathogen colonizes the site of interest, isolating other microbes that colonize the same site and produce metabolites that inhibit or kill the pathogen, and verifying that when introduced to pathogen-free animals,

colonization of the pathogen is reduced or prevented (Zhao et al. 1995). The commercial implication of this technique is an essential part of the Hazard Analysis Critical Control Point (HACCP) plan of animal production, as it reduces the presence of the pathogen from the animals (Zhao et al. 1995).

Cattle are a reservoir for *S.* Typhimurium DT104, which may move into the human food chain subsequently leading to increased risk of foodborne disease caused by this pathogen. One approach to reduce carriage of *S.* Typhimurium DT104 by cattle is the use of competitive exclusion bacteria that produce antimicrobials and compete with *S.* Typhimurium DT104 at sites of colonization. Potential competitive exclusion bacteria should be isolated from the fresh feces of cattle that are not shedding *Salmonella* spp. and evaluated for anti-*S.* Typhimurium DT104 activity in vitro. Isolates showing anti-*S.* Typhimurium DT104 activity in vitro should be evaluated for the ability to inhibit *S.* Typhimurium DT104 within a natural environment, such as feces. Results of these studies would be useful as a first step to the application of competitive exclusion bacteria into cattle to reduce their carriage of *S.* Typhimurium DT104.

MATERIALS AND METHODS

Sample collection. A total of 108 fecal samples were collected in the middle Georgia region from September 2001 though January 2002. Samples were obtained from 28 dairy cattle, 80 beef cattle and five calves between four months and one year of age. Ten grams of feces was collected into Cary Blair with indicator fecal transport system

(Corpimex, Miami, Fl), and immediately transported to the Center for Food Safety at 5°C. Samples were stored at 4°C for 0 to 7 days until use.

***Salmonella* isolation and identification.** Each fecal sample (10 g) was preenriched in 90 ml of lactose broth (Becton Dickinson, Sparks, Md.) for 24 hours at 35°C. After preenrichment, 1-ml volumes of enrichment culture were transferred, for selective enrichment, to 10 ml of selenite cystine broth (Becton Dickinson, Sparks, Md.) and incubated for 24 hours at 37°C, to 10 ml of tetrathionate broth (Becton Dickinson, Sparks, Md.) and incubated for 48 hours at 37°C, and to 10 ml of Rappaport-Vassiliadis R10 broth (Becton Dickinson, Sparks, Md.) and incubated for 24 hours at 42°C. After selective enrichment, a 10-□l loopful from each broth was plated in duplicate on to the surface of bismuth sulfite agar (BSA), Hektoen enteric agar (HEA), xylose lysine deoxycholate agar (XLD) and xylose lysine tergitol 4 agar (XLT4) (all Becton Dickinson, Sparks, Md.) plates. Plates were incubated for 24 hours at 37°C. Colonies with typical *Salmonella* spp. morphology were selected from all plates, no more than 10 colonies per plate, and transferred into triple sugar iron agar and lysine iron agar (both Becton Dickinson, Sparks, Md.) slants and incubated for 24 hours at 35°C. All presumptive *Salmonella* isolates were tested by the *Salmonella* latex agglutination assay (Oxoid Ltd., Basingstoke, Hampshire, UK). All isolates positive with the *Salmonella* latex agglutination assay were tested with the API 20E assay (bioMerieux, Hazelwood, Mo.) for biochemical characteristics for the identification of *Salmonella* (Zhao et al. 2002). Serotyping was conducted at the U.S. Department of Agriculture- Animal and Plant Health Inspection Service (APHIS) National Veterinary Services Laboratories, Ames,

Iowa. Antibiotic resistance profiles were conducted at the U.S. Department of Agriculture-Agricultural Research Service, Athens, GA.

Isolation of potential competitive inhibition bacteria. *Salmonella*-negative fecal samples were serially diluted (1:10) in 0.1% peptone buffer, 0.1 ml of each dilution was plated in duplicate onto MacConkey agar (MAC) and tryptic soy agar (TSA) (both Becton Dickinson, Sparks, Md.), and the plates were incubated for 24 hours at 37°C. Seven colonies were randomly selected from MAC agar plates, and three colonies were randomly selected from TSA plates. Each colony was transferred to a test tube containing 10 ml of trypic soy broth (TSB) (Becton Dickinson, Sparks, Md.) and incubated for 24 hours at 37°C.

Screening of cultures for anti-*Salmonella* Typhimurium DT104 properties. A three-strain mixture of *Salmonella enteritidis* serovar Typhimurium DT104 from our culture collection, including strains 8748A-1 (cattle isolate, R-type ACSSuT), 11942A-1 (cattle isolate, R-type ACSSuT), and 62 (ground beef isolate, R-type ASSuT), were initially used to screen cultures for anti-*Salmonella* Typhimurium DT104 activity. Two methods were used to screen for activity, the disk method (Zhao et al. 1998) and the agar spot test (Schillinger and Lucke 1989).

Approximately 10^7 *S.* Typhimurium DT104 cells of approximately equal populations of each strain in 0.1 ml were plated onto the surfaces of XLD and TSA plates and allowed to dry for at least 30 minutes. Supernatant fluid from each culture was filter sterilized (0.2-□l-pore-size cellulose acetate membrane: Nalgene Co., Rochester N. Y.) for determination of anti-*S.* Typhimurium DT104 activity. Two disks (12 mm diameter, Dispens-O-Disc, Difco Laboratories, Detroit, Mich.) were placed on the surface of both

the TSA and XLD plates, and 0.1 ml of the filter-sterilized supernatant fluid from a single culture was applied to the surface of the disk. In addition, filter-sterilized supernatant fluid from *E. coli* ATCC 14763 (produces colicin V) and 70% ethanol were used as positive controls, and filter-sterilized TSB was used as the negative control. Cultures were incubated for 24 hours at 37°C and observed for zones of growth inhibition. Competitive inhibition bacteria were selected as those that produced a clear zone of at least 1mm around the disk.

Isolates were streaked onto TSA for single colonies and incubated for 24 hours at 37°C. Single colonies were spot inoculated onto TSA and MAC plates and incubated for 24 hours at 37°C for colony development. Five milliliters of brain heart infusion broth (BHI) (Becton Dickinson, Sparks, Md.) with 0.5% agar (Becton Dickinson, Sparks, Md.) containing approximately 10^6 CFU of the three strain S. Typhimurium DT104 mixture at 50°C was applied onto the surface of each plate, without disturbing the colony, and allowed to cool. Plates were incubated for 24 hours at 37°C, and observed for zones of growth inhibition. Competitive inhibition bacteria were selected as those that produced a clear zone of at least 1 mm around the disk.

Competitive inhibition cultures were then screened, using the methods described above, against nine additional strains of *S.* Typhimurium DT104, obtained from the collection of P.J. Fedorka-Cray, U.S. Department of Agriculture-Agricultural Research Service, Athens, Ga. All strains were cattle isolates, and included from 1998 strains 526-K, 2848-K and 12993-K, from 1999 strains MH25382, 99-103712-5 and 12-410 and from 2000 strains 4698-K, NE14055, IA45025. As well, competitive inhibition cultures

were screened against 10 *Salmonella* spp. isolates obtained during the screening process of this study.

The pH of TSB was determined before and after culture growth. The pH of TSB and MAC plates were determined before colony growth, and after colony growth both near the colony and two centimeters away from the colony following 24 hours of growth at 37°C. Growth curves for the competitive inhibition isolates strains and *Salmonella* were performed and generation times were calculated.

Identification of Competitive inhibition Bacteria. Initially competive inhibition isolates were characterized by Gram staining. Gram-positive strains were subjected to catalase tests, oxidase tests, and to biochemical testing using the API 50CH Assay (bioMerieux, Hazelwood, Mo.) with both the CHL media for lactic acid bacteria, and the CHB/E media for *Bacillus* spp. Spore formation was determined by holding overnight cultures at 80°C with agitation at 190 rpm for 10 minutes, then streaking the cultures in duplicate onto TSA plates and incubating for 24 hours at 37°C.

Gram-negative isolates were subjected to biochemical testing using the API 20E Assay (bioMerieux, Hazelwood, Mo.), and subtyping by PFGE using procedures similar to those described previously (Meng et al. 1995) and those used by the Centers for Disease Control and Prevention. This involved growing isolates on TSA plates for 24 hours at 37°C, then suspending cells of each culture in Cell Suspension Buffer (CSB) (100mM Tris:100mM EDTA, pH 8.0) with a sterile swab to a cell populations having an optical density of 1.3-1.4 at 610 nm (SPEC). The bacterial suspension, 0.2 ml, was mixed with 10 □l of 20 mg proteinase K/ml and 0.2 ml 1% SeaKem Gold: 1% SDS agarose in TE buffer (1mM Tris:1mM EDTA, pH 8.0). The mixture was dispensed into sample

moulds and the agarose plugs were digested with 0.1 mg proteinase K/ml in lysis buffer (20 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosine at 54°C for 2 hours. The plugs were then washed at 50°C, three times in sterile water and three times in TE buffer. Plugs were cut to 2.5 mm wide, prerestricted with 1X restriction buffer for 10 minutes at 37°C, then restricted using 50U Xba1 for 2 hours at 37°C. The reaction was stopped by the removal of reaction buffer and the addition of 0.5X Tris-borate EDTA buffer (TBE). The DNA samples were electrophoresed in 1% SeaKem gold agarose in 0.5X TBE buffer with a contour-clamped homogeneous electric field device (CHEF MAPPER, Bio-Rad, Hecules, Ca.). After electrophoresis for 18 hours at 6.0 V/cm with pulse times of 2.16 to 54.17 seconds, linear ramping and an electric field angle of 120 at 14°C, the gels were stained with ethidium bromide. The bands were visualized and photographed with UV transillumination.

Antibiotic resistance profiles of all unique isolates were obtained using Sensititre gram-positive and gram-negative MIC plates (TREK Diagnostic Systems, Inc.Westlake, Ohio).

Preparation of competitive inhibition bacteria for inoculation into feces. To facilitate enumeration of the competitive inhibition bacteria, all gram-negative bacterial isolates were selected for resistance to nalidixic acid (50 μg/ml) by exposure to serially (1:2) increased concentrations (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25 and 50 μg/ml) of nalidixic acid in TSB every 24 hours at 37°C. A single colony of each strain of nalidixic acid-resistant, gram-negative, competitive inhibition bacteria was transferred to 10 ml of TSB containing nalidixic acid (50 μg/ml) and incubated for 24 hours at 37°C. A 0.1 ml portion was transferred to 10 ml of TSB and incubated for 16 hours at 37°C. Bacteria

were then sedimented by centrifugation (4000 X g, 10 min), washed three times in 0.1% phosphate buffered saline, pH 7.2 (PBS) and then resuspended in PBS to an optical density of 0.5 at 640 nm (ca. 10^8 CFU/ml). Nineteen gram-negative isolates were combined at equal populations. Two levels of inocula (10^5 and 10^8 CFU of gram-negative competitive inhibition isolates strains per g of feces) were used.

Gram-positive competitive inhibition isolates were transferred to 10 ml of TSB and incubated for 16 hours at 37°C. Bacteria were then sedemented by centrifugation (4000 X g, 10 min), washed three times in 0.1% phosphate buffered saline, pH 7.2 (PBS) and then resuspended in PBS to an optical density of 0.5 at 640 nm (ca. 10^8 CFU/ml). Six gram-positive strains were combined at equal populations. Two levels of inocula (10^5 and 10^8 CFU of gram-positive competitive inhibition isolates per g of feces) were used.

A four-strain mixture of *S.* Typhmurium DT104, including strains 4698-K, 11942A1, 8748A1 and 62, which were previously described, was used. Each strain was grown in 10 ml of TSB held for 16 hours at 37°C. Bacteria were then sedimented by centrifugation (4000 X g, 10 min), washed three times in 0.1% phosphate buffered saline, pH 7.2 (PBS) and then resuspended in PBS to an optical density of 0.5 at 640 nm (ca. 10^8 CFU/ml). The four *S.* Typhimurium DT104 strains were combined at equal populations. Two levels of inocula (10^3 and 10^5 CFU of *S.* Typhimurium DT104 per g of feces) were used.

A four strain mixture of *Salmonella enteritidis* serovar Newport, including, strains S55, S57, S78, and S88, which were all characterized in this study, was used. The four-strain cell suspension was prepared according to the same procedures described above for

S. Typhimurium DT104.Two levels of inocula (10^5 and 10^8 CFU of S. Newport per g of feces) were used.

Feces. Ten healthy beef cattle over the age of one year were used as the sources of feces. Fecal samples, which were obtained in June, were collected into 50 ml Falcan tubes, and transported to the laboratory at 5°C. All samples were screened for *Salmonella* spp. by the procedure described above. All feces were mixed well in stomacher bags at medium speed for 5 minutes.

Inoculation of the feces with S. Typhimurium DT104, S. Newport and competitive inhibition bacteria. The inocula of S. Typhimurium DT104 or S. Newport, and gram-negative competitive inhibition bacteria or gram-positive competitive inhibition bacteria (total 2 ml) mixtures at the appropriate dilution were added to 18 g of feces in sterile stomacher bags and mixed in a stomacher at medium speed for five minutes to obtain the desired bacterial concentrations.

Fecal sample inoculations included, 10^5 *S.* Typhimurium DT104/g and 10^8 gram-negative competitive inhibition bacteria/g, 10^3 *S.* Typhimurium DT104/g and 10^5 gram-negative competitive inhibition bacteria/g, 10^5 *S.* Typhimurium DT104/g and 10^8 gram-positive competitive inhibition bacteria/g, 10^3 *S.* Typhimurium DT104/g and 10^5 gram-positive competitive inhibition bacteria/g, 10^5 S. Newport/g and gram-positive competitive inhibition bacteria/g, and 10^3 S. Newport/g and 10^5 gram-positive competitive inhibition bacteria/g. Controls included both inoculation levels of gram-negative competitive inhibition bacteria, gram-positive competitive inhibition bacteria, S. Typhimurium DT104, S. Newport and total aerobic counts.

Incubation and Sampling. Inoculated fecal samples were held under aerobic conditions at 21° and 37°C. Duplicate samples were obtained at 0, 1, 3, 5, 7, 14 and 21 days post-inoculation. Fecal samples (1 g) were serially diluted (1:10) in 0.1% peptone and assayed for *S.* Typhimurium DT104 or *S.* Newport counts by direct plating 0.1 ml portions onto XLD containing ampicillin (32 μg/ml), tetracycline (16 μg/ml) and streptomycin (64 μg/ml) (XLD+). Plates were incubated for 24 hours at 37°C. When *Salmonella* was not detectable by direct plating, 1 g samples of feces mixed with 0.1% peptone were added to 10 ml of double strength lactose broth for enrichment cultures at 35°C for 24 hours. Enrichment cultures were subsequently plated onto XLD+ and incubated at 37°C for 24 hours. Gram-negative competitive inhibition bacteria were enumerated by direct plating 0.1 ml portions onto MAC containing nalidixic acid (50 μg/ml). Plates were incubated for 24 hours at 37°C. Gram-positive competitive inhibition bacteria were enumerated by direct plating 0.1 ml portions onto TSA, incubating for 24 hours at 37°C, and subtracting total aerobic counts obtained for that day of the study. pH values were determined for 1 g fecal samples mixed with 9 ml of 0.1% peptone. All tests were performed in duplicate and the entire study was performed in triplicate.

Statistical analysis. The Statistical Analysis System (SAS) computer statistical package (SAS Institute, Cary, NC) was used for analysis of data with Duncan's multiple range tests to determine if significant differences ($P<0.05$) in populations of S. Typhimurium DT104 exist between mean population values.

RESULTS

Isolatioin of *Salmonella* spp. from bovine feces. *Salmonella* spp. were isolated from 10 of 108 fecal samples. All positive samples were from beef cattle over one year of age (Table 1). Two samples were collected on October 16, 2001, three samples were collected January 15, 2002, and five samples were collected January 29, 2002.

The positive isolates included serogroups B, C1, C2, and E, four of which were serotyped as *Salmonella* Newport, two as monophasic *Salmonella* sp., one as *Salmonella* Bareilly, one as *Salmonella* Mbandaka, one as *Salmonella* Montevideo, and one as *Salmonella* Meleagridis.

Antimicrobial resistance profiles indicated that all four of the *Salmonella* Newport isolates were resistant to amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, cephalothin, chloramphenicol, streptomycin, and tetracycline. Isolates S55 and S57 were additionally resistant to sulphamethoxazole, and isolate S78 was also resistant to trimethoprim/ sulphamethoxazole. Isolate S88 was additionally resistant to sulphamethoxazole and trimethoprim/sulphamethoxazole, and had intermediate resistance to ceftriazone. All other *Samonella* isolates had intermediate resistance to tetracycline, but were sensitive to all other antibiotcs. All isolates were sensitive to amikaxin, apramycin, ciprofloxacin, gentamicin, imipenem, kanamycin, and naladixic acid.

In vitro screening and identification of potential competitive inhibition bacteria inhibitory to S. Typhimurium DT104. A total of 1097 bacterial colonies were

isolated from the feces of cattle determined not to excrete *Salmonella* spp. in their feces. These bacteria were initially screened for their ability to inhibit the growth of, or kill a three-strain mixture of *S.* Typhimurium DT104 in vitro, and 45 were determined to be inhibitory (Table 2), one through the paper disk assay (Fig. 1.), and 44 via the agar-spot test 9 (Fig. 2.). The size and clarity of the zones of inhibition varied with the type of media and the competitive inhibition candidate.

The 45 candidates were screened for in vitro inhibitory activity against an additional nine isolates of *S.* Typhimurium DT104, and 30 were determined to be inhibitory (Table3, Table 4), one through the paper disk assay, and 29 through the agar-spot assay. These 30 candidates were then screened for antimicrobial activity against the isolated *Salmonella* spp. isolates from the bovine feces. Only six gram-positive bacteria produced any degree of inhibitory activity against all 10 strains as demonstrated by the agar spot test. Three gram-negative isolates were inhibitory to five of the ten isolated strains (Table 5, Table 6).

The initial pH of TSB was 7.38, the pH of the medium decreased from 0.82 to 1.70 pH units occurred with the growth of each of the 30 competitive inhibition isolates that were active against all 12 strains of *S.* Typhimurium DT104. A decrease in pH also occurred with the growth of 12 *S.* Typhimurium DT104 strains and ranged from 1.10 to 1.37 pH units. The pH on MAC plates either increased slightly or decreased slightly near the competitive inhibition colonies, with a maximum pH increase of 0.22 pH units, and a maximum pH decrease of 0.54 pH units. The pH on TSA plates also increased slightly with the growth of some competitive inhibition candidates and decreased slightly with

others, with a maximum pH increase of 0.34 pH units, and a maximum pH decrease of 0.08 pH units.

Generation times of *S*. Typhimurium DT104 in TSB averaged 25 minutes, those of gram-negative competitive inhibition isolates ranged from 25 minutes to 50 minutes. The generation times of gram-positive competitive inhibition isolates ranged from 38 minutes to 52 minutes (data not shown).

The six gram-positive bacteria isolates were catalase-positive and oxidase-negative (Table 7). The API 50CH gave only doubtful profiles with the CHL media. With the CHB/E media gave very good identification of isolate 71-8 as *Bacillus circulans*, good identification of isolates 58-9 and 76-9 as *Bacillus circulans*, acceptable identification of 47-10 as *Bacillus circulans*, low discrimination for isolate 59-9 small *as Bacillus circulans,* and a doubtful profile for 59-9 big as *Bacillus circulans*. Each isolate was confirmed to be a spore producer. Antibiotic resistance profiles varied, with all strains being resistant to cefoxitin and ceftiofur, some strains being resistant to streptomycin, varying strains having intermediate resistance and resistance to ceftriaxone, all strains having intermediate resistance to tetracycline and some strains having intermediate resistance to chloroamphenicol.

The 24 gram-negative competitive inhibition bacteria were identified through biochemical testing using the API 20E Assay (Table 8). Twenty-two *E. coli*, one *Serratia fonticola*, and one *Enterobacter cloacae* were identified. Genomic DNA subtyping revealed 17 different profiles among the 22 *E. coli* isolates (Fig. 3). Antibiotic resistance profiling revealed that all the strains had some level of resistance to tetracycline. Other resistance to antibiotics varied among strains.

Competitive Growth in Feces. The average initial aerobic plate count of the fecal samples was 2.6 x 10^9 CFU/g, and the average initial pH was 7.1. No *Salmonella* spp. was detected in the feces before inoculation. At 37°C, all the *Salmonella* populations increased about 2 $\log_{10}$ CFU/g one-day post inoculation (Fig. 4). No significant differences were observed with either of the inoculations of competitive inhibition bacteria against *S*. Typhimurium DT104 at both inoculation levels during the 21-day period. At 37°C, a significant difference (P>0.05) was observed with the low-level inoculations of gram-positive competitive inhibition isolates with *S*. Newport at days 3 and 5, and at the high level inoculation at day 21. The pH of the feces increased slightly (ca. pH of *Salmonella* only=7.25, pH *Salmonella* and CI bacteria= 7.58) for all samples during the incubation period (data not shown).

At 21°C, a population increase of 1 to 4 $\log_{10}$ *Salmonella*/g was seen following the first day of growth (Fig. 5). The low-level inoculation of gram-negative competitive inhibition bacteria did not significantly reduce (P>0.05) *S*. Typhimurium DT104 growth when compared to the control. The high inoculation level of gram-negative competitive inhibition bacteria significantly reduced *S.* Typhimurium DT104 populations at day 5 only. A significant reduction (P>0.05) of *S*. Typhimurium DT104 occurred at day five of the low inoculation level of gram-positive competitive inhibition bacteria. No significant reductions occurred at this temperature with the high inoculation level of gram-positive competitive inhibition bacteria. The gram-positive competitive inhibition bacteria did not significantly reduce (P>0.05) the growth/survival of *S*. Newport at 21°C. The pH increase slightly for all *Salmonella* control samples (ca. pH= 7.49), and decreased slightly for

Salmonella and CI bacteria samples (ca. pH=7.02) during the incubation period (data not shown).

DISCUSSION

Ten *Salmonella* spp. were isolated from the feces of beef cows, none were found from dairy cows or calves. These data indicate that the prevalence of asymptomatic fecal shedding of *Salmonella* spp. by beef cows in Georgia is 12.5 %. Other reports indicate that 1.4 % of fecal samples were positive for fecal shedding of *Salmonella* within beef cow-calf operations nationally (Dargatz et al. 2000), and that 5.5 % of fecal samples collected in feedlots were positive for *Salmonella* spp. nationally (Fedoka-Cray et al. 1998). Dargatz et al. (2000) reported no positive samples from the 96 fecal samples collected from Georgia. Our data indicate that this number is higher. Other studies indicate that 5.4% of fecal samples from dairy cows, in 20 selected states representing 83% of U. S. dairy cows, on farms were positive for *Salmonella* (Wells et al. 1998). However, we did not isolate *Salmonella* spp. from any fecal samples of 24 dairy cows assayed. Several factors may have influenced the outcome of these results. Shopping and relocation stress has been shown to increase the fecal shedding of *Salmonella* spp. by cattle from 0 % at the farm to 1.5 % at entry to the auction market (Corrier et al. 1990). The time of year the samples were collected may also influence the prevalence of *Salmonella* spp. Seasonal variations in the fecal shedding of *Salmonella* spp. have been reported (Wray et al. 1987), with increased shedding in warmer months. One may have expected this trend during the course of this, study with more *Salmonella* spp. isolated from samples collected in the summer and early fall and than isolated in the winter.

However fewer Salmonella spp. isolates were collected in the summer and early fall than in the winter, possibly related to the warm weather conditions that were present at all sampling times.

Of the 10 Salmonella spp. isolates obtained in this study, five were of serotypes among the ten most commonly associated with human illness, including *Salmonella* Newport (n=4) and *Salmonella* Montevideo (n=1). These isolates were also among the 20 serotypes most commonly associated with non-human clinical isolates, as were *Salmonella* Mbandaka and *Salmonella* Meleagridis (CDC 2000). The presence of these organisms in the feces of beef cattle may lead to contamination of beef through the slaughter, transportation, handling and preparation process (Corrier et al. 1990). *S*. Newport infections have increased from 5% of reported *Salmonella* infections in 1997, to 10% in 2001, making it the third most common *Salmonella* serotype in the United States. Between January and April 2002, a multi-resistant strain of *Salmonella* Newport was isolated from 47 ill persons in five states. A culture of *S*. Newport from ground beef revealed an indistinguishable PFGE pattern from the outbreak strain, implicating exposure to raw or undercooked ground beef as the vehicle of transmission (MMWR Weekly 2002). The outbreak strain was resistant to at least 9 antibiotics. In our study *S*. Newport resistant to at least nine antibiotics, were obtained from 3.7% of cattle assayed on three separate dates. These findings provide further evidence for the emergence of multi-resistant *S*. Newport in the United States, and indicate that cattle are a reservoir through which *S*. Newport can enter the food supply.

Cattle are also reservoirs for other foodborne pathogens, such as *Escherichia coli* O157:H7 (Borczyk et al. 1987). Some strains of *E. coli* can produce colicins that are

inhibitory to other closely related strains (Bradley et al. 1991). Colicins could be one of many metabolites produced by probiotic bacteria in the rumen and secretions of the gastrointestinal tract. Studies by Zhao et al. (1998) determined that the administration of probiotic *E. coli* to calves prior to exposure to *E. coli* O157:H7 decreases the duration of ruminal carriage of *E. coli* O157:H7. More than one administration may be necessary to provide protection for all animals. *Bacillus subtilis* spores are also currently being used as probiotics for both human and animal consumption (Casula and Cutting, 2002). They are available as over-the-counter prophylactics for mild gastrointestinal disorders, as health foods, and nutritional supplements. In Vietnam, however, *B. subtilis* spores are being used for oral bacteriotherapy of gastrointestinal disorders under clinical supervision (Casula and Cutting, 2002). Spore germination in the gastrointestinal tract of mice has been demonstrated. Recent studies by Hoa et al. (2001) revealed that *B. subtilius* can suppress of *E. coli* O78:K80 infection in day–old-chicks.

The administration of competitive exclusion bacteria to prevent *Salmonella* spp. infection has been examined in chickens and mice. Pascual et al. (1999) determined a rifampin-resistant *Lactobacillus salivarius* strain CTC2197 prevented *Salmonella* Enteritidis C-114 colonization in chickens, when administered by oral gavage or through feed and drinking water, to day old birds. The strain, however, could not be detected in some birds at 21 and 28 days post administration indicating more than one dose may be necessary for the competitive exclusion bacteria to be maintained in the gastrointestinal tract. Similar results were observed by Audisio et al. (1999) who determined that *Enterococcus faecium* J 96 isolated from free-range chickens inhibited *Salmonella* Gallinarum, *S*. Pullorum, *S*. Enteritidis and *S*. Typhimurium in mixed fermentation

cultures. When orally adiministered to chickens, they survived a challenge of *S.* Pullorum, but the probiotic strain was not effective as a theraputic agent (Audisio et al. 2000). Seo et al. (2000) eliminated *S*. Enteritidis infection in chicks with normal avian gut flora derived from a pathogen-free adult; however, no significant differences were observed for organ infection. A culture mixture of *Lactobacillus crispatus* and *Clostridium lactaifermentans* has also been evaluated as a competitive exclusion culture for *S*. Enteritidis in chickens (van der Wielen et al. 2002). The mechanism of exclusion is hypothesized to be to undissociated forms of acetic and propionic acid present at pH 5.8 that are produced by *C. lactaifermentans* fermentation. Filho-Lima et al. (2000) orally administered *Lactobacillus acidophilus, Saccharomyces boulardii* and *E. coli* to gnotobiotic mice prior to challenge with *S*. Typhimurium but were unnable to affect colonization by *S*. Typhimurium.

In our study, 25 potential competitive inhibition candidates were identified with different levels of inhibitory activity to *S*. Typhimurium DT104 based on spot on agar assay and paper disk assay. Only one isolate tested positive by the paper disk assay, whereas the other 24 were active by the spot on agar test. The inability of cell-free supernatants of competitive inhibition isolates to inhibit *S*. Typhimurium DT104 may be due to excretion of anti-*S*. Typhimurium DT104 compounds at concentrations below the level of detection when assayed by a paper disk method (Schillinger et al. 1993) or that the anti-*S*. Typhimurium DT104 compounds were bound to the cell surface and not excreted into the liquid medium (Leriche et al. 1999).

Nineteen of the competitive inhibition were identified as gram negative rods. Seventeen of these competitive inhibition bacteria isolated were *E. coli*, one was *Serratia*

fonticola and one *Enterobacter cloacae*. Eleven of the isolates (59.9%), including nine *E. coli*, and both the *S. fonticola* and *E. cloacae*, were isolated from dairy cows, 1 isolate (3.3%) was obtained from a beef calf, and the remaining 7 isolates (36.8%) were isolated from beef cattle. Previous studies indicate *E. coli* are capable of competitive inhibition in cattle against *E. coli* O157:H7 (Zhao et al. 1998) and against S. Typhimurium in gnotobiotic mice (Filho-Lima et al. 2000).

Antibiotic testing revealed varying resistance patterns among the different isolates. An intermediate resistance to chloroamphenicol was observed in three of the E. coli isolates, which is suprising because this antibiotic is not approved for feedlot animals (beef cattle), and does not target *E. coli*. Isolates 13-2 and 13-6, with identical pulsotypes, were resistant to sulphamethoxazole and tetracycline, both of which are active against *E. coli* and are approved for use in feedlot animals. These isolates also were resistant to streptomycin, which is not approved to feed to cattle. However, its mode of action is similar to another aminoglyoside, spectinomycin, which is approved for beef catle, and prolonged exposure may result in resistance to both (Brock, 1997). Isolate 66-3 was resisitant to sulphamethoxazole. The resistance seen in these isolates to the antimicrobials approved for usage in beef cattle may be due to exposure to the antibiotic on the feedlot. Isolate101-1 was resistant to ampicillin and cephalothin. Ampicillin is approved for use in beef cattle and is active against *E. coli*, therefore it's resistance may be due to exposure in the environment. Cephalothin, although it is specific for *E. coli*, is not approved for feedlot animals. Cephalothin is not approved for beef cattle thus resistance to this antibiotic is not due to prolonged exposure in the environment. Resistance to this antimicrobial may be due to exchange of resistance factors among promiscuous bacteria.

All isolates were intermediately resistant to tetracycline, this is not surprising, because tetracyclines have been used in humans and animals for 50 years (Chopra and Roberts 2001). Isolate 5-3, a strain of *Enterobacter cloacae*, was resistant to four antibiotics. *E. cloacae* has natural occurring resistance to ampicillin, amoxicillin/clavulanic acid and cefoxitin (Stick et al. 2001).

The remaining six strains were identified as *Bacillus circulans*. These isolates all had antimicrobial activity by the spot on agar assay. *B. circulans* spores frequently occur in soil, and have also been isolated from the intestinal tract of free-living small mammals (Swiecika 2001). This species of *Bacillus* is very heterogeneous, with growth requirements ranging from minimal nutrients for some strains to complex nutrients for others. Nakamura and Swezey (1983) suggest that *B. ciculans* should be named a complex rather than a species. Studies have revealed that some environmental, food, and clinical isolates of *B. circulans* are toxigenic, producing enterotoxin similar to those of *B. cereus* (Beattie and Williams 1999; Rowan et al. 2001; Phelps and McKillip 2002). Some clinical conditions in humans that have been associated with *B. circulans* include meningitis (Boyette and Rights 1957), endocarditis (Krause et al. 1999) and endophthalmitis (Tandon et al. 2001).

Hyronimus et al. (2002) isolated from poultry houses four strains of *Bacillus subtilus* that showed a wide anti-*Salmonella* spectrum. Their results suggest that the anti-*Salmonella* metabolites possess an essential lipopeptidic moiety for bactericidal activity, with surface-active properties. Strains of *B. circulans* are known to produce butirosin, an aminoglycosidic antibiotic (Howells et al. 1972; Dion et al. 1972; Heifetz et al. 1972). Nam and Ryu (1985) suggest that spore formation is related to antibiotic production. Two

types of butirocin have characterised, A and B, as well as many analogs that exhibit broad-spectrum antibacterial activities (Takeda et al. 1978). Ota et al. (2000) have characterized the butirosin-biosynthetic gene cluster in *B. circulans*.

All six B. circulans isolates were resistant to cefoxitin, cefiofur and streptomycin. Strain 58-9 was also resistant to chloramphenicol, and strains 47-10 and 71-8 exhibited additional resistance to ceftriaxone. Cefoxitin and ceftriaxone are in the cephalosporin family of antibiotics that primarilly targets gram negative-bacteria, thus resistance of the gram-positive *B. circulans* isolates is not surprising. Ceftiofur is approved for use in beef cattle, and targets *E. coli*, hence resistance to this antibiotic by all strains is also not surprising. Streptomycin resistance by all isolates is also not unexpected, and is similar to spectinomycin, which is approved for beef cattle, and prolonged exposure may result in resistance to both (Brock, 1997). As well if the *B. circulans* isolates are producing butirocin, another aminoglycoside, resistance to streptomycin may be infered. Some strains of *B. circulans* that carry the vanA gene cluster, which confers resistance to vancomycin, have been identified (Ligozzi et al. 1998), and may be linked to vancomycin resistance in enterococci (Fontana et al. 1997).

Our candidate competitive inhibition bacteria, when inoculated into cattle feces artifically contaminated with *S.* Typhimurium DT104 and *S.* Newport, were unable to substantially decrease the duration of survival of the *Salmonella* spp. The initial increase of *S.* Typhimurium DT104 and *S.* Newport observed one day post-inoculation was similar to the increase observed for *E. coli* O157:H7 (Wang et al. 1996) and indicates growth of the pathogen in feces at both 21 and 37°C. At 37°C, a significant reduction of *S.* Newport was observed with the *B. circulans* at days 3 and 5 for the low inoculum, and at day 21

for the high inoculum. At 21°C significant inhibition of *S*. Typhimurium DT104 by the high inoculation of gram-negative competitive inhibition bacteria occurred at day 5 only, and no significant reductions were observed for *S*. Newport. Although inconsistent reductions were observed throughout the 21-day incubation period, some reductions did occur in feces. These reductions, however, indicate that the competitive inhibition bacterial isolates can competitively decrease the growth of *S*. Typhimurium DT104 and *S*. Newport in a natural environment. It would be useful to test their utility as competitive inhibition agents in an animal model.

The mechanism(s) by which our competitive inhibition bacteria inhibit the growth of *S*. Typhimurium DT104 and *S*. Newport in vitro remains to be determined. Competitive inhibition bacteria are thought to prevent colonization of the gastrointestinal tract by pathogenic organisms by three basic mechanism, including immune exclusion, competitive adhesion and synthesis of an antimicrobial substrate to impair colonization (Tannok 1999). The generation times of the competitive inhibition isolates the same as those of *S*. Typhimurium DT104 or longer, thus the mechanism of action is unlikely to be due to the rate of growth of the organism. As well pH changes of media used were similar with growth of the competitive inhibition isolates and *S*. Typhimurium DT104, thus it is unlikely changes in pH played a role in the mechanism of action of the competitive inhibition isolates.

The efficacy of the treatment of cattle with competitive inhibition bacteria to reduce the level of intestinal carriage and fecal shedding of *S*. Typhimurium DT104 and *S*. Newport by cattle needs to be determined. However, if effective, reducing the intestinal carriage of these pathogens by cattle should decrease the potential for

contamination of meat, fruits and vegetables, and water, thereby decreasing the risk of *S.* Typhimurium DT104 and *S.* Newport infection in humans.

REFERENCES

Anonymous. 2002. Outbreak of multidrug-resistant *Salmonella* Newport --- United States, January – April 2002. MMWR Weekly. 51:545-548.

Anonymous. 2000. National increase in *Salmonella typhimurium* DT104—update. Communicable Disease Report CDR Weekly.10:311,314,323,326.

Audisio, M. C., G. Oliver, M. C. Appella. 2000. Protective effect of *Enterococcus faecium* J96, a potential probiotic strain, on chicks infected with *Salmonella pullorum*. J.Food Prot. 63:1333-1337.

Audisio, M. C., G. Oliver, M. C. Apella. 1999. Antagonistic Effect of *Enterococcus faecium* J96 against Human and Poultry Pathogenic *Salmonella* spp. J. Food Prot. 62(7):751-755.

Beattie, S. H., A. G. Williams. 1999. Detection of toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. with an improved cytotoxicity assay. Vet. Appl. Microbiol. 28:221-225.

Besser, T. E., M. Goldoft, L. C. Pritchett, R. Khakhria, D. D. Hancock, D. H, Rice, J. M. Gay, W. Johnson, C. C. Gay. 2000, Multiresistant *Salmonella* Typhimurium DT104 infections of humans and domestic animals in the Pacific Northwest of the United States. Epidemiol. Infection. 124:193-200.

Besser, T. E., C. C. Gay, J. M. Gay, D. D. Hancock, D. Rice, L. C. Pichett, E. D. Erickson. 1997. Salmonellosis associated with *Salmonella typhimurium* DT104 in the United States. Vet. Rec. 140:75.

Borczyk, A. A., M. A. Karmali, H. Lior, and L. M. C. Cuncan. 1987. Bovine resevoir for verotoxin-producing *Escherichia coli* O157:H7. Lancet i:98.

Boyette, D. P. and F. L. Rights. 1952. Heretofore undescribed aerobic sporeforming bacillus in a child with meningetis, J. Am. Med Assoc. 148:1223-1224.

Bradley, D. E., S. P. Howard, H. Lior. 1991. Colicinogeny of O157:H7 enterohemorrhagic *Escherichia coli* and the shielding of colicin and phage receptors by their O-antigenic side chains. Can. J. Microbiol. 37:97-104.

Brock. 1997. Biology of Microorganisms, 8th. Prentice Hall. Upper Saddle River NJ. P.423-424.

Casula, G. and S. M. Cutting. 2002. *Bacillus* probiotics: spore germination in the gastrointestinal tract. Appl. Environ. Microbiol. 68:2344-2352.

CDC. *Salmonella* surveillance: Annual summary, 2000. Atlanta, Georgia: US Department of Health and Human Services, CDC, 2001.

Chopra, I. And M. Roberts. 2001. Tetracycline antibiotics: mode of action, applications, molecular bioloby , epidemiology of bacterial resistance. Review. Microbiol. Molec. Biol. Rev. 65:232-260.

Corrier, D. E., C. W. Purdy, J. R. DeLoach. 1990. Effects of marketing stress on fecal excretion of *Salmonella* spp. in feeder calves. Am J Vet Res 51:866-869.

Crerar, S. K., T. J. Nicholls, M. D. Barton. 1999. Multidrug-resistant *Salmonella typhimurium* DT104—implications for animal industries and the veterinary profession. Aust. Vet. J. 77(3):170-171.

Dargatz, D. A., P. J. Fedorka-Cray, S. R. Ladely, K. K. Ferris. 2000. Survey of *Salmonella* serotypes shed in feces of beef cows and their antimicrobial susceptibility patterns. J. Food Prot. 63:1648-1653.

De Vaux, A., M. Morrison, R. W. Hutkins. 2002. Displacement of *Escherichia coli* O157:H7 from rumen medium containing prebiotic sugars. Appl. Environ. Microbiol. 68:519-524.

Dion, H. W., P. W. K. Woo, N. E. Willmer, D. L. Ker, J. Onaga, S. A. Fusari. 1972. Butirosin, a new aminoglycosidic antibiotic complex: isolation and characterization. Antimicrob. Agents Chemother. 2:84-88.

Fedorka-Cray, P. J., D. A. Dargatz, L. A. Thomas, J. T. Gray. 1998. Survey of *Salmonella* serotypes in feedlot cattle. J. Food Prot. 61:525-530.

Filho-Lima, J. V. M., E. C.Vieira, J. R. Nicoli. 1999. Antagonistic effect of *Lactobacillus acidophilus, Saccharomyces boulardii* and *Escherichia coli* combinations against experimental infections with *Shigella flexneri* and *Salmonella entertitdis* subsp. Typhimurium in gnotobiotic mice. J. of Appl. Microbiol. 88:365-370.

Fontana, R., M. Ligozzi, C. Pedrotti, E. M. Padovani, G. Cornaglia. 1997. Vancomycin-resistant *Bacillus circulans* carrying the vanA gene responsible for vancomycin resistance in enterococci. Euro. J. Clin. Microbiol. 16:473-4.

Glynn, M. K., C. Bopp, W. Dewitt, P. Dabney, M. Mokhtar, F. J. Angulo. 1998. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. N. Engl. J. Med. 338:1333-1338.

Heifetz, C. L., M. W. Fisher, J. A. Chodubski, M. O'. DeCarlo. 1972. Butirosin: a new aminoglycosidic antibiotic complex: antibacterial activity in vitro and in mice. Antimicrob. Agents and Chemother. 2:89-94.

Hoa, T. T. , L. H. Duc, R. Isticato, L. Baccigalupi, E. Ricca, P. H. Van, S. M. Cutting. 2001. The fate and dissemination of *Bacillus subtilis* spores in a murine model. Appl. Environ, Microbiol. 67:3819-3823.

Howells, J. D., L. E. Anderson, G. L. Coffey, G. D. Senos, M. A. Underhill, D. L. Vogler, J. Ehrlich. 1972. Butirosin , a new aminoglycosidic antibiotic complex: bacterial origin and some microbiological studies. Antimicrob. Agents Chemother. 2:79-83.

Hyronimus, B., L. Dhetz, C. Moquet, F. Roblet, A. Rosique, J. Benaud. 2002. Selection of spore forming bacteria producing anti-*Salmonella* compounds. In *Salmonella* & salmonellosis proceedings. P 621.

Krause, A. F. K. Gould, J. Forty. 1999. Prosthetic heart valve endocarditis caused by *Bacillus circulans*. J. Infect. 39:160-162.

Leriche, V. D., B.. Chasaing, B. Carpentier. 1999. Behavior of *Listeria monocytogenes* in an artifically made biofilm of a nisin-producing strain of *Lactococcus lactis*. Int. J. Food Microbiol. 51:169-182.

Ligozzi, M., G. Lo Cascio, R. Fontana, 1998. van A gene cluster in a vancomycin-resistant clinical isolate of *Bacillus circulans*. Antimicrob. Agents Cnemother. 42:2055-9.

Meng, J., S. Zhao, T. Zhao, M. P. Doyle. 1995. Molecular characterization *of Escherichia coli* O157:H7 isolates by pulsed-field gel electrophoresis and plasmid DNA analysis. J. Med. Microbiol. 42:258-263.

Nakamura, L. K. and J. Sweeny. 1983. Taxonomy of *Bacillus circulans* Jordan 1890: base composition and reassociation of deoxyribonucleic acid. Int. J. Sys. Bacteriol. 33:703-708.

Nam, D. H. and D. D. Y. Ryu. 1985. Relationship between butirosin biosynthesis and sporulation in *Bacillus circulans*. Antimicrob. Agents Chemother. 27:798-801.

Ota, Y., H. Tamegai, F. Kudo, H. Kuriki, A. Koike-Takeshita, T. Eguchi, K. Kakinuma. 2000. Butirosin-biosynthetic gene cluster from *Bacillus circulans*. J. Antibio. 53:1158-1167.

Phelps, R. J. and J. L. McKillip. 2002. Enterotoxin production in natural isolates of Bacillaceae outside of the *Bacillus cereus* group. Appl. Environ. Microbiol. 68:3147-3151.

Pascual, M., M. Hugas, J. I. Badiola, J. M. Monfort, M. Garriga. 1999. *Lactobacillus salivarius* CTC2197 prevents *Salmonella enteritidis* colonization in chickens. Appl. Environ. Microbiol. 65:4981-4986.

Rantala, M., and E. Nurmi. 1973. Prevention of the growth of *Salmonella infantis* in chick by the flora of the alimentary tract of chicks. Br. Poultr. Sci. 14:627-630.

Rowan, N. J., K. Deans, J. G. Anderson, C. G. Gemmell, I. D. Hunter, T. Chaithong. 2001. Putative virulance factor expression by clinical and food isolates of *Bacillus* spp. after growth in reconstituted infant milk formulae. Appl. Environ. Microbiol. 67:3873-3881.

Schillinger, U., M. E. Stiles, W. H. Holapfel. 1993. Bacteriocin production by *Carnobacterium piscicola* LV-61. Int. J. Food Microbiol. 20:131-147.

Schillinger, U. and F.-K. Lucke. 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. 55:1901-1906.

Seo, K. H., P. S. Holt, R. K. Gast, C. L.Hofacre. 2000. Elimination of early *Salmonella enteritidis* infection after treatment with competitive-exclusion culture and enrofloxacin in experimentally infected chicks. Poult. Sci. 79:1408-1413.

Stock, I., T. Gruger, B. Wiedemann. 2001. Natural antibiotic susceptibility of strains of the *Enterobacter cloacae* complex. Int. J. Antimicrob. Agents. 18:537-545.

Swiecicka, I. 2001. Protein profile and biochemical properties of *Bacillus circulans* isolated from intestines of small free-living animals in Poland. Folia microbiologica. 46:165-71.

Takeda, K., A. Kinumaki, H. Hayasaka, T. Yamaguchi, Y. Ito. 1978. Mutational biosynthesis of butirosin analogs. II. 3', 4'-Dideoxy-6'-N-methylbutirosins, new synthetic aminoglycosides. J. Antibiot. 31:1031-1038.

Tandon, A., M. L. Tay-Kearney, C. Metcalf, L. McAllister. 2001. *Bacillus circulans* endophthalmitis. Clin. Exp. Ophthal. 29:92-3.

Tannock, G. W. (ed.). 1999. Probiotics: a critical review. Horizon Scientific Press, Norfolk, United Kingdom.

Threlfall, E. J., J. A. Frost, L. R. Ward, B. Rowe. 1994. Epidemic in cattle and humans of *Salmonella typhimurium* DT104 with chromosomally integrated multiple durg resistance.Vet. Rec. 134:577.

van der Wielen, P. W. J. J., L. J. A. Lipman, F. van Knapen, S. Biesterveld. 2002. Competitive exclusion *of Salmonella enterica* serovarEnteritidis *by Lactobacillus crispatus* and *Clostridium lactatifermentans* in a sequencing fed-batch culture. Appl. Environ. Microbiol. 68(2):555-559.

Wang, G., T. Zhao, M. P. Doyle. 1996. Fate of Enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. Appl. Environ. Microbiol. 62:2567-2570.

Wells, S. J., P. J. Fedorka-Cray, D. A. Dargatz, K. Ferris, A. Green. 2001. Fecal shedding of *Salmonella* spp. by dairy cows on farm and at cull cow markets. J. Food Prot. 64(1):3-11.

Wray, C., J. N. Todd, M. Hinton. 1987. Epidemiology of *Salmonella typhimurium* infection in calves: excretion of *S.* Typhimurium in the feces of calves in different management systems. Vet. Rec. 121:293-296.

Zhao, S., J. Meng, T. Zhao, M. P. Doyle. 1995. Use of vaccine and biological control techniques to control pathogens in animals used for foods. J. Food Safety 15:193-199.

Zhao, T., M. P. Doyle, B. G. Harmon, C. A. Brown, P. O. E. Mueller, A. H. Parks. 1998. Reduction of carriage of enterohemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. J. Clin. Microbiol. 36:641-647.

Zhao, T., M. P. Doyle, P. J. Fedorka-Cray, P. Zhao, S. Ladely. 2002. Occurrence of *Salmonella enterica* serotype Typhimurium DT104a in retail ground beef. J. Food Prot. 65:403-407.

TABLE 1. Serotype, serogroup and antibiotic resistance of *Salmonella* spp. isolates

Isolate No.	Date isolated	Serotype	Serogroup	Antibiotic resistance[ab]
55	10-16-01	Newport	C2	AAmCeCfCpCSSuT
57	10-16-01	Newport	C2	AAmCeCfCpCSSuT
73	01-15-02	Bareilly	C1	None
74	01-15-02	Mbandaka	C1	None
78	01-15-02	Newport	C2	AAmCeCfCpCSTTr
88	01-29-02	Newport	C2	AAmCeCfCpCSSuTTr
90	01-29-02	Montevideo	C1	None
92	01-29-02	Meleagridis	E	None
102	01-29-02	Monophasic	B	None
103	01-29-02	Monophasic	B	None

[a] A=ampicillin, Am=amoxicillin/clavulanic acid, Ce=cefoxitin, Cf-ceftiofur, Cp=cephalothin, C=chloramphenicol, S=streptomycin, Su=sulphamethoxazole, T=tetracycline Tr=trimethoprim/sulphamethoxzole

[b] Screened against, amikacin, amoxicillin/clavulanic acid, ampicillin, apramycin, cefoxitin, ceftiofur, ceftriazone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, inipenem, kanamycin, nalidixic acid, streptomycin, sulphamethoxazole, tetracycline, trimethoprim/sulphamethoxazole

TABLE 2. Initial screening of potential competitive inhibition bacteria with inhibitory activity against 3 strains of *S.* Typhimurium DT104[a].

Isolate No.	Source	Date of Isolation	Disk Assay [b] XLD	Disk Assay [b] TSA	Overlay Assay [c] MAC	Overlay Assay [c] TSA
1-1	Dairy cow	09/10/01	-	-	+	-
3-7	Dairy cow	09/10/01	-	-	-	+
4-4	Dairy cow	09/10/01	-	+	+	-
4-5	Dairy cow	09/10/01	-	-	+	+
5-3	Dairy cow	09/10/01	-	-	+	-
6-8	Dairy cow	09/10/01	-	-	-	+
7-7	Dairy cow	09/10/01	-	-	+	-
8-7	Dairy cow	09/10/01	-	-	+	-
9-2	Dairy cow	09/10/01	-	-	+	-
11-1	Dairy cow	09/10/01	-	-	+	-
12-5	Dairy cow	09/10/01	-	-	+	-
13-2	Dairy cow	09/10/01	-	-	+	-
13-6	Dairy cow	09/10/01	-	-	+	-
15-3	Dairy cow	09/10/01	-	-	+	-
15-6	Dairy cow	09/10/01	-	-	+	-
16-2	Dairy cow	09/10/01	-	-	+	-
16-6	Dairy cow	09/10/01	-	-	+	-
16-10	Dairy cow	09/10/01	-	-	+	-
18-4	Dairy cow	09/10/01	-	-	+	-
18-6	Dairy cow	09/10/01	-	-	+	-
21-6	Dairy cow	09/10/01	-	-	+	-
21-9	Dairy cow	09/10/01	-	-	+	-
23-5	Dairy cow	09/10/01	-	-	+	-
24-2	Dairy cow	09/10/01	-	-	+	-
25-10	Beef calf	10/16/01	-	-	+	+
29-5	Beef calf	10/16/01	-	-	+	+
30-1	Beef calf	10/16/01	-	-	+	+
30-5	Beef calf	10/16/01	-	-	+	+
31-6	Beef cow	10/16/01	++	++	-	-
35-3	Beef cow	10/16/01	-	-	+	-
39-3	Beef cow	10/16/01	-	-	+	-
44-2	Beef cow	10/16/01	-	-	+	-
44-4	Beef cow	10/16/01	-	-	+	-
47-10	Beef cow	10/16/01	-	-	-	++
50-10	Beef cow	10/16/01	-	-	-	++
51-2	Beef cow	10/16/01	-	-	+	+
58-7	Beef cow	10/16/01	-	-	+	+
58-9	Beef cow	10/16/01	-	-	-	++
59-9 small	Beef cow	10/16/01	-	-	-	++
59-9 big	Beef cow	10/16/01	-	-	-	++
66-3	Beef cow	01/15/02	-	-	+	-

Isolate No.	Source	Date of Isolation	Disk Assay [b] XLD	Disk Assay [b] TSA	Overlay Assay [c] MAC	Overlay Assay [c] TSA
71-8	Beef cow	01/15/02	-	-	+	+++
76-9	Beef cow	01/15/02	-	-		++
101-1	Beef cow	01/29/02	-	-	+	+
106-2	Beef cow	01/29/02	-	-	+	+

[a] Strains include: 8748A-1, 11942A-1, 62
[b] Zone of inhibition: ++ = > 2.0mm, + = <2.0mm, - = no zone
[c] Zone of inhibition: +++ = > 10 mm, ++ = >5.0mm, + = <5.0mm, - = no zone

TABLE 3. Screening of potential competitive inhibition bacteria with inhibitory activity against 5 strains of *S.* Typhimurium DT104[a].

Isolate	Disk Assay [b]		Overlay Assay [c]	
No.	XLD	TSA	MAC	TSA
1-1	-	-	+	-
3-7	-	-	+	-
4-4	-	-	+	-
4-5	-	-	+	-
5-3	-	-	+	+
6-8	-	-	+	-
7-7	-	-	+	-
8-7	-	-	-	-
9-2	-	-	+	-
11-1	-	-	+	-
12-5	-	-	+	-
13-2	-	-	+	-
13-6	-	-	+	-
15-3	-	-	+	-
15-6	-	-	+	-
16-2	-	-	+	-
16-6	-	-	+	-
16-10	-	-	-	-
18-4	-	-	+	-
18-6	-	-	+	-
21-6	-	-	+	-
21-9	-	-	-	+
23-5	-	-	+	-
24-2	-	-	+	+
25-10	-	-	+	-
29-5	-	-	+	-
30-1	-	-	+	-
30-5	-	-	+	+
31-6	++	++	+	-
35-3	-	-	+	-
39-3	-	-	-	-
44-2	-	-	-	-
44-4	-	-	-	-
47-10	-	-	-	+++
50-10	-	-	-	-
51-2	-	-	+	-
58-7	-	-	+	-
58-9	-	-	-	+++
59-9 small	-	-	-	+++
59-9 big	-	-	-	+++
66-3	-	-	+	-

Isolate	Disk Assay [b]		Overlay Assay [c]	
No.	XLD	TSA	MAC	TSA
71-8	-	-	-	+++
76-9	-	-	-	+++
101-1	-	-	+	-
106-2	-	-	+	-

[a] Strains include: AI45025, MH2538, 99-103712-5, NE14055, 12-410
[b] Zone of inhibition: ++ = > 2.0mm, + = <2.0mm, - = no zone
[c] Zone of inhibition: +++ = > 10 mm, ++ = >5.0mm, + = <5.0mm, - = no zone

TABLE 4. Screening of potential competitive inhibition bacteria with inhibitory activity against 4 strains of *S.* Typhimurium DT104[a].

Isolate	Disk Assay [b]		Overlay Assay [c]	
No.	XLD	TSA	MAC	TSA
1-1	-	-	+	-
3-7	-	-	+	+
4-4	-	-	+	-
4-5	-	-	+	-
5-3	-	-	-	+
6-8	-	-	+	-
7-7	-	-	+	-
8-7	-	-	-	-
9-2	-	-	+	-
11-1	-	-	+	-
12-5	-	-	+	-
13-2	-	-	+	-
13-6	-	-	+	-
15-3	-	-	-	+
15-6	-	-	+	-
16-2	-	-	+	-
16-6	-	-	+	+
16-10	-	-	-	-
18-4	-	-	+	-
18-6	-	-	-	-
21-6	-	-	-	-
21-9	-	-	-	+
23-5	-	-	-	-
24-2	-	-	-	-
25-10	-	-	-	-
29-5	-	-	-	-
30-1	-	-	+	-
30-5	-	-	+	-
31-6	++	++	+	-
35-3	-	-	+	-
39-3	-	-	+	-
44-2	-	-	+	-
44-4	-	-	-	-
47-10	-	-	-	+++
50-10	-	-	-	-
51-2	-	-	+	-
58-7	-	-	+	-
58-9	-	-	-	+++
59-9 small	-	-	-	+++
59-9 big	-	-	-	+++
66-3	-	-	+	-

Isolate	Disk Assay [b]		Overlay Assay [c]	
No.	XLD	TSA	MAC	TSA
71-8	-	-	-	+++
76-9	-	-	-	+++
101-1	-	-	+	+
106-2	-	-	+	-

[a] Strains include: 12993-k, 2748-k, 520-k, 4698-k
[b] Zone of inhibition: ++ = > 2.0mm, + = <2.0mm, - = no zone
[c] Zone of inhibition: +++ = > 10 mm, ++ = >5.0mm, + = <5.0mm, - = no zone

TABLE 5. Screening of potential competitive inhibition bacteria with inhibitory activity against 5 strains of *Salmonella* spp.[a] isolated from beef cattle in Georgia.

Isolate	Disk Assay[b]		Overlay Assay[c]	
No.	XLD	TSA	MAC	TSA
1-1	-	-	-	-
3-7	-	-	-	-
4-4	-	-	-	-
4-5	-	-	-	-
5-3	-	-	-	-
6-8	-	-	-	-
7-7	-	-	-	-
8-7	-	-	-	-
9-2	-	-	-	-
11-1	-	-	-	-
12-5	-	-	+	-
13-2	-	-	-	-
13-6	-	-	-	-
15-3	-	-	-	-
15-6	-	-	-	-
16-2	-	-	-	-
16-6	-	-	-	-
16-10	-	-	-	-
18-4	-	-	-	-
18-6	-	-	-	-
21-6	-	-	-	-
21-9	-	-	-	-
23-5	-	-	-	-
24-2	-	-	-	-
25-10	-	-	-	-
29-5	-	-	-	-
30-1	-	-	-	-
30-5	-	-	-	-
31-6	++	++	+	-
35-3	-	-	-	-
39-3	-	-	-	-
44-2	-	-	-	-
44-4	-	-	-	-
47-10	-	-	-	+++
50-10	-	-	-	-
51-2	-	-	-	-
58-7	-	-	-	-
58-9	-	-	-	+++
59-9 small	-	-	-	+++
59-9 big	-	-	-	+++
66-3	-	-	-	-

Isolate	Disk Assay [b]		Overlay Assay [c]	
No.	XLD	TSA	MAC	TSA
71-8	-	-	-	+++
76-9	-	-	-	+++
101-1	-	-	+	-
106-2	-	-	-	-

[a] Strains include: *S*. Newport 55, *S*. Newport 57, *S*. Bareilly 73, *S*. Mbandaka 74, *S*. Newport 88

[b] Zone of inhibition: ++ = > 2.0mm, + = <2.0mm, - = no zone

[c] Zone of inhibition: +++ = > 10 mm, ++ = >5.0mm, + = <5.0mm, - = no zone

TABLE 6. Screening of potential competitive inhibition bacteria with inhibitory activity against 5 strains of *Salmonella* spp.[a] isolated from beef cattle in Georgia.

Isolate	Disk Assay[b]		Overlay Assay[c]	
No.	XLD	TSA	MAC	TSA
1-1	-	-	-	-
3-7	-	-	-	-
4-4	-	-	-	-
4-5	-	-	-	-
5-3	-	-	-	-
6-8	-	-	-	-
7-7	-	-	-	-
8-7	-	-	-	-
9-2	-	-	-	-
11-1	-	-	-	-
12-5	-	-	-	-
13-2	-	-	-	-
13-6	-	-	-	-
15-3	-	-	-	-
15-6	-	-	-	-
16-2	-	-	-	-
16-6	-	-	-	-
16-10	-	-	-	-
18-4	-	-	-	-
18-6	-	-	-	-
21-6	-	-	-	-
21-9	-	-	-	-
23-5	-	-	-	-
24-2	-	-	-	-
25-10	-	-	-	-
29-5	-	-	-	-
30-1	-	-	-	-
30-5	-	-	-	-
31-6	-	-	-	-
35-3	-	-	-	-
39-3	-	-	-	-
44-2	-	-	-	-
44-4	-	-	-	-
47-10	-	-	-	+++
50-10	-	-	-	-
51-2	-	-	-	-
58-7	-	-	-	-
58-9	-	-	-	+++
59-9 small	-	-	-	+++
59-9 big	-	-	-	+++
66-3	-	-	-	-

Isolate	Disk Assay [b]		Overlay Assay [c]	
No.	XLD	TSA	MAC	TSA
71-8	-	-	-	+++
76-9	-	-	-	+++
101-1	-	-	-	-
106-2	-	-	-	-

[a] Strains include: *S*. Newport 88, *S*. Montevideo 90, *S*. Meleagridis 92, and monophasic *Salmonella* spp. 102 and 103.
[b] Zone of inhibition: ++ = > 2.0mm, + = <2.0mm, - = no zone
[c] Zone of inhibition: +++ = > 10 mm, ++ = >5.0mm, + = <5.0mm, - = no zone

TABLE 7. Selected characteristics of potential gram-positive competitive inhibition bacteria with inhibitory activity against 12 strains of *S.* Typhimurium DT104, and 10 strains of *Salmonella* spp. isolated from cattle.

Isolate No.	Identification	Antibiotic resistance[ab]
47-10	*Bacillus circulans*	CeCfCtS
58-9	*Bacillus circulans*	CeCfCS
59-9 small	*Bacillus circulans**	CeCfS
59-9 big	*Bacillus circulans**	CeCfS
71-8	*Bacillus circulans*	CeCfCtS
76-9	*Bacillus circulans*	CeCfS

[a] Ce=cefoxitin, Cf=ceftiofur, Ct=ceftriaxone, S=streptomycin, C= chloroamphenicol
[b] Screened against, amikacin, amoxicillin/clavulanic acid, ampicillin, apramycin, cefoxitin, ceftiofur, ceftriazone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, inipenem, kanamycin, nalidixic acid, streptomycin, sulphamethoxazole, tetracycline, trimethoprim/sulphamethoxazole
* Doubtful profiles/low discrimination by API 50 CH screening

TABLE 8. Selected characteristics of potential gram-negative competitive inhibition bacteria with inhibitory activity against 12 strains of *S*. Typhimurium DT104 and isolated from cattle.

Isolate No.	Identification	PFGE DNA subtype[a]	Antibiotic resistance[bc]
1-1	*E. coli*	Unique	None
3-7	*S. fonticola*	Unique	None
4-5	*E. cloacae*	Unique	Am, A, Ce, Cp
5-3	*E. coli*	Unique	None
6-8	*E. coli*	Unique	None
7-7	*E. coli*	Unique	None
9-2	*E. coli*	Unique	None
11-1	*E. coli*	Unique	None
12-5	*E. coli*	Same as 5-3	None
13-2	*E. coli*	Unique	SSuT
13-6	*E. coli*	Same as 13-2	SSuT
15-6	*E. coli*	Unique	None
16-2	*E. coli*	Same as 15-6	None
16-6	*E. coli*	Unique	None
18-4	*E. coli*	Same as 15-6	None
30-1	*E. coli*	Unique	Intermediate C
30-5	*E. coli*	Same as 30-5	Intermediate C
31-6	*E. coli*	Unique	T
44-2	*E. coli*	Unique	Intermediate C
51-2	*E. coli*	Unique	None
58-7	*E. coli*	Unique	None
66-3	*E. coli*	Unique	Su
101-1	*E. coli*	Unique	A, Cp
106-2	*E. coli*	Unique	None

[a] DNA subtyping as determined by PFGE; unique indicates that the PFGE pulsotype is different from those of the other strains in this study

[b] Am= amoxicillin/clavulancic acid, A=ampicillin, C=chloramphenicol, Ce=cefoxitin, Cp= cephalothin, S= streptomycin, Su=sulphamethoxazole, T=tetracycline

[c] Screened against, amikacin, amoxicillin/clavulanic acid, ampicillin, apramycin, cefoxitin, ceftiofur, ceftriazone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, inipenem, kanamycin, nalidixic acid, streptomycin, sulphamethoxazole, tetracycline, trimethoprim/sulphamethoxazole

A

B

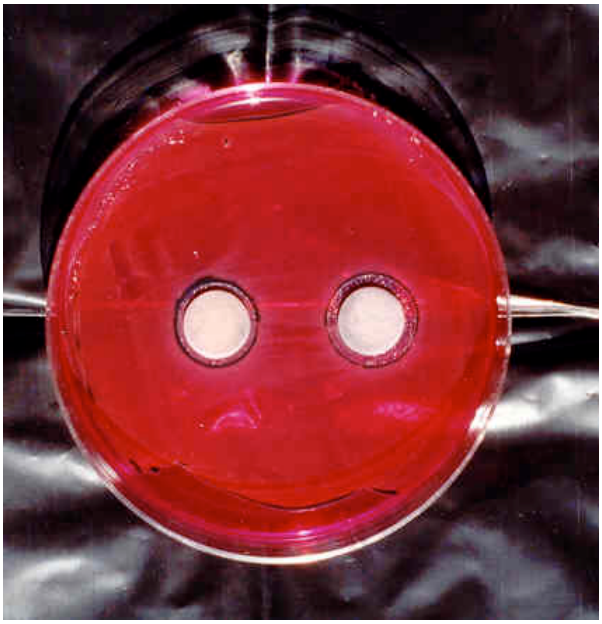
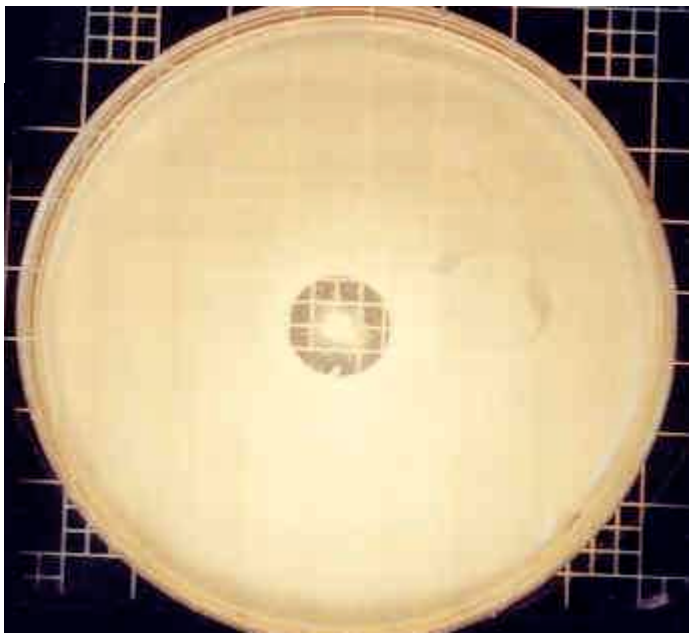


FIG. 1. Zones of inhibition, paper disk assay, sterile supernatent from isolate 31-6. (A) S. Typhimurium DT104, strains 8748A-1, 11942A-1 and 62, on TSA. (B) S. Typhimurium DT104, strains 8748A-1, 11942A-1 and 62, on XLD.

A



B

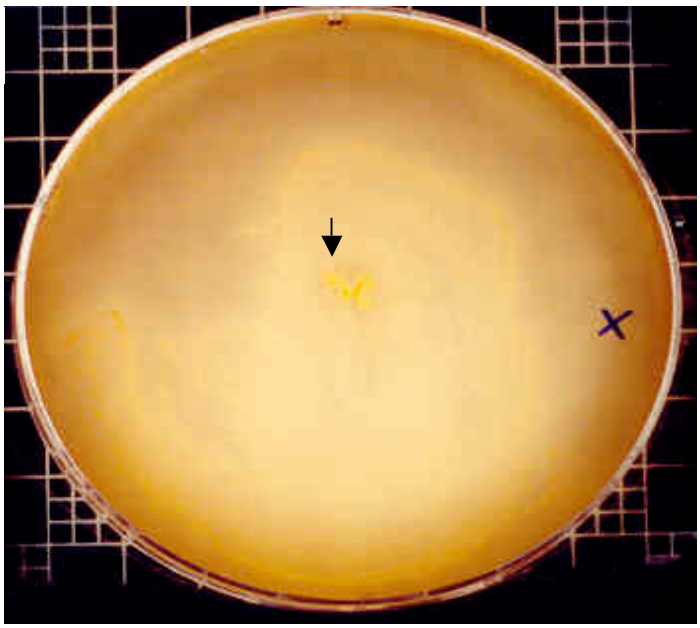
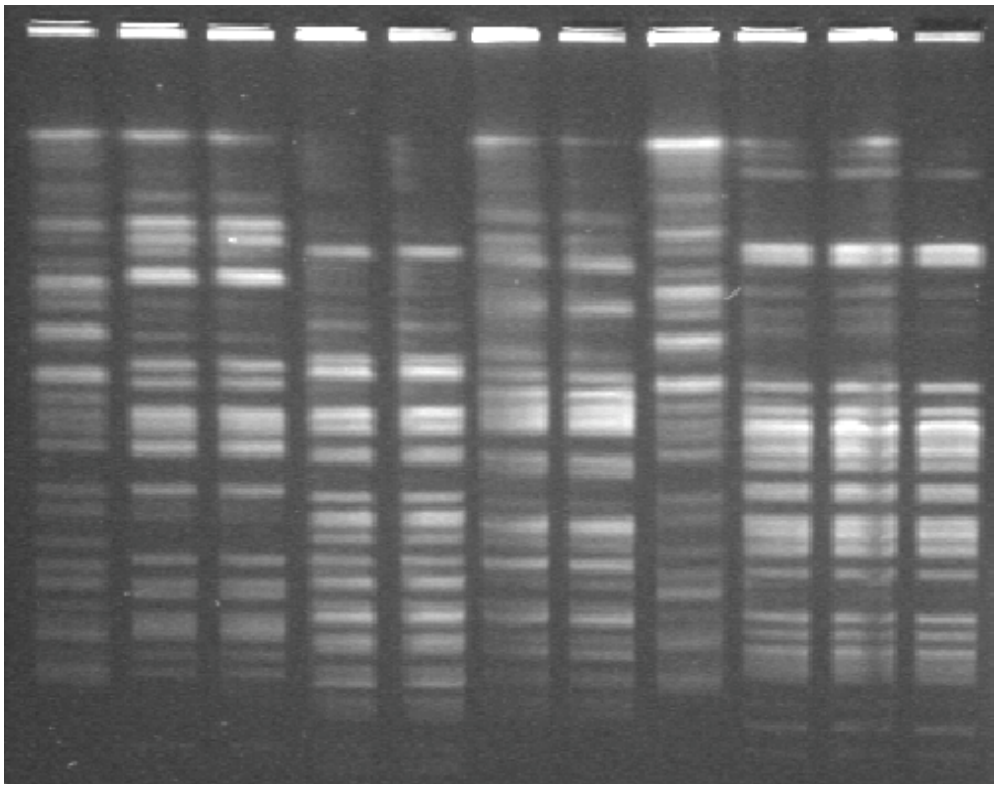


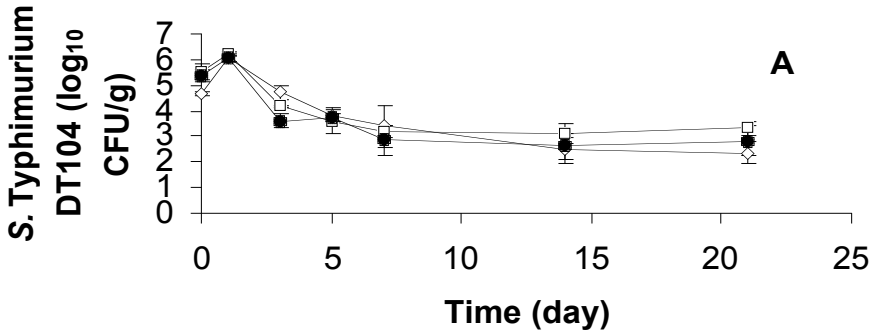
FIG. 2. Zones of inhibition, agar-spot assay. (A) Isolate 59-9 small, S. Typhimurium DT104, strains 8748A-1, 11942A-1 and 62, on TSA. (B) Isolate 66-3, S. Typhimurium DT104, strains 8748A-1, 11942A-1 and 62, on MAC.

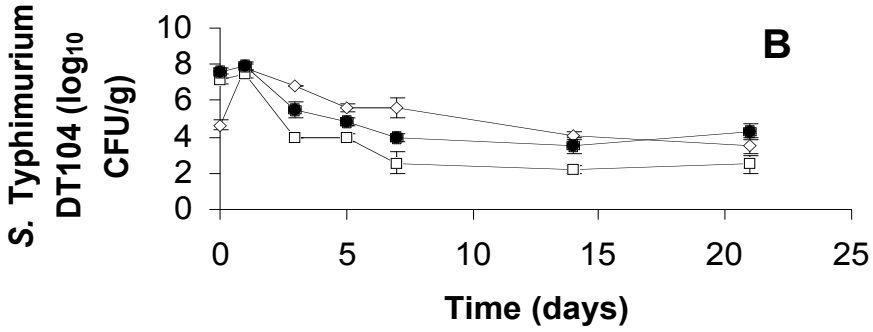


1 2 3 4 5 6 7 8 9 10 11

FIG. 3. Comparison of PFGE DNA pulsotype of gram-negative competitive inhibition isolates from cattle. Lanes 1 and 8, E. coli O157:H7, G5244; lane 2, isolate 5-3; lane 3, isolate 12-5; lane 4, isolate 13-6; lane 5, isolate 13-2; lane 6, isolate 30-5; lane 7, isolate 30-1; lane 9, isolate 15-6; lane 10, isolate 16-2; lane 11, isolate 18-4.

FIG. 4. Growth of *Salmonella* sp. at 37°C in bovine feces. *S*. Typhimurium DT104 only (_), DT104 with gram-negative CI bacteria (_), DT104 with gram-positive CI bacteria (_), *S*. Newport low inoculum (_), *S.* Newport high inoculum (_), *S*. Newport with gram-positive CI low inoculum(□), *S*. Newport with gram-positive CI high inoculum(✶). (A) Low inoculum DT104 and CI bacteria; (B) high inoculum DT104 and CI bacteria; (C) S. Newport and CI bacteria. Error bars represent standard deviations (n=3).


S. Typhimurium DT104 (log10 CFU/g)
A
Time (day)


S. Typhimurium DT104 (log10 CFU/g)
B
Time (days)

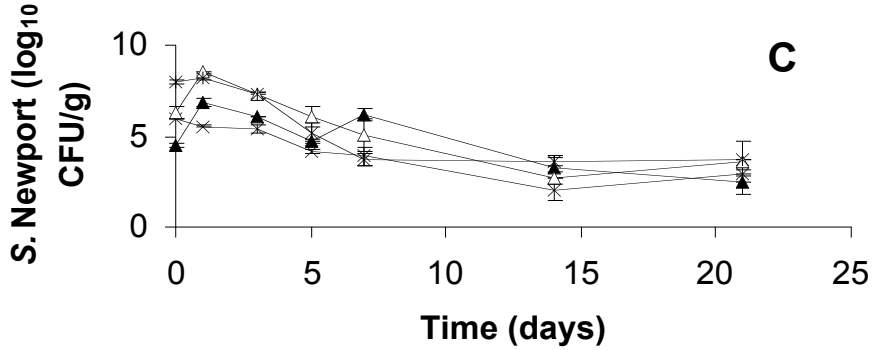
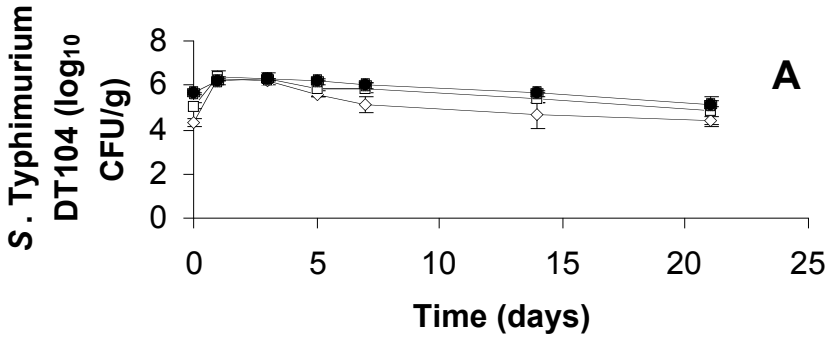
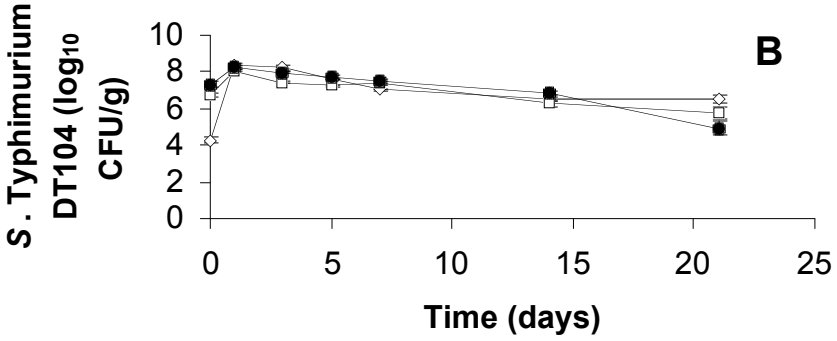
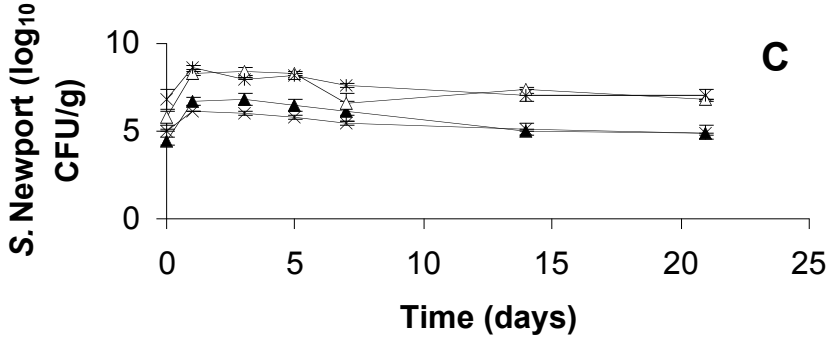

S. Newport (log10 CFU/g)
C
Time (days)

FIG. 5. Growth of *Salmonella* sp. at 21°C in bovine feces. *S*. Typhimurium DT104 only (_), DT104 with gram-negative CI bacteria (_), DT104 with gram-positive CI bacteria (_), *S*. Newport low inoculum (_), *S.* Newport high inoculum (_), *S*. Newport with gram-positive CI low inoculum(□), *S*. Newport with gram-positive CI high inoculum(✶). (A) Low inoculum DT104 and CI bacteria; (B) high inoculum DT104 and CI bacteria; (C) S. Newport and CI bacteria. Error bars represent standard deviations (n=3).


S. Typhimurium DT104 (log10 CFU/g)
A
0
2
4
6
8
0
5
10
15
20
25
Time (days)


S. Typhimurium DT104 (log10 CFU/g)
B
0
2
4
6
8
10
0
5
10
15
20
25
Time (days)


S. Newport (log10 CFU/g)
C
0
5
10
0
5
10
15
20
25
Time (days)

CHAPTER 4

SUMMARY AND CONCLUSION

The objectives of the research in this thesis were to isolate and identify potential competitive exclusion bacteria from the feces of cattle that are not shedding *Salmonella* spp. and evaluste them for anti-*S*. Typhimurium DT104 activity in vitro and in cattle feces artificially contaminated with *S*. Typhimurium DT104 and S. Newport.

This research identified *Salmonella* spp. in 10 (9.25%) fecal samples collected from beef cattle. When serotyped, six serotypes were found *S*. Newport (4), *S*. Bareilly (1), *S*. Mbandaka (1), *S*. Montevideo (1), *S*. Meleagris (1), and two monophasic *Salmonella* spp. All four *S*. Newport isolates showed resistance to at least nine antibiotics, including ampicillin, amoxicillin/clavuanic acid, defoxitin, ceftiofur, cephalothin, chloramphenicol, streptomycin, sulphamethoxazole and tetracycline. These findings provide further evidence for the emergence of multi-resistant *S*. Newport in the United States, and imply that cattle are a reservoir through which *S*. Newport can enter the food supply.

Of the 1097 bacterial isolates from cattle feces screened thirty were determined to inhibit the growth of *S. Typhimurium* DT104 in vitro. Following identification 22 *E. coli*, six *Bacillus circulans*, one *Serratia fonticola*, and one *Enterobacter colacae* were identified. Genomic DNA identification of the 24 gram-negative organisms by pulsed field electrophoresis revealed 19 distinguishable profiles. Only one isolate tested positive by the paper disk assay the other 24 were active by the spot on agar rest. The inability of isolates to inhibit *S*. Typhimurium DT104 using cell-free extracts may be due to excretion of anti-*S*. Typhimurium DT104 below the level of detection necessary when assayed by the paper disk method, or that the anti-*S*. Typhimurium DT104 compounds produced were bound to the cell surface.

Inconsistent significant reductions of *S*. Typhimurium DT104 and *S*. Newport were seen during the 21-day incubation period in the artificially contaminated cattle feces at both 21 and 37°C. The reductions, however, indicate that the competitive inhibition candidates can competitively decrease the growth of *S*. Typhimurium DT104 and *S*. Newport in a natural environment.

The competitive inhibition bacteria identified in this study should be examined for anti-*S*. Typhimurium DT104 and anti-*S*. Newport activity in an animal model. More consistant reductions of *Salmonella* spp. may be seen in this environment as the organisms will have an opportunity to grow and produce the anti-*Salmonella* compounds. The mechanisms by which the competitive inhibition candidates inhibit the growth of *S*. Typhimurium DT104 and *S*. Newport remain to be determined. Determination of these mechanisms would aide in the determination of their use as competitive inhibition bacteria in an animal model.

The 25 competitive inhibition bacteria identified in this study offer the first step in the treatment of cattle with competitive inhibition bacteria to reduce the level of intestinal carriage and fecal shedding of *S*. Typhimurium DT104 and *S*. Newport. This method has the potential to decrease the risk of *S*. Typhimurium DT104 and *S*. Newport infections in humans, by reducing the contamination of these pathogens in food.