Prevalence of Nalidixic Acid Resistant *Salmonella* Species on Chicken Skin with Commercial Marinades

by

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Abstract

Marination of poultry meat is widely done in the industry not only for value addition but also to enhance the shelf life. In addition to increased consumer acceptance the combination of spices in marinades have a potential to inhibit microbial growth in poultry products. A series of experiments were conducted to determine the efficacy of commercial teriyaki and lemon pepper marinades on viability of multiple strains of nalidixic acid (NAL) resistant *Salmonella*. NAL resistant *Salmonella* (Typhimurium, Heidelberg and Senftenberg) cultures were developed by inoculating BHI broth and incubating for 24 h at 37 °C; subjecting strains to increasing concentrations of NAL in XLT4. As a result, *S.* Typhimurium and *S.* Heidelberg resistant to 60 *µgm* and *S.* Senftenberg resistant to 35 *µgm* of NAL were obtained. These strains were inoculated in 9 ml of BHI and incubated for 24 h at 37 °C; followed by transferring 100 µl of inoculum into 40 ml of BHI and incubating for 20 h at 37 °C. Each strain was individually inoculated (ca. $10^{6\text{-}8}$ CFU/ ml) into either teriyaki and lemon pepper marinade, maintained at 4 and 25 °C, and samples were drawn after 0, 4, 8, 16, 24, and 32 h. Serial dilutions of inoculated marinade were made and surviving populations ($\log_{10}$ CFU/ ml) of *Salmonella* were enumerated by plating 0.1 ml onto XLT4 agar. Plates were incubated at 37 °C for 24 h. Teriyaki marinade significantly ($p < 0.05$) lowered the counts of *Salmonella* throughout the study as compared to Lemon pepper irrespective of the time and temperature of storage. *S.* Heidelberg and

Typhimurium populations were significantly lowered ($p<0.05$) as a result of an interaction effect of marination time and type of marinade used whereas survival populations of *S.* Senftenberg were significantly lowered ($p<0.05$) as a result of a three way interaction between marination time, type of marinade, and temperature at which the marinades were stored. These findings suggest that marination would be a promising approach in developing antimicrobial systems for poultry products.

Based on the results of this experiment, an experiment was conducted to determine the prevalence of *Salmonella* strains on the chicken skin when marinated with Teriyaki and Lemon pepper marinades. The NAL resistant strains were cultured individually in BHI ay 37 °C for 20 h. These cultures were then serially diluted in peptone water and 10^1, 10^2, 10^3 and 10^4 CFU/ml were used as inoculum levels to inoculate the skin. Chicken skin from the breast was inoculated with 100 µl of inoculum from 0.6 to 3.14 $\log_{10}$ CFU/ g in a 12-well titer plate and placed under a bio-safety hood for 30 minutes to allow bacterial attachment followed by marination with teriyaki or lemon pepper marinades, respectively. Marinated samples were stored at room temperature (25 ºC) and refrigeration temperature (4 °C) for up to 32 h. Samples were removed from the marinades at 0, 4, 8, 16, 24 and 32h and plated in duplicates onto XLT4 followed by incubation of the plates at 37 ºC for 24 h. The results were analyzed using Analysis of variance (ANOVA) in SAS and the significance was reported at $p < 0.05$. Prevalence of *Salmonella* was significantly reduced ($p < 0.05$) by teriyaki marinade at all levels of contamination. Lemon pepper reduced the prevalence at low levels of contamination (10^1 and 10^2 CFU/gm) whereas no significant effect ($p > 0.05$) was observed at higher levels of contamination. There was no significant ($p > 0.05$) difference observed at the two storage temperatures, 4 and 25 °C, except for *S.* Typhimurium which show significant reduction with lemon pepper marinade at 4 °C.

Marination of chicken skin greatly helped in the reduction of prevalence of *Salmonella* spp irrespective of the temperature of storage indicating antimicrobial potential in addition to enhancing shelf life of poultry and meat products.

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1 – INTRODUCTION

Poultry and poultry products are being increasingly consumed in the United States. There is competitive pricing of the poultry products as compared to the other meat products in the market. Factors like live production, efficiency, flock health and carcass and meat quality have been the primary economic drivers for the broiler industry. The rapidly changing consumption patterns in the markets, awareness about foodborne infections, scientific advances, changes in food technology and distribution and changes in the virulence of the microorganisms challenges the food processing industry to develop new products. The consumer trends towards convenience foods, natural, microbiologically safe and with longer shelf-lives has generated an interest in the development of spices and other natural antimicrobials as methods of food preservation.

Bacterial contamination of raw processed poultry is of concern not only to the consumers but also to the regulatory and health agencies. Procedures to reduce surface contamination of carcasses by *Salmonella* and other foodborne pathogens during processing have been investigated in various studies. The microbiological safety and the sensory quality of food products is the based on the application of the preservative factors. The physiological responses of microorganisms to food preservation are the basis for food preservation techniques. Spraying the carcasses with antibacterial solutions, physical treatments like steam, dry heat and UV light have been researched but these treatments may result in deterioration in taste, texture and odor of chicken. Also the increased line speeds may reduce the contact time of the antimicrobial spray with the target microorganism and reduce its effectiveness. Processed and manufactured food

products are perishable and thus require protection from foodborne and spoilage organisms during preparation, storage and distribution and have an adequate shelf-life until consumption. Preservation systems like heating and refrigeration are used to reduce the risk of foodborne pathogens but these methods alone cannot assure the complete safety and quality of the food products. There may be adverse effects associated with the changes in sensory quality and decrease in nutrients in the food.

Convenience foods are becoming more popular and often these are products which have undergone value-addition processing, such as marination, to improve the quality of the saleable yield and also to enhance the sensory attribute of the product. They are convenient for consumers as meal preparation only requires heat treatment with minimal handling and spicing of the meat. Marinades have become more complex with the addition of various spices and essential oil extracts. Traditionally, marination was done for tenderizing and flavoring the meat products. Marinades have now been studied to have preservative, medicinal and anti-oxidant properties which apart from value-addition enhance the safety and shelf-life of meat products by inhibiting the microbial growth.

The antimicrobial agents can be directly added to the food during processing or can be applied by spraying, immersion or injection techniques. The anti-microbial activity of the spices and other natural antimicrobials depends on the target pathogen, microbial load, the food product system, and the storage and handling conditions. Fat content of food, temperature of storage and pH of the food are the most important factors that can influence the antimicrobial activity of marinades. Traditionally, the meats were immersed in the marinade solution to allow the components to passively penetrate in the muscle fibers. Today the poultry industry uses injection marination and vacuum tumbling for commercial marination.

Spice and herbs are GRAS (Generally Recognized As Safe) compounds and have been approved by FDA as food additives. They have been used as culinary additives and have no documented adverse effects. Several studies have reported the bactericidal and bacteristatic effects of spices and essential oils on various foodborne pathogens. The combination of spices in marinade sauces can influence the safety of poultry products and will also have consumer acceptability for being natural food additives. There are different types of marinades available in the markets including curry marinades, Italian, Honey-based, Chinese and barbeque marinades, which have an effect on the appearance and taste of the product. Marination improves the quality of the meat products by increasing the tenderness, juiciness and decreases water loss during cooking. The pH of the marinades is usually acidic and that may affect the survival of the microorganisms.

Chicken skin is the major component of the retail chicken and is the source of *Salmonella* contamination and transmission. Several microbiological surveys indicate that chicken skin is usually contaminated with low numbers of *Salmonella* which can grow to higher numbers when subjected to temperature abuse. Our study was undertaken to investigate the viability of three different strains of Nalidixic acid resistant *Salmonella* species in commercial Teriyaki and Lemon pepper marinades and to determine their prevalence on chicken skin with immersion marination at refrigeration (4 °C) and abuse temperature (25 °C) after 32 h of exposure.

2 – LITERATURE REVIEW

2.1 - *Salmonella*

Salmonella spp. are motile, Gram-negative, aerobic, rod-shaped bacilli belonging to the bacterial family *Enterobacteriaceae*. These organisms have peritrichous flagella except for *S.* Pullorum and *S.* Gallinarum which do not have flagella (D' Aoust, 2000). The genus *Salmonella* is obligate pathogenic and is divided into five subgenera that include approximately 2,600 serovars of gram-negative facultative anaerobic bacilli which have been detected and classified according to the serology and the phage susceptibility assays (Barnes, 2007). The earlier classification nomenclature was based upon the names of the places where the organism was first discovered, such as *S.* Miami, *S.* Kentucky, *S.* Dublin, etc. *Salmonella* can be classified based on their susceptibility to different bacteriophages, known as phage typing. Nearly 4,500 different ds-DNA phages capable of infecting a large diversity of bacterial hosts have been described and more than 200 have been reported as definitive phage types (Hendrix et al, 1999). Phage typing has been reported to be extremely useful in epidemiological investigation (Maher et al, 1986) and has also been proven useful regarding distribution of *Salmonella* in North American poultry industry (Anderson and Ziprin, 1994). Resistance to different antibiotics has also been used as a method of classification of *Salmonella*. According to Center of Disease Control, 2009 most DT104 isolates are resistant to at least Ampicillin, Chloramphenicol, Streptomycin,

Sulfonamides, Tetracycline, Florifenicol, Tetracyclines, Nalidixic acid and Ciprofloxacin. Presently *Salmonella* genus consists of two species *S*. Enterica which is divided into six sub-species, enteric, salamae, arizonae, diarizonae, houtenae and indica and *S*. Bongori (D'Aoust, 2000).

The World health Organization (WHO) has recognized Kauffman-White diagnostic scheme which arranges *Salmonella* into groups and serotypes according to antigens present in the envelope, Vi antigens, the specific somatic (O) antigen in the cell wall or flagellar (H) antigens. Originally, the extensive serological variation in O antigen according to Kauffman-White scheme was used to divide *Salmonella* into different species (Xiang et al, 1994). The O antigen is a polysaccharide which forms a lipopolysacchride (LPS) along with lipid A and the core in the gram negative bacteria. It is composed of repeating oligosaccharide units the structure of which determines the major immuno-chemical properties. The somatic antigens are heat stable, alcohol resistant and are made of lipid-polysacchride-polypetide complexes which make up the endotoxins found in the *Salmonella* cells Vi antigen is more susceptible to heat and is present in a few strains like *S*. typhi, *S*. paratyphi A and *S*. paratyphi C indicating virulence of the organisms. Vi antigen of *Salmonella* typhi, which is the agent of human typhoid fever is a capsular polysaccharide (Virlogeux, 1995). It is present in freshly isolated organisms and is lost within few transfers in artificial cultures. Presence of large amounts of Vi antigen completely blocks agglutination of cells in O antiserum, which may be covered or masked. *Salmonella* have been observed to undergo smooth-rough (S-R) dissociation which causes modifications in the colony morphology, loss of virulence, loss of specificity of the O antigens on the cell surface but the H antigens are not affected (Guthrie, 1992).

Salmonellae are mesophilic, heterotrophic organisms and require simple inorganic salts containing nitrogen, sulphur, phosphorus and an organic source of carbon and energy for sustaining growth and biochemical reactions (Minor, 1991). Temperature, pH, water activity and salt concentration in the environment influence the adaptability and growth kinetics of the microorganism. *Salmonella* can grow at an optimum temperature of 35 – 37°C, a moderate pH range of 6.5 – 7.5; high water activity conditions of above 0.94 and can catabolize carbohydrates into acids and gas using citrate as a carbon source (D'Aoust, 2000; Minor 1991; Anderson and Ziprin, 1994). These organisms are non-spore forming, oxidase negative, do not produce indole, do not hydrolyze urea and are unable to deaminate phenylalanine to tryptophan (Anderson and Ziprin, 1994). The properties of *Salmonella* species to produce abundant hydrogen sulphide, decarboxylate lysine, arginine and ornithine and inability to ferment lactose have been utilized in various selective and differential media such as XLD (Xylose lysine decarboxylase), RA (Rambach agar), SS agar (*Salmonella-Shigella*), BGA (Brilliant green agar), BGS (Brilliant green sulphite agar), MacConkey's agar (Anderson and Ziprin, 1994).

Recent evidence suggests that a single cell of *Salmonella* may constitute an infectious dose for humans (D'Aoust, 1997). Samples cultured for *Salmonella* often may contain few cells to allow detection by plating on selective and differential agars. Pre-enrichment and enrichment procedures are done to increase the sensitivity of detection of the organism (D'Aoust, 1989). Pre-enrichment is done in non-selective media such as buffered peptone water (BPW) or TSB (Tryptic soy broth) to allow the injured cells to recover and increase in numbers (D'Aoust, 1989). Enrichment procedures are done to inhibit the growth of undesired competing background flora (Ewing, 1986). Following enrichment the cultures are spread on selective media which is incubated for 24 – 48 hours and is examined for presence of colonies. Biochemical and

serological tests are performed to confirm the *Salmonella* colonies. Enrichment media used for *Salmonella* include Muller's tetrathionate (TT) broth, Muller-Kauffman's tetrathionate brilliant green (BG), Leifson's selenite cystine (SC) broth and the most commonly used Rappaport-Vassilidis (RV) broth (Anderson and Ziprin, 1994).

Salmonellae are responsive to marked changes in temperature, oxygen tension, osmolarity, oxidative stress and low nutrient levels in their micro-environment. The production of heat-shock proteins in prokaryotic and eukaryotic cells is induced by stress of extreme temperature variations, shifts in pH and release of toxic products from the oxidative burst of phagocytes (Gross, 1996). Heat shock proteins are important for the survival of *Salmonella* during the infectious cycle and studies have shown that low levels of oxygen conditions such as that found in the host intestinal lumen increase the invasiveness of *S.* Typhimurium in cultured mammalian cells and reduced invasiveness under high oxygen tension (Mahan, 1996). *Salmonella* species have the ability to enter mammalian cells which allows these organisms to reach deeper in the tissues to a more permissive environment. The virulence properties of toxin production, invasion of mammalian epithelial cells and survival within macrophages and resistance to the antibiotics distinguish salmonellae from other species of enteric bacteria (Allaoui, 1993). Pathogenicity and antibiotic resistance of *Salmonella* is carried through the virulence plasmids which are present in low numbers and replicate independently from the bacterial chromosome. These are present in very few serovars and absent in the typhoid and paratyphoid strains (D'Aoust, 1991). These promote systemic spread of *Salmonellae* in the host tissues and proliferation in the macrophages is induced on exposure to acidic conditions within phagocytes and other stress conditions. *Salmonella* species produce several toxins, endotoxin, enterotoxin, cytotoxin, zot (zonula occludans toxin) – like protein. The endotoxin has an outer

hexose polysaccharide chain which provides the serotype differentiation of strains, a core polysaccharide chain made up of outer hexose and inner heptose units and the innermost lipid A complex which triggers the endotoxic response in host. Most strains of *S. enterica* possess the enterotoxin gene but none is present in the strains of *S. bongori.* The cytotoxin produced by these organisms is thermo-labile and is associated with the outer cell membrane (D'Aoust, 2000).

2.2 - *Salmonella* related Human Illnesses

In 1885, Theobald Smith, research-assistant to Daniel E. Salmon, discovered the first strain of *Salmonella* – *Salmonella cholerae suis*, and since then number of serotypes of *Salmonella* known to cause salmonellosis has increased to over 2,300. The enteric bacteria classified as *Salmonella* have been recovered from a wide variety of mammalian and non-mammalian species and are the principal etiological agents of gastroenteritis and enteric fever in humans and domesticated animals. The U. S. Department of Agriculture stated that the most commonly identified paratyphoid serotypes in chickens were *S.* Heidelberg, *S.* Kentucky, *S.* Senftenberg, *S.* Enteritidis and *S.* Thompson (Ferris at al, 1999). There are approximately 40,000 reported cases of salmonellosis in the US each year with an estimated annual cost of over $2 billion dollars (Richard and Martin, 1988). The increase in incidence of salmonellosis in the United States may be due to changing agricultural and food distribution methods, increased consumption of raw or slightly cooked foods, an increase in the number of immuno-compromised or chronically ill people and deterioration of the public health infrastructure (Altekruse, 1997).

Salmonellosis is typically a food-borne illness acquired from contaminated raw poultry, eggs, and unpasteurized milk and cheese products or water, human-to-human and direct animal-

to-human transmission (Prost, 1967). It is most commonly recognized in children, elderly and people with a compromised immune system. *Salmonella* proliferation within eukaryotic cells represents an extraordinary example of microbial exploitation of host cell functions. The outcome of which depends on the dose of *Salmonella* ingested specifically the number of viable organisms reaching the small intestine, the characteristics of the organism and status of the host (Hook, 1961).

There are two major clinical presentations of foodborne infections with *Salmonella*, enteric fever syndrome following infection with typhoid or paratyphoid strains or the non-typhoid dependent gastroenteritis which can progress into systemic infection (D'Aoust, 2000). *Salmonella* pathogenesis is initiated by oral ingestion of contaminated food and penetration of bacteria into the intestinal epithelium before induction of disease. Invasion into the host intestinal cells results in morphological changes in the cell that are due to exploitation of the host cytoskeleton. Bacterial pathogens have evolved numerous strategies to exploit their host's cellular processes so that they can survive and persist. The genomic *phoP/phoQ* regulon facilitates the survival of the organism in acidic environment of phagocytes by inducing acid resistance (Anderson and Ziprin, 1994). *Salmonella* induce degeneration of enterocyte microvilli and the loss in microvillar structure is followed by extensive membrane ruffling in the area of bacterial-host cell contact, followed by profuse macropinocytosis with internalization of bacteria into the host cells where it resides within membrane-bound vesicles (Goosney et al, 1999).

The clinical symptoms of enteric fever appear within 7 to 28 days of exposure with watery diarrhea, fever, abdominal cramps, headache, nausea and a rash of rose spots may occur over the chest or abdomen. Intestinal bleeding may occur from ileal ulcers or intestinal perforation from hyperplasia of epithelio-lymphoid Peyer's patches (D'Aoust, 2000). Widal

immunological test is done for diagnosis of enteric fever, where antibodies in the sera of the patient agglutinate with the somatic (O) and the flagellar (H) antigens (Mandal, 1989). Clinical management of the infection is with an aggressive course of antibiotic therapy. Earlier, chloramphenicol, ampicillin and trimethoprim were used for treatment of human typhoid and paratyphoid infections but with the emergence of antibiotic resistance to these drug their use in limited (Rodriguez et al. 1989; Rowe et al, 1997).

The symptoms of non-typhoid salmonellosis results after 8 to 72 h of exposure to the bacterium and include nausea, abdominal cramps, diarrhea, fever of short duration and vomiting. It can progress from enterocolitis to septicemia because of the migration of the bacteria into deeper tissues through the vascular and lymphatic system (D'Aoust, 2000). Fluoroquinolones are used for the treatment of septicemic conditions (Cohen et al, 1987). There are several important sequelae associated with non-typhoidal salmonellosis which include arthropathies such as, reactive aseptic arthritis (RA), Reiter's syndrome (RS), Ankylosing spondylitis (AS), Rheumatoid arthritis and deficiencies in nutrition and intestinal absorption (D'Aoust, 2000).

2.3- *Salmonella* in the Environment and related Outbreaks

The ubiquity of *Salmonella* spp. in the natural environment contributes to its continued presence in a wide variety of mammals, birds, reptiles, and other animals (Wigley, 2004). It can survive, even though it may not actively grow, in many environmental waters. The organism is present in the feces of humans and birds thus are often present in waters polluted with fecal matter (Casner, 2001). Reptiles are a well-established source of human salmonellosis and in 1996-97, a study estimated that 6% of all human, laboratory-confirmed, sporadic *Salmonella* infections in the United States (and 11% of infections among persons aged < 21 years) were

attributed to contact with reptiles and amphibians (Mermin et al, 2004). *Salmonella* are difficult to eradicate from the environment as most serovars are not host adapted and hence are found in a variety of animal products, fruits, vegetables and processed foods. *S.* enteritidis is associated with chicken and *S.* Senftenberg found in turkeys are capable of causing severe human illness. *S.* Choleraesuis causes septicemia in swine and *S.* Dublin affects cattle and can be transmitted to humans through raw milk and unpasteurized dairy products (Ziprin and Hume, 1994). Poultry, pork and beef products are important reservoirs of human foodborne salmonellosis and reduction in the numbers of *Salmonella* harbored in these animals would significantly reduce human exposure.

Chickens can be infected with different types of *Salmonella* serotypes. Pullorum diseases caused by S. *pullorum* and fowl typhoid caused by S. *gallinarum* are lethal diseases in chickens (Nisbet and Ziprin, 1994) which can be introduced through various routes. Raw materials in the animal feeds can be of vegetable or animal origin and can introduce *Salmonella* in the finished feed products. Studies show that the incidence of *Salmonella* in feeds with animal protein is 56% and with vegetable protein it is 36% (Anon, 1994).

Salmonella is also widespread in the processing environment as this bacterium can be present on the feet, feathers, skin and feces of the birds. Contamination of birds in the holding pens and stress induced shedding of *Salmonella* during transport from the farm to the slaughter house amplifies the bacterial contamination at the processing plant (D' Aoust, 2000). Scalding, de-feathering and evisceration are critical control points in poultry processing but still it is difficult to completely remove the organism from carcasses and cross contamination occurs (Slavik et al, 1995). The high prevalence of *Salmonella* (42% positive) in retail poultry reflects

the inability to completely prevent the spread of this organism during production and marketing of raw poultry meat (D' Aoust, 2000).

There has been an increase in human salmonellosis due to *S.* Enteritidis, with 82% of the outbreaks in 1985-98 associated with raw and undercooked shell eggs (CDC, 2000). Grade-A shell eggs have been attributed to human salmonellosis due to the ability of *S.* Enteritidis to infect ovarian tissues and to be deposited in the developing eggs. Eggs can be contaminated through cracks in the shell or trans-ovarally from an infected ovary or oviduct to the yolk prior to deposition of the shell (St. Louis et al, 1988). This mode of transmission can be especially difficult to control because egg-laying hens are usually asymptomatic. Trans-ovarian infection of eggs has been associated with *S.* Thompson, *S.* Menston, *S.* Typhimurium, *S.* Pullorum and *S.* Gallinarum (Rodrigue et al, 1990). The prevalence of internally contaminated shell eggs is generally very low, 0.03% in the content and 1.1% on the shell (Humphrey et al, 1989). Contaminated turkey has also been implicated in outbreaks of human salmonellosis. *S.* Reading, *S.* Heidelberg and *S.* Saint-paul are the most commonly isolated serotypes accounting for approximately 42% of the turkey isolates (Ferris and Miller, 1990).

Salmonella remains a primary cause of food poisoning worldwide with an estimated 1.4 million foodborne illnesses annually (Mead et al, 1999), related to human gastroenteritis resulting from the ingestion of contaminated food products, such as undercooked beef, pork, chicken, eggs and seafood. Although foodborne diseases are common, only a fraction of these illnesses are routinely reported to CDC because a complex chain of events must occur before a foodborne infection is reported and any break in the chain will result in a case not being reported. Most reported foodborne illnesses are sporadic in nature; only a small number are identified as being part of an outbreak and thus are reported through the Foodborne-Disease Outbreak

Surveillance System. Thus, the system greatly underestimates the burden of foodborne diseases (Mead et al, 1999). During 1993-1997, a total of 2,751 outbreaks of foodborne diseases were reported in which 86,058 people became ill and bacterial pathogens were the cause of 75% of outbreaks with the largest percentage of cases (86%). In 1980-90, *Salmonella* serotype Enteritidis was an important cause of a number of outbreaks, cases, and deaths, most of which are attributed to eating eggs (CDC, 2000). Approximately 40,000 of the laboratory confirmed cases of *Salmonella* infections are reported to the National *Salmonella* Surveillance System annually and *S.* Typhimurium is the most commonly reported serotype (CDC, 2006). In 2006, 19% of all reported salmonellosis cases for which a serotype was identified were caused by *S.* Typhimurium. Although only an estimated 3% of *Salmonella* infections are laboratory confirmed and officially reported to surveillance systems (Voetsch et al, 2004), many milder cases that are never diagnosed suggest that the true incidence of the infection is undoubtedly much higher (Mead, 1999). During 2003--2007, an annual average of 18 outbreaks caused by *S.* Typhimurium were reported to CDC. Approximately 600 deaths are caused by *Salmonella* infections in the U.S. every year, accounting for 31 percent of all food-related deaths (CDC, 2005).

Many foodborne outbreaks have been witnessed in the recent past and several types of foods, including, sprouts (Taormina et al, 1999), peanut butter (CDC, 2009), tomatoes (Voetsch, 2004), produce, beef, poultry and pork products have been implicated. The multistate outbreak of human infections in peanut butter and its products due to *Salmonella* serotype Typhimurium, infected 714 people with the outbreak strain of *Salmonella* Typhimurium reported from 46 states in the United States (CDC, 2009). Three outbreaks of *Salmonella* infections associated with eating Roma tomatoes were detected in the summer of 2004 with 561 outbreak-related illnesses from 18 states in the United States and one province in Canada and multiple *Salmonella* strains

were isolated (CDC, 2005). Tomato-associated *Salmonella* outbreaks have increased in frequency and magnitude in recent years and have caused 1,616 reported illnesses in nine outbreaks during 1990-2004, representing approximately 60,000 illnesses (Voetsch, 2004). The first cases of *Salmonella* Saint-paul outbreak were identified in Nebraska affecting 228 cases in 13 states and implicated the source as alfalfa sprouts produced at multiple facilities using seeds that likely originated from a common grower. Raw and lightly cooked sprouts have been recognized as a source of foodborne illness in the United States since 1995 (Taormina et al, 1999) and the conditions suitable for sprouting are also ideal for markedly increasing counts of bacteria that might be present on seeds (Winthrop et al, 2003).

Changing animal slaughtering practices to reduce cross-contamination of animal carcasses, protecting processed foods from contamination, training in hygienic practices for all food-handling personnel in slaughterhouses, food processing plants, and restaurants; cooking and refrigerating foods adequately in food processing plants, restaurants, and homes; and expanding government enteric disease surveillance programs can be helpful in controlling *Salmonella* (Rabsch et al, 2001). Regulatory agencies are making efforts towards reducing the risk of *Salmonella* outbreaks and taking measures to enhance food safety. The current *Salmonella* performance standards for broilers are 20%, 12 positive samples in a set of 51 and efforts are being made to reduce it to half. FSIS has set *Salmonella* performance standards to verify the efficacy of HACCP systems in controlling contamination by this pathogenic microorganism. *Salmonella* was selected because it is a pathogen of food safety concern and is present on virtually all classes of raw food products in detectable numbers. FSIS posts updated results of completed *Salmonella* verification sample sets for young chicken (broiler) slaughter establishments on or about the 15th of each month which replaces previous month's posting.

Category 1 establishments are those which have results from their two most recent completed sample sets that are ≤ 10% (6 positive samples out of a set of 51). **Category 2** establishments have results from their most recent completed sample set that are ≥ 10% (more than 6 positive samples out of a set of 51) and ≤ 20% (12 positive samples out of 51). **Category 3** establishments have results from their most recent completed sample set that exceed the 20% standard for *Salmonella* in young chickens, 13 or more positive samples out of 51 (USDA, FSIS, 2008).

2.4 – Marinades as Antimicrobial agents

Food preservation is done in an attempt to maintain quality and functionality of foods while maintaining the safety of products that have a low spoilage potential. Food preservation has evolved from salting and drying methods to a wide range of processing techniques including thermal processes such as canning, non-thermal processes like irradiation, UV rays, aseptic procedures and freezing. Recent consumer trends demand natural, wholesome and microbiologically safe food products with no artificial additives that have led to an increasing interest in natural ingredients as antimicrobial agents. Numerous studies have been published on the antimicrobial effects of plant extracts against different foodborne pathogens (Marques et al, 2008; Delaquis et al, 2002) emphasizing the natural antimicrobial properties of spices, essential oils and organic acids and their potential for use in the food industry. These compounds can be exploited to meet consumer demands for fresh, additive-free and natural-tasting products which are also microbiologically safe. The increase in demand for convenience foods has resulted in expansion of the processed meat and poultry industry.

Essential oils, components of herbs, and spices are widely used in food preparation. Spices are aromatic compounds derived from woody shrubs, vines, aromatic lichens, tree barks, roots, flowers, seeds of herbaceous plants (Farrell, 1999). These compounds are generally recognized as safe (GRAS) by the FDA and their use may inhibit the growth of foodborne pathogens (Hao et al, 1998). Spices and herbs are natural and are GRAS - because of their traditional use without any documented cases of sensitization or allergies except for the occasional alteration in taste due to aging of the spices (Furia, 1980). Several studies document the antibacterial, antifungal and antiviral activities of certain extracts (Ferrell, 1990). Compounds with plant extracts, herbs, spice extract and essential oils can be incorporated in foods without adversely affecting the sensory, nutritional and safety characteristics and without increasing significantly the formulation, processing or marketing costs of minimally processed food to which they are added (Beuchat and Golden, 1989).

Marination of meat is an emerging industrial technology which is being used to improve tenderness, flavor and prolong the shelf-life of the products. There are 3 methods used in marination; 1) immersion, 2) injection marination, and 3) vacuum tumbling. Originally, meat was immersed in marinades allowing the marinade components to passively penetrate the meat over a period of time. Immersion marination does not provide regular distribution of the marinade and is a longer process (Alvarado and McKee, 2007). Hence, the poultry industry uses injection marination where a known quantity of marinade can be deposited regularly over the product and is easy to control and requires less time (Smith and Acton, 2001). Vacuum tumbling is done to provide a ready-to-cook (RTC), value added product. The process causes extraction of proteins which increase the cohesiveness during thermal processing or cooking. This facilitates

moisture retention during cooking and improves the juiciness and slicing characteristics of meats (Alvarado and McKee, 2007).

The poultry industry commonly uses salt-water-sodium tripolyphosphate marinades which have been shown to increase meat yield and juiciness while cooking processes (Alvarado and McKee, 2007). Commercial marinades are a complex combination of spices and have a great effect on product appearance and taste. They are typically water-oil emulsions containing phosphates, salt, sugar, acids (acetic, citric), and additives (Xanthan, Guar gum), antimicrobial agents (sorbate, benzoate) and aroma enhancers. The main aim of marinating has been considered to be flavoring and enhancing safety and shelf life of meat products by inhibiting growth of micro-organisms. Micro-organisms are suppressed by the acidic pH of sorbates and benzoates in the marinades (Bernard et al, 1990). The pH of marinades is usually acidic so sugar is added to counter the acidic flavor. The basic flavor is obtained by adding pepper, onions, tomatoes and spices (Bjorkroth, 2005). Marinades can enhance food safety while retaining the sensory properties of the product such as, color, flavor, texture and the nutritional value.

Use of natural antimicrobial compounds, like organic acids and aromatic compounds is of interest to the food industry. Acids can be naturally present as a constituent in the food or can be added as a formulation. The acidity of poultry and meats is due to presence of lactic acid during rigor mortis (Lund and Eklund, 2000). The un-dissociated form of acid is responsible for the antimicrobial activity. Many food antimicrobials are weak acids which are able to penetrate the cytoplasmic membrane of the bacterial cell. The commonly used acidic antimicrobials are acetic, lactic, benzoic and sorbic acid. Preservatives have an optimal inhibitory activity at low pH as this allows the un-dissociated form to pass through the cell membrane and enter the cell. High pH inside the bacterial cell causes the molecule to dissociate resulting in accumulation of anions and

protons inside the cell (Booth and Kroll, 1989). The inhibition of bacteria in an acidic medium is due to the disruption of the cell wall (Stratford and Anslow, 1998), inhibition of essential metabolic functions in the bacterial cell (Kreb's et al, 1983), stress on the intracellular pH homeostasis (Bracey et al, 1998) and the accumulation of toxic anions (Eklund, 1985).

In a preservation technique, susceptibility to an antimicrobial is dependent upon the conditions of application. The presence of any interacting conditions such as, pH and temperature may increase or decrease the susceptibility of bacterial cells. Food composition and pH has a major influence on the activity and effectiveness of marinades. The combination of technologies with preservative effects is known as the "Hurdle Technology" concept in food safety (Leistner, 1985), which is a way to combine different preservative techniques to prevent the growth of pathogens while still maintaining the flavor quality of food. The pH may be influenced by presence of available sugars and amino acids in the food and also by the temperature at which the foods are maintained (Lund and Eklund, 2000). Antimicrobial effects of preservatives are liable to be reduced by the presence of fats in the food and are greater in acidic foods. The presence of fats in foods provides a protective layer around the bacterial cells in a micelles formation and studies have reported that higher levels of fat in beef resulted in increased heat resistance in *S*. Typhimurium DT104 (Juneja and Eblen, 2000; Line et al, 1991; Ahmed et al, 1995).

Refrigeration is the most common factor used in control of foodborne pathogens and temperature abuse of refrigerated foods could result in food borne illness. Addition of naturally derived antimicrobial ingredients in combination with refrigeration may have an application in controlling the pathogens in food. Marinades can influence the safety and quality of foods, by acting as antimicrobial hurdles and at the same time improving the flavor of products. Industrial

marination is becoming increasingly popular and about 80% of the poultry products, such as skinned meats, fillets, skin-on breast fillets and leg cuts sold at retail level are marinated in Finland (Bjorkroth, 2005).

Studies have reported inhibition of *Salmonella* spp. by essential oils of thyme, oregano, orange, lemon, grapefruit, bay, clove, coriander, cinnamon and allspice tested with disc agar diffusion method (Tassou et al, 1995). A number of traditional antimicrobials, like acetic and benzoic acid, are approved for use in foods by most international regulatory agencies but there is limited available data on their effects in commercial practice (Theron and Lues, 2007). Several studies have shown reduction in the numbers of microorganisms by immersion of meat in acidic solutions of vinegar, wine or fruit juice (Lewis and Purslow, 1991) and the antibacterial activity of spices on few serotypes of *Salmonella*, such as Typhimurium (Kim et al, 1995). Orange oils from citrus fruits have been found to have antimicrobial effects against *Salmonella* spp. on the disc diffusion assay (O'Bryan et al, 2008). A rapid and drastic reduction in the viable cells of *S.* Typhimurium on nutrient agar was observed after addition of either the essential oil of thyme or its constituent thymol, especially under anaerobic conditions (Juven et al, 1994).

Tassou et al (1995) reported a synergistic effect of pH and mint essential oils in inhibition of *S.* Enteritidis and *L. monocytogenes* in food and laboratory media. Mint essential oil in a concentration of as low as 0.4% also has an inhibitory action against, *Salmonella* Enteritidis and *Staphylococcus aureus* and reduced the total viable counts by 3 and 6-7 logs respectively (Tassou et al, 2000). Nazer et al (2005) have documented the inhibitory effect of aromatic compounds like thymol, carvacarol, citral, eugenol and geraniol in combination with acids on growth of *Salmonella* Typhimurium in laboratory media. Yogurt is also used as a marinating ingredient in many Middle Eastern and Indian cuisines and has shown significant reduction of 2-

logs of *Campylobacter jejuni* on pork medallions (Birk and Knochel, 2009). Studies have also reported that marination can be used in synergism with irradiation to reduce the load of *Salmonella* on poultry meat (Mahrour et al, 2003). While numerous *in vitro* studies have been conducted on the antimicrobial activities of spices and their extracts, further food application studies are needed to estimate the concentrations required in food systems as amounts effective in culture media may not be sufficient to cause the same activity in foods.

2.5 - Teriyaki chicken and Lemon Pepper marinades

Teriyaki and Lemon pepper marinades are manufactured and commercialized by The Great American Spice, Fort Wayne, IN. Teriyaki marinade is a highly acidic marinade which is combination of powdered soy sauce, wheat soybeans, salt, maltodextrins, sugar, onion and garlic powder, sodium phosphates, spice and caramel coloring. The lemon pepper marinade is a combination of ground black pepper, sugar, lemon peel granules, oil of lemon, salt and extractives of spice in it.

'Teriyaki' is a Japanese word, where 'teri', means the luster or shine which is from the sugar content and 'yaki' is the cooking method after marination in soy sauce (Merriam-Webster Dictionary, 1962). Teriyaki sauces and marinades are extremely popular flavorings for poultry and meats and can be also easily prepared at home by mixing corn starch soy sauce, cider vinegar, sugar, garlic, ginger and black pepper. Several different companies manufacture Teriyaki marinade for commercial industrial and consumer use. There is Annie's Natural's an organic brand, a gluten free La Choy Teriyaki marinade and sauce, Carb Options® Asian Teriyaki marinade, Japanese Kikkoman Teriyaki marinade which is low sodium basted glaze,

Lawry's teriyaki marinade, World Harbor's teriyaki, Sable and Rosenfeld Tipsy, Barefoot Contessa Maple, Stonewall Kitchen Garlic, Roland Fusion, Shrimp teriyaki, Teriyaki chicken and many other brands and types. McCormick lemon pepper salt free seasoning, StarKist® Tuna Creations™ – Zesty Lemon pepper, Simply Organic Grilling Seasons® Lemon pepper marinade, Lawry's Lemon pepper marinade are few of the brands of Lemon pepper marinade available in the grocery stores.

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3 – ANTIMICROBIAL ACTIVITY OF COMMERCIAL MARINADES AGAINST MULTIPLE STRAINS OF SALMONELLA SPECIES

3.1 - Abstract

Marination of poultry meat is widely done for value addition, enhancing shelf life, and increasing consumer acceptance. This study was conducted to determine *in vitro* the efficacy of commercially available teriyaki and lemon pepper marinades on the survivability of multiple strains of nalidixic acid (NAL) resistant *Salmonella* spp. *S.* Typhimurium and *S.* Heidelberg resistant to 60 *µgm* of NAL and *S.* Senftenberg resistant to 35 *µgm* of NAL were individually inoculated into the marinades (ca. 10^8 CFU/ ml) and maintained at 4 and 25 °C for up to 32 h. Teriyaki marinade significantly ($p < 0.05$) reduced the populations of all three strains of *Salmonella* over the 32 h period as compared to lemon pepper, irrespective of the storage temperature. Following the 32 h storage, irrespective of the storage temperature, surviving populations of *S.* Heidelberg, Typhimurium, and Senftenberg were reduced ($p<0.05$) by 3.55, 4.62 and 2.27 $\log_{10}$ CFU/ ml respectively at 0 h and subsequently were reduced ($p< 0.05$) below detectable limits after 32 h whereas no significant reductions ($p>0.05$) were observed in the lemon pepper marinade. These findings suggest that, in addition to the potential for improving the sensory attributes of poultry products, marination can enhance their safety irrespective of the storage temperature. The findings from this study suggest a promising approach in developing antimicrobial systems for poultry products.

3.2 - Introduction

Foodborne illnesses remain a major concern in the developed countries with an estimated 6.5-33 million illnesses and 900 deaths occurring annually from bacteria, viruses, parasites, and fungi in the United States (Roberts, 2000). *Salmonella* spp. are Gram negative, heterotrophic, mesophilic bacteria present in warm-blooded animal hosts and is an important human pathogen associated with poultry and poultry products (Bryan and Doyle, 1995). Although most outbreaks cause mild to moderate self limited illness, serious disease resulting in death does occur particularly in elderly and immuno-compromised populations. *Salmonella* is the most commonly identified bacterial agent causing illnesses such as typhoid fever in humans (Winfield and Groisman, 2003; Marques *et al*., 2008) and an annual estimate of 800,000 to 4,000,000 of non-typhoidal infections (Voetsch *et al.*, 2004). Meat, milk, poultry, and eggs are primary vehicles that lead to human illnesses due to undercooking or cross contamination and Pang *et al*. (1995) had reported approximately 1.3 billion annual cases of *Salmonella* related human gastroenteritis resulting from ingestion of contaminated food products such as undercooked beef, pork, chicken, seafood and eggs. In the year 2006, there were 121 *Salmonella* outbreaks causing more than 3,300 illnesses reported to the Centers for Disease Control and Prevention (CDC) in which the most common outbreak serotypes involved were *S*. Enteriditis, Typhimurium, Newport and Heidelberg (CDC, 2006). According to the Food-Net data, incidence of human infections by *S.* Heidelberg has increased by 25% from 1996 to 2005, while the overall number of cases of salmonellosis has decreased by 9% (CDC, 2006). This report also identified that of the 5,869 isolates of *Salmonella* serotyped, six accounted for 61 % of the infections as follows: Typhimurium (19%), Enteriditis (18%), Newport (10%), Heidelberg (6%), and Javiana (5%). *Salmonella* serovars are a significant hazard in raw meat and poultry products and processors

may have limited control over the presence of this pathogen in raw meat received for processing (Ingham *et al.*, 2004).

In 1999, the U.S. Department of Agricultures' Food Safety and Inspection Service (USDA-FSIS) established lethality regulations for fully and partially cooked meat and poultry products (USDA-FSIS, 1999). A 6.5-log unit reduction of *Salmonella* in cooked beef and roast beef and a 7.0-log unit reduction in certain fully and partially cooked poultry products were set as a performance standard for lethality, stabilization, and product handling (Weche *et al.*, 2005). Although foodborne pathogens are subjected to physical, chemical, and nutritional stresses during processing (Yousef and Courtney, 2003), their elimination/ inhibition poses a challenge to processors. Although significant advances have been made in developing thermal and non-thermal intervention technologies to reduce *Salmonella* spp. in meat and poultry product, the ability of some strains of this pathogen to grow at temperatures of up to 54 °C and survive in foods stored at colder temperatures (2-4 °C) (D'Aoust, 1991) along with its ability to grow over a wide range of pH from 4.5 to 9.5 (D'Aoust, 1989) is a concern during food preparation, storage and distribution. Increasing consumer demands for convenience foods with longer shelf lives have generated interest in the use of spices, essential oils, and organic acids in marinades to enhance safety of foods (Shelef, 1984; Sabah *et al*, 2004). Marinades are water-oil emulsions containing combinations of sugar, salt, acids (acetic, citric acids), additives (Xanthan and guar gum), spices, sorbates and benzoates and aroma enhancers (Bjorkroth, 2005). Mainly salt-water and phosphate formulations are used for commercial marination to increase tenderness, juiciness and yield of meat with current practices in the food industry employing injector machines, immersion and vacuum tumbling for marination (Alvarado and McKee, 2007). Spice extracts are being widely used in the industry as they are easily soluble and are compatible with injection

marination systems as they do not clog the needles (Ferrell, 1990; Carlos and Harrison, 1999). Marketing of marinated poultry products has become one of the fastest growing segments of the food industry around the world.

Industrial marination is becoming increasingly popular and about 80 % of the poultry products, such as skinned meats, fillets, skin-on breast fillets and leg cuts sold at retail level are marinated in Finland (Bjorkroth, 2005). Bremer and Osbourne (1995) reported anti-listerial activities of organic acid marinades in green shell mussels and can potentially be used for developing effective marination methods to reduce *Listeria monocytogenes*. Yogurt is also used as a marinating ingredient and has shown reductions of 2-logs of *Campylobacter jejuni* on pork medallions (Birk and Knochel, 2009). According to Bjorkroth (2005), marinade sauces prevent the growth of spoilage microorganisms based on a low pH, high concentration of salts (NaCl), sorbates and benzoates, and various spices. Perko-Makela *et al.* (2000) reported reductions of *C. jejuni* (2.4 $\log_{10}$ CFU/ ml after 24 h and none detected after 48 h at 4 °C) in a marinade emulsion of vegetable oil and water with spices, and NaCl whereas, no changes in the survival of *C. jejuni* were reported on the product (marinated chicken drumsticks and breast fillets). Antibacterial activity of marinades and spices depends on several factors such as pH, temperature, and duration of storage. Kim *et al.* (1995) reported antibacterial activity of essential oil components of spices against *S.* Typhimurium while Nazer *et al.* (2005) documented the inhibitory effect of aromatic compounds like thymol, carvacrol, citral, eugenol and geraniol in combination with acids on the growth of *S.* Typhimurium in laboratory media. Limited data on the survival of *Salmonella* spp. in marinades and marinated products have been published. Thus, the goal of this study was to determine the effect of commercially available teriyaki and lemon pepper

marinades on survival of *Salmonella* (serotypes Heidelberg, Typhimurium and Senftenberg) at refrigerated (4 °C) and room (25 °C) temperatures.

3.3 – Materials and Methods

Bacterial Cultures. Three strains of *Salmonella* [Heidelberg, Typhimurium and Senftenberg (obtained from Nelson Cox, USDA-ARS, Athens, Georgia)] were used individually for this study and maintained on Tryptic Soy Agar slants (TSA; Neogen Cooperation, Lansing, Michigan) at 4 °C. The selected strains were independently cultured in Brain Heart Infusion broth (BHI; Neogen Cooperation, Lansing, Michigan) at 37 °C for 24 h. *S.* Heidelberg and Typhimurium were used in this study as these are the most common serotypes causing foodborne infections (CDC, 2006), while *S.* Senftenberg was used as it is the most heat resistant of all the *Salmonella* serotypes (Jay, 2000). The strains were then subjected to increasing concentrations of nalidixic acid (NA; Sigma Aldriech, St. Louis, MO) on Xylose Lysine Tergitol4 (XLT4; Neogen Cooperation, Lansing, Michigan) to develop antibiotic resistance. The concentration of NAL in XLT4 agar was gradually increased from 5 to 65 µgm/ml in 5 µgm/ml increments until no *Salmonella* growth was observed. As a result, *S.* Typhimurium and *S.* Heidelberg resistant to 60 µgm and *S.* Senftenberg resistant to 35 µgm of nalidixic acid were obtained.

These nalidixic acid resistant (NAL) strains were maintained on TSA slants at 4 °C. A loop-ful of the nalidixic acid resistant strain was then transferred individually in 9 ml of BHI with two consecutive 24 h transfers at 37 °C followed by a 100µl transfer of each culture in 40 ml BHI tubes. These tubes were then incubated for 20 h at 37 °C, centrifuged at 10,967 x g (FiberLite® centrifuge, F13-14x50c rotor, Piramoon Technologies Inc. Santa Clara, CA) for 10 min at 4 °C, the supernatant was decanted and the pellet suspended in 10 ml of 0.1% peptone

water (PW; Neogen Cooperation, Lansing, Michigan) and re-centrifuged. The pellet obtained was then re-suspended in 10 ml of 0.1% PW and final concentrations of approximately 10^{8-9} CFU/ml of pure cultures for each of the three strains were obtained.

Marinade Preparation. Teriyaki (The Great American Spice, Fort Wayne, IN) and lemon pepper marinade (The Great American Spice, Fort Wayne, IN) were used in the study and prepared separately in sterile beakers (autoclaved; 121°C, 20 min) according to manufacturer's instructions on the labels. Accordingly, one part of teriyaki marinade was reconstituted with two parts of water (10 g of teriyaki marinade powder in 20 ml of water) and 0.66 g of lemon pepper was mixed with 20 ml of water. The pH of the marinades was recorded at each sampling time interval and is shown in Table 1.

Inoculation and Salmonella Enumeration. Teriyaki and lemon pepper marinade were inoculated by adding 1 ml of the nalidixic acid resistant *Salmonella* strains individually into 20 ml of each marinade. The marinade solutions were homogenized by vortexing, divided into 10-ml aliquots in sterile beakers for each strain, and then stored at 4 or 25 °C for up to 32 h. For enumeration of initial (0 h) *Salmonella* populations, 0.1 ml of the inoculated marinade was taken after 30 min and serially diluted in 9.9 ml of 0.1% PW. Dilutions were plated onto XLT4+60µg NAL agar for *S.* Heidelberg and Typhimurium, while *S.* Senftenberg was plated onto XLT4+35µg NAL agar; incubated at 37 °C for 24 h. Similarly, serial dilutions of 0.1 ml of each marinade were made in 0.1% PW blanks and plated on XLT4+60µg NAL agar for *S.* Heidelberg and Typhimurium and XLT4+35µg NAL agar for *S.* Senftenberg at 4, 8, 16, 24 and 32 h of storage at each temperature. Each dilution was plated in duplicate and incubated at 37 °C for 24 h to enumerate the survival populations. Survival populations were reported as colony forming unit per ml of the marinade (CFU/ ml). Non-inoculated teriyaki and lemon pepper marinade

solutions were plated at each time interval to ensure that no bacterial growth occurred on the negative controls.

Statistical Analysis. Survival populations of *Salmonella* spp. in the marinades (2 marinades x 6 time interval x 2 temperatures) were analyzed using PROC GLM procedures in SAS (2002-03 SAS Institute Inc., Gary, NC). Main and interaction effects on residual *Salmonella* populations were analyzed and Least Square Means were differentiated at $\alpha = 0.05$. Three replications of the experiment were performed and survival populations ($\log_{10}$ CFU/ ml) of each strain of *Salmonella* spp. are reported as an average of the three replications.

3.4 – Results and Discussion

Analysis of variance (ANOVA) of survival populations ($\log_{10}$ CFU/ml) of nalidixic acid resistant *S.* Heidelberg showed significant reductions ($p \leq 0.05$) following treatment with teriyaki from 5.89 $\log_{10}$ CFU/ ml at 0 h to below detection limits (<5 CFU/ ml) over a 32 h period irrespective of storage temperature (4 and 25 °C) (Table 2). On the other hand, the survival populations ($\log_{10}$ CFU/ml) of nalidixic acid resistant *S.* Heidelberg following treatment with lemon pepper marinade showed an initial reduction ($p<0.05$) from 9.73 $\log_{10}$ CFU/ ml at 0 h to 8.05 and 8.43 $\log_{10}$ CFU/ ml at 4 and 25 °C respectively after 4 h. Following this initial reduction, no significant reductions ($p > 0.05$) of the survival populations ($\log_{10}$ CFU/ml) of nalidixic acid resistant *S.* Heidelberg were observed with lemon pepper marinade over a 32 h period irrespective of the storage temperature (Table 2). Comparing survival populations ($\log_{10}$ CFU/ml) of nalidixic acid resistant *S.* Heidelberg in the teriyaki marinade stored at 4 and 25 °C

showed no significant differences ($p<0.05$) at 0, 4, 8, and 32 h, while significant differences ($p<0.05$) were observed at 16 and 24 h of storage (Table 2).

Survival populations (log_{10} CFU/ml) of nalidixic acid resistant *S.* Typhimurium were reduced ($p<0.05$) from 5.65 log_{10} CFU/ml at 0 h to below detection limits over the 32 h storage period at 4 and 25 °C (Table 3). Although, no significant reductions ($p>0.05$) of nalidixic acid resistant *S.* Typhimurium populations (log_{10} CFU/ml) were observed in lemon pepper marinade stored at 25 °C, maintaining the marinade at 4 °C resulted in significant ($p<0.05$) reductions over the 32 h storage period. Comparison of specific significant differences ($p<0.05$) in the survival populations of nalidixic acid resistant *S.* Typhimurium over time are shown in Table 3. There was no significant difference ($p>0.05$) in the survival populations at 0, 4, 24, and 32 h interval, while at 8 and 16 h interval significant differences ($p<0.05$) were observed between the 4 and 25 °C storage temperature for *S.* Typhimurium in the teriyaki marinade. On the other hand, surviving populations of *S.* Typhimurium were not significantly different ($p>0.05$) in the lemon pepper marinade stored at 4 and 25 °C for up to 24 h, while, a significant difference ($p<0.05$) was observed at 32 h of storage.

Survival populations (log_{10} CFU/ ml) of nalidixic acid resistant *S.* Senftenberg in teriyaki marinade were significantly reduced ($p<0.05$),whereas, for lemon pepper marinade no significant reductions ($p>0.05$) were observed over the 32 h storage period at 4 and 25 °C (Table 4). Storage of teriyaki marinade at 4 and 25 °C showed significant differences ($p<0.05$) at 8, 16, and 24 h interval, while no differences ($p>0.05$) were observed in the survival populations of *S.* Senftenberg for the initial 4 h period. In lemon pepper marinade, there was no difference ($p>0.05$) in the survival populations of *S.* Senftenberg at 4 and 25 °C over the 32 h period. Throughout the 32 h period, survival populations of all three strains of nalidixic acid resistant

Salmonella (Heidelberg, Typhimurium, and Senftenberg) were lower ($p<0.05$) in teriyaki as compared to the lemon pepper marinade irrespective of the storage temperature. This can be attributed to the lower pH of teriyaki (3.71 – 3.78) observed over the 32 h period as compared to lemon pepper (5.02 – 5.94) (data not shown) and the results from our study are in agreement with previous studies where it has been reported that a single ecological factor such as pH can reduce survival populations of bacteria such as *Campylobacter jejuni* in marinated chicken products (Perko-Makela *et al.*, 2000).

Results from our study indicated higher survival populations of all three strains of *Salmonella* spp. in the lemon pepper marinade as compared to teriyaki. Although, not compared in our study, it has been reported that Gram positive bacteria are more sensitive to the citrus essential oils *in vitro* than Gram negative bacteria (Fisher and Phillips, 2006). Similarly, Dabbah *et al*. (1970) reported higher activity of orange, lemon, grapefruit and mandarin citrus oils and their derivatives *in vitro* against *Staphylococcus aureus*, whereas the least activity was observed against *S.* Senftenberg in a study in which *Escherichia coli* and *Pseudomonas* spp. were also tested. Although, survival populations were lower in teriyaki as compared to lemon pepper marinade, results from our study showed no differences in the growth patterns of all three strains of *Salmonella* spp. in either of the marinade. Populations of *S.* Typhimurium were reduced below detection limit after 8 h while *S.* Heidelberg and Senftenberg were not detected after 16 h in teriyaki stored at 25 °C. These results are consistent with the findings of Nanasombat and Lohasupthawee (2005) according to which, the antibacterial activity of ethanolic extracts and essential oils of spices were different among multiple strains of *Salmonella* belonging to serotypes Derby, Rissen, Agona, and Typhimurium. Furthermore, Smith-Palmer *et al.* (1998) reported higher efficacy of essential oils obtained from plant extracts against *Campylobacter*

jejuni, *S. enteritidis*, *E. coli*¸ *S. aureus*, and *Listeria monocytogenes* at 35 °C as compared to 4 °C which are similar to those observed in our study where survival populations of all three strains of *Salmonella* spp. (Heidelberg, Typhimurium, and Senftenberg) were found to be higher (not significant) at 4 °C than at 25 °C in teriyaki marinade.

Teriyaki marinade powder used in our study had garlic, NaCl, and sodium polyphosphates as its ingredients which may contribute towards its bactericidal effects and hence to its higher efficacy in reducing populations of *Salmonella* spp. as compared to lemon pepper marinade. Studies have shown that marinade sauces with paprika, garlic, coriander, NaCl, and sodium polyphosphates have been effective in reducing the survival of *Campylobacter* cells (Perko-Makela *et al*., 2000). Teriyaki marinade is a highly acidic thick sauce with powdered onion, garlic, spices, and powdered soy sauce as the dominant ingredients that can contribute to its bactericidal activity. The bactericidal activity of garlic in reducing 93% of *Staphylococcus epidermis* and *S.* Typhi populations within 3 h of exposure have been reported by Arora and Kaur (1999) while, soy sauce has been shown to have antimicrobial activity against *Salmonella, Shigella, Staphylococcus* and *Proteus* (Kataoka, 2005). The pH of lemon pepper marinade was less acidic as compared to teriyaki with oils of lemon, ground black pepper, and lemon peel granules as its main ingredients. The milder activity of lemon pepper marinade may be due to the presence of oils which can potentially protect the bacterial cells hence resulting in higher survival populations.

Thus, marination has the potential to increase the shelf life of poultry and meat products. Value-addition procedures along with other thermal and non-thermal intervention technologies can enhance poultry and meat products safety and quality by acting as hurdles for bacterial growth and at the same time improve flavor, juiciness and convenience of handling. Our study

revealed that teriyaki marinade was more effective in reducing *Salmonella* populations as compared to lemon pepper both at 4 and 25 °C. There may be limitations in the use of marinades as they can alter the taste of food and eventually reduce consumer acceptability. Research done in laboratory media may differ from the actual concentrations of marinades needed in poultry and meat products to inhibit bacterial growth. Hence, further research needs to be done to investigate the inhibitory concentrations of marinades and their application in food matrices.

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Tables and Figures

Table 1: pH of Teriyaki and lemon pepper marinade at 4 and 25 °C over 32 h of storage.

Time (h)	Teriyaki		Lemon Pepper	
	4 °C	**25 °C**	**4 °C**	**25 °C**
0	3.78	3.78	5.15	5.15
4	3.73	3.71	5.08	5.19
8	3.72	3.78	5.10	5.04
16	3.74	3.73	5.07	5.94
24	3.75	3.75	5.04	5.88
32	3.71	3.72	5.02	5.74

Table 2: Survival populations[@] (log_{10} CFU/ ml) of *Salmonella* Heidelberg in commercial marinades stored at refrigerated and room temperature.

Time (h)	Control[*]	Teriyaki		Lemon Pepper	
		4 °C	25 °C	4 °C	25 °C
0	9.44	5.89(0.25)^{d,x,A}	5.89(0.25)^{d,A,X}	9.73(0.25)^{b,y,A}	9.73(0.25)^{b,A,Y}
4	9.47	3.58(0.25)^{c,x,A}	3.23(0.25)^{c,A,X}	8.05(0.25)^{a,y,A}	8.43(0.25)^{a,A,Y}
8	9.23	3.24(0.25)^{c,x,A}	2.67(0.25)^{bc,A,X}	8.10(0.25)^{a,y,A}	8.00(0.25)^{a,A,Y}
16	9.56	3.09(0.25)^{c,x,B}	2.09(0.25)^{b,A,X}	8.29(0.25)^{a,y,A}	8.37(0.25)^{a,A,Y}
24	9.29	1.7(0.25)^{b,x,B}	ND(0.25)^{a,A,X}	8.11(0.25)^{a,y,A}	8.30(0.25)^{a,A,Y}
32	9.43	ND(0.25)^{a,x,A}	ND(0.25)^{a,A,X}	8.29(0.25)^{a,y,A}	8.17(0.25)^{a,A,Y}

[@]Least square means (standard error)

[*]Populations (log_{10} CFU/ ml) of *S.* Typhimurium inoculum without marinade over time

abc -Means with different superscripts indicate significant difference ($p<0.05$) within a column

AB - Means with different superscripts indicate significant difference ($p<0.05$) at 4 and 25 °C within a marinade

xy - Means with different superscripts indicate significant difference ($p<0.05$) between the two marinades at 4 °C for each time interval

XY - Means with different superscripts indicate significant difference ($p<0.05$) between the two marinades at 25 °C for each time interval

ND - Indicates survival populations were below detection limit (≤ 5 CFU/ ml)

Table 3: Survival populations[@] (log_{10} CFU/ ml) of *Salmonella* Typhimurium in commercial marinades stored at refrigerated and room temperature.

Time (h)	Control[*]	Teriyaki		Lemon Pepper	
		4 °C	25 °C	4 °C	25 °C
0	10.27	$5.65(0.23)^{c,x,A}$	$5.65(0.23)^{c,A,X}$	$9.76\ (0.23)^{c,y,A}$	$9.76(0.23)^{b,A,Y}$
4	10.12	$2.74(0.23)^{b,x,A}$	$2.51(0.23)^{b,A,X}$	$8.08(0.23)^{b,y,A}$	$8.17(0.23)^{a,A,Y}$
8	9.88	$2.15(0.23)^{b,x,B}$	$1.53(0.23)^{a,A,X}$	$7.65(0.23)^{ab,y,A}$	$8.27(0.23)^{a,A,Y}$
16	10.01	$2.23(0.23)^{b,x,B}$	$ND(0.23)^{a,A,X}$	$7.85(0.23)^{ab,y,A}$	$8.33(0.23)^{a,A,Y}$
24	10.14	$0.9(0.23)^{a,x,A}$	$ND(0.23)^{a,A,X}$	$7.65(0.23)^{ab,y,A}$	$8.09(0.23)^{a,A,Y}$
32	9.21	$ND(0.23)^{a,x,A}$	$ND(0.23)^{a,A,X}$	$7.42(0.23)^{a,y,A}$	$8.16(0.23)^{a,B,Y}$

[@]Least square means (standard error)

[*]Populations (log_{10} CFU/ ml) of *S.* Typhimurium inoculum without marinade over time

abc -Means with different superscripts indicate significant difference ($p<0.05$) within a column

AB - Means with different superscripts indicate significant difference ($p<0.05$) at 4 and 25 °C within a marinade

xy - Means with different superscripts indicate significant difference ($p<0.05$) between the two marinades at 4 °C for each time interval

XY - Means with different superscripts indicate significant difference ($p<0.05$) between the two marinades at 25 °C for each time interval

Table 4: Survival populations[@] (log_{10} CFU/ ml) of *Salmonella* Senftenberg in commercial marinades stored at refrigerated and room temperature.

Time (h)	Control[*]	Teriyaki Marinade		Lemon Pepper	
		4 °C	25 °C	4 °C	25 °C
0	9.86	$7.59(0.29)^{c,x,A}$	$7.59(0.29)^{e,A,X}$	$8.84(0.29)^{b,y,A}$	$8.84(0.29)^{b,A,Y}$
4	9.77	$4.21(0.29)^{b,x,A}$	$3.91(0.29)^{d,A,X}$	$8.4(0.29)^{ab,y,B}$	$7.14(0.29)^{a,A,Y}$
8	9.76	$3.86(0.29)^{b,x,B}$	$2.87(0.29)^{c,A,X}$	$7.69(0.29)^{a,y,A}$	$8.16(0.29)^{b,A,Y}$
16	9.46	$3.75(0.29)^{b,x,B}$	$1.78(0.29)^{b,A,X}$	$7.91(0.29)^{a,y,A}$	$8.57(0.29)^{b,A,Y}$
24	9.38	$1.57(0.29)^{a,x,B}$	$ND(0.29)^{a,A,X}$	$8.57(0.29)^{ab,y,A}$	$8.61(0.29)^{b,A,Y}$
32	9.59	$0.85(0.29)^{a,x,A}$	$ND(0.29)^{a,A,X}$	$8.35(0.29)^{ab,y,A}$	$8.56(0.29)^{b,A,Y}$

[@]Least square means (standard error)

[*]Populations (log_{10} CFU/ ml) of *S.* Typhimurium inoculum without marinade over time

abcde -Means with different superscripts indicate significant difference ($p<0.05$) within a column.

AB - Means with different superscripts indicate significant difference ($p<0.05$) at 4 and 25 °C within a marinade.

xy - Means with different superscripts indicate significant difference ($p<0.05$) between the two marinades at 4 °C for each time interval.

XY - Means with different superscripts indicate significant difference ($p<0.05$) between the two marinades at 25 °C for each time interval.

4 – INHIBITION OF NALIDIXIC ACID RESISTANT *SALMONELLA* SPECIES ON MARINATED CHICKEN SKIN

4.1 - Abstract

Marination is widely done to enhance flavor and increase consumer acceptability of meat and poultry products. The impact of such marination on safety and shelf-life of poultry meat was evaluated in this study. A series of experiments were conducted to determine the efficacy of Teriyaki and Lemon pepper marinades against multiple strains of Nalidixic acid (NAL) resistant *Salmonella*. Nalidixic acid resistant *Salmonella* (Typhimurium, Heidelberg, and Senftenberg) cultures were inoculated onto chicken skin at levels varying from 0.6 to 3.14 $\log_{10}$ CFU/ gm in a 12-well titer plate. Inoculated chicken skin was exposed to teriyaki or lemon pepper marinades for up to 32 h and stored at 4 and 25 °C to determine the prevalence of *Salmonella*. Simultaneously, to enumerate survival populations of *Salmonella* a three-strain cocktail of *Salmonella* was inoculated at low (Ca. 4 $\log_{10}$ CFU/ gm) and high (8 $\log_{10}$ CFU/ gm) levels onto chicken skin and marinated with either teriyaki or lemon pepper marinade that was stored at 4 and 25 °C for up to 32 h. Prevalence of *Salmonella* was significantly reduced ($p \leq 0.05$) by teriyaki marinade at all levels of contamination irrespective of storage temperature. Lemon pepper marinade reduced ($p \leq 0.05$) the prevalence at low levels of contamination (10^1 and 10^2 CFU/gm) whereas no significant effect ($p>0.05$) was observed at higher levels of contamination. Marination of chicken skin with teriyaki marinade greatly helped in reducing ($p \leq 0.05$) the

prevalence and survival populations of *Salmonella* irrespective of the storage temperature indicating its antimicrobial potential in poultry and meat products.

4.2 - Introduction

Foodborne diseases result in an estimated 76 million illnesses, 325,000 hospitalizations and 5,000 deaths with an annual cost of 7 billion dollars in the United States (WHO, 2007). An estimated 1.4 million cases of salmonellosis are reported annually in United States (Mead at al, 1999) which is commonly associated with consumption of contaminated poultry due to improper handling or inadequate cooking procedures. Most reported foodborne illnesses are sporadic in nature; only a small number are identified as being part of an outbreak and thus are reported through the Foodborne-Disease Outbreak Surveillance System. Thus, the system greatly underestimates the burden of foodborne diseases (Mead et al, 1999). The increase in incidence of salmonellosis in the United States may be due to changing agricultural and food distribution methods, increased consumption of raw or slightly cooked foods, an increase in the number of immuno-compromised or chronically ill people and deterioration of the public health infrastructure (Altekruse, 1997).

The ubiquity of *Salmonella* spp. in the natural environment contributes to its continued presence in a wide variety of mammals, birds, reptiles, and other animals (Wigley, 2004). *Salmonella* are difficult to eradicate from the environment as most serovars are not host adapted and hence are found in a variety of animal products, fruits, vegetables and processed foods. According to the U. S. Department of Agriculture the most commonly identified paratyphoid serotypes in chickens are *S.* Heidelberg, *S.* Kentucky, *S.* Senftenberg, *S.* Enteritidis and *S.*

Thompson (Ferris at al, 1999). Contamination of birds in the holding pens and stress induced shedding of *Salmonella* during transport from farms to slaughter houses amplifies the bacterial contamination at the processing plant (D' Aoust, 2000). The high prevalence of *Salmonella* (42% positive) in retail poultry reflects the inability to completely prevent the spread of this organism during production and marketing of raw poultry meat (D' Aoust, 2000). The microbial contamination of raw chicken can be minimized by good manufacturing practices, but the total elimination of foodborne pathogens is difficult. Poultry has become an important source of food in most countries, which has also led to an increase in poultry-borne diseases, particularly salmonellosis (Todd, 1980). Raw chicken is a potential source of bacterial pathogens such as *Salmonella*, *Listeria monocytogenes* and *Campylobacter jejuni* (Carlos and Harrison, 1999), however, poultry and its products have been identified as one of the important reservoirs of *Salmonella* as it can occur naturally in the intestinal tracts of chickens and are widespread throughout live production and processing environments.

Salmonella is present on the surface of skin, feathers and feet in addition to the gastrointestinal tract of the birds (Bianchi et al, 1994). Chicken skin is an ideal surface for bacterial attachment (Firstenberg-Eden et al, 1978; Conner and Bilgili, 1994) as the feather follicles can trap bacteria making it difficult for their removal by chemicals and washing (McMeekin et al, 1984). They can also form colonies in the muscle via the natural pores in meat or they can create pores by hydrolysis of the Myofibrillar proteins (Sikes and Maxcy, 1980). Microorganisms trapped in feather follicles and skin folds are protected from surface antimicrobials (Mehyar et al, 2005) and moisture present on the chicken skin surface can also act as a diluent and reduce effectiveness (Oyarzabal et al, 2004). Scalding removes skin cuticle and affects its adhesiveness by making it more hydrophilic, facilitating in microbial contamination

(Suderman and Cunningham, 1980). The rate and firmness of bacterial attachment is time dependent (Firstenberg-Eden et al, 1978) and is affected by bacterial strain, pH and temperature (Notermans and Kampelmacher, 1974).

Many physical and chemical methods of decontaminating meat surfaces and skin have been investigated (Bolder, 1997). Washing processes swell the collagen in dermal layers and shields removal of surface contaminants (Thomas and McMeekin, 1982), therefore, bacterial destruction should not only ensure safety of foods but also involve application of intense treatments which may cause additional food quality losses (Ray, 1986). Temperature, water activity, addition of preservatives, and pH are all parameters used to inhibit or destroy the microorganisms and their spores and aid in food preservation (Marechal et al, 1999). A significant reduction in total bacterial numbers is achieved during processing by scalding, washing, and chilling operations but many frozen broiler chickens at retail outlets still contain some *Salmonella* strains at low levels (Izat et al, 1991). Treatments with organic acids (Van der Marel et al, 1988), ozone (Sheldon and Brown, 1986), potassium sorbate (Robach and Sofos, 1982), chlorine dioxide (Thiessen, 1983), lactic acid/ sodium lactate (Zeitoun and Debevere, 1980) and glutaraldehyde (Thomson et al, 1977) have shown to reduce microbial populations on poultry carcasses. Ionizing radiation is effective in decontaminating poultry carcasses (Patterson, 1989), but its application may be limited by consumer acceptance. Recently, consumer interest in minimally processed foods preserved with natural ingredients has significantly increased (Cagri et al, 2004). Hence, marination of meat is an emerging industrial technology which is being used to improve tenderness, flavor and prolong the shelf-life of meat and poultry products. Originally, meat was immersed in the marinades allowing its components to passively penetrate the meat over a period of time (Alvarado and McKee, 2007).

Present day marination technology involves methods such as injection marination and vacuum tumbling that are widely practiced in the food industry. The use of natural antimicrobials produced from herbs and spices lends a favorable consumer acceptance along with microbiological safety (Ferrell, 1990). Marinades are a complex combination of spices; typically water-oil emulsions containing salt, sugar, acids (acetic and citric), additives (Xanthan and Guar gum), antimicrobial agents (sorbates and benzoate), and aroma enhancers. Essential oils, spices and their extracts have preservative and antioxidant properties along with antibacterial, antifungal and antiviral activities that have been well documented (Ferrell, 1990; Hammer et. al, 1999). Essential oils derived from the steam distillation of *Pelargonium* species have been demonstrated to be effective against *Salmonella* Enteriditis and *Listeria* innocua (Lis-Balchin et al, 2003). It has been reported that the growth of *S.* Enteritidis is inhibited by essential oils of oregano in homemade tarama-salad (Tassou et al, 1995) and Koutsoumanis et al (1998) reported antimicrobial effect of essential oils of orange, lemon, grapefruit, oregano, bay, cloves, coriander, cinnamon, all-spice and thyme against *Salmonella* by disc diffusion methods. The objective of marination is not only to improve flavor but also enhance microbial safety and shelf life of meat products by inhibiting growth of microorganisms due to acidic pH of the sorbates and benzoates in the marinades (Bernard et al, 1990). It is of interest to quantify the antimicrobial effect of commercial marinades against microorganisms present on poultry meat. Therefore, the objective of this research was to determine the ability of commercial marinades (Teriyaki and Lemon Pepper marinade) to inhibit, inactivate, or reverse the attachment of *Salmonella* spp. on chicken skin.

4.3 - Materials and Methods

Bacterial Strains. The three strains of *Salmonella* [Heidelberg, Typhimurium and Senftenberg (obtained from Nelson Cox, USDA-ARS, Athens, Georgia)] used in the study were separately maintained on Tryptic Soy Agar slants (TSA; Neogen Cooperation, Lansing, Michigan) at 4 °C. The selected strains were independently cultured in Brain Heart Infusion broth (BHI; Neogen Cooperation, Lansing, Michigan) at 37 °C for 24 h. *S.* Heidelberg and Typhimurium were used as these are the most common serotypes causing foodborne infections (CDC, 2006), while *S.* Senftenberg was used as it is the most heat resistant of all the *Salmonella* serotypes (Jay, 2000). These strains were subjected to increasing concentrations of nalidixic acid (NAL; Sigma Aldriech, St. Louis, MO) on Xylose Lysine Tergitol4 (XLT4; Neogen Cooperation, Lansing, Michigan) agar to develop antibiotic resistance. The concentration of NAL in XLT4 agar was gradually increased from 5 to 65 µgm/ml in 5 µgm/ml increments until no *Salmonella* growth was observed. As a result, *S.* Typhimurium and *S.* Heidelberg resistant to 60 µgm and *S.* Senftenberg resistant to 35 µgm of nalidixic acid were obtained. The NAL resistant strains were then maintained on TSA slants at 4 °C, before each strain was cultured separately in 9 ml of BHI and incubated for 24 h at 37 °C. Antibiotic resistant strains were used in this study because it is possible to study their low levels of inoculation on chicken skin in presence of other natural micro flora by using selective media (XLT4 agar) along with antibiotics (Oscar, 2008).

Bacterial growth and preparation of inocula. Nalidixic acid resistant *Salmonella* (Typhimurium, Heidelberg and Senftenberg) were grown separately in BHI for 18 h (approximately 10^8 CFU/ml). To determine prevalence of *Salmonella* spp. each strain was diluted 8-fold in 0.1% peptone water (PW; Neogen Cooperation, Lansing, Michigan) to obtain

inoculum levels of 10^4, 10^3, 10^2 and 10^1 CFU/ml that were used for inoculating the chicken skin. Lower levels of the inoculum used in our study were to simulate natural contamination levels that are likely to occur on chicken products at the retail stores. To determine antimicrobial efficacy of the marinades a cocktail of the three strains was used at two levels; 1) high (Ca. ~ 8 $\log_{10}$ CFU/ ml) and 2) low (Ca. ~ 4 $\log_{10}$ CFU/ ml) on the chicken skin.

***Skin Sample Preparation*.** Chicken breast skin was obtained from the broilers processed at Auburn University Poultry Research Farm simulating commercial processing procedures. Following evisceration carcasses were placed in a chill tank and skin was obtained after manual deboning. The skin was spread on sterile Styrofoam trays and covered with Serrano wrap. Following freezing at –20 °C for 15 min. the skin was cut into 1 g pieces prior to inoculation. Freezing was done to facilitate cutting of the skin.

***Marinade Preparation*.** Teriyaki and lemon pepper marinade (The Great American Spice, Fort Wayne, IN) were prepared separately in sterile beakers according to manufacturer's instructions. Accordingly, one part of teriyaki marinade was reconstituted with two parts of water and 394 g of lemon pepper was mixed with 3,785 ml of water. The pH of the marinades was recorded at each sampling time interval (Table 1).

***Salmonella Inoculation on chicken skin*.** Chicken skin was cut into 1 g pieces and placed in each well of a 12-well titer-plate. Of the 12 skin pieces, 10 were spot inoculated with the respective inoculum and exposed to teriyaki and lemon pepper marinade for up to 32 h, while one well each served as a positive and a negative control. Inoculation was done by adding 0.1 ml of 10^4, 10^3, 10^2 or 10^1 CFU/ml of the appropriate *Salmonella* strain and placed under the biosafety hood (Nuaire Inc., Plymouth, MN) for 30 min. to allow bacterial attachment. The

positive control comprised of 1 g chicken skin inoculated with the bacterial strain with no exposure to any marinade solution, whereas the negative control consisted of non-inoculated 1 g chicken skin without any marinade.

***Marinade Application*.** The inoculated skin samples were marinated in the same 12-well plates by immersing the samples in either the teriyaki or lemon pepper marinade solutions. For each inoculum level (10^4, 10^3, 10^2 or 10^1 CFU/ml) of an individual strain (Typhimurium, Heidelberg and Senftenberg), two sets of titer plates were prepared with teriyaki and lemon pepper marinade and stored at 4 and 25 °C for up to 32 h.

***Salmonella Prevalence Assay*.** Marinated chicken skin samples were removed after 0, 4, 8, 16, 24, and 32 h of exposure and transferred into 9 ml of buffered peptone water (BPW; Neogen Cooperation, Lansing, Michigan). The samples were then vortexed for 10 s and 0.1 ml was plated onto XLT4 agar containing NAL. The 0 h samples in our study were actually exposed to the respective marinades for 30 min. at 4 and 25 °C prior to plating on XLT4 agar containing appropriate levels of NAL. The plates were incubated at 37 °C for 24 h. After 24 h of incubation the plates were observed for the presence or absence of characteristic black colonies of *Salmonella*. Results were recorded as “positive” or “negative” to determine the prevalence. For the “negative” results, samples enriched with BPW were incubated at 37 °C for an additional 24 h for confirmation. The prevalence of *Salmonella* spp. on the chicken skin was calculated as [number of positive samples/ total number of samples*100] and reported as percentage (%).

***Salmonella Enumeration on Chicken Skin*.** To determine the survival populations of *Salmonella* spp. following marination for up to 32 h, marinated skin samples were removed at each time interval and added to 9 ml PW. Samples were then serially diluted and plated onto

XLT4+35µg NAL agar; incubated at 37 °C for 24 h. Survival populations were reported as colony forming units per gram of the skin (log_{10} CFU/ g). Non-marinated, inoculated skin samples served as positive controls for the enumeration study.

***Statistical analysis*.** Survival populations and prevalence of *Salmonella* spp. in the marinades (2 marinades x 6 time interval x 2 temperatures) were analyzed using PROC GLM procedures in SAS (2002-03 SAS Institute Inc., Gary, NC). Three replications of the experiment were performed for the prevalence and survival populations (log_{10} CFU/ ml) of *Salmonella* spp. and reported as an average. Main and interaction effects on residual *Salmonella* populations (log_{10} CFU/ g) and prevalence (%) were analyzed and Least Square Means were differentiated at $\alpha = 0.05$.

4.4 - Results and Discussion

***Enumeration of Salmonella*.** Analysis of variance (ANOVA) of survival populations (log_{10} CFU/gm) of NAL resistant *Salmonella* cocktail (Heidelberg, Typhimurium and Senftenberg) showed significantly lower ($p<0.05$) residual populations following treatment with teriyaki as compared to the lemon pepper marinade at time intervals of 16, 24, and 32 h when stored at 25 °C, while no differences ($p>0.05$) were observed for the initial 8 h (Table 2). Contrastingly, no significant differences ($p>0.05$) in the survival populations of the *Salmonella* spp. were observed between the teriyaki and lemon pepper marinade over the 32 h storage period at 4 °C. While comparing effects of storage temperature (4 and 25 °C), no significant differences ($p>0.05$) were observed in the survival populations of *Salmonella* spp. for the initial 8 h following which, significantly lower ($p<0.05$) survivors were observed at 16, 24, and 32 h of

storage when the chicken skin was immersed in lemon pepper marinade. For the chicken skin marinated in teriyaki, no significant differences ($p<0.05$) were observed in the survival populations of *Salmonella* spp. for up to 24 h irrespective of the storage temperature, whereas, at 32 h lower ($p<0.05$) survivors were observed at 25 °C as compared to 4 °C.

Comparing survival populations (log_{10} CFU/gm) of low inoculum level (Ca. ~ 3 to 4 log_{10} CFU/ml) of NAL resistant *Salmonella* cocktail (Heidelberg, Typhimurium and Senftenberg), no significant ($p>0.05$) reductions were observed for either of the marinades (teriyaki and lemon pepper) irrespective of the storage time at 4 °C. At 25 °C, no differences ($p>0.05$) were observed between the teriyaki and lemon pepper marinade at 0 h, while consistently lower ($p<0.05$) survival populations of *Salmonella* spp. were observed on the chicken skin marinated with teriyaki as compared to the lemon pepper marinade for time intervals 4, 8, 16, 24, and 32 h (table 3). The pH of the marinades over 32 h of exposure showed that teriyaki marinade was highly acidic (3.91 - 4.04) as compared to lemon pepper which ranged from 5.65 - 5.81 (Table 1). There were no significant difference ($p>0.05$) in pH of the marinades at 4 and 25 °C.

Prevalence of Salmonella. Prevalence studies were conducted with low levels of inoculum; 0.46 log_{10} CFU/gm to 3.67 log_{10} CFU/gm of *Salmonella* spp. on marinated chicken skin. These low levels of inoculum were used to simulate natural contamination levels of chicken products. Analysis of variance (ANOVA) suggested that teriyaki marinade significantly reduced ($p<0.05$) the prevalence of *S.* Heidelberg as compared to lemon pepper marinade over the 32 h marination period at all levels of inoculation irrespective of the storage temperature (Figures 1 and 2). On chicken skin inoculated with 10^1 and 10^2 CFU/gm of *S.* Heidelberg, lemon pepper marinade reduced ($p<0.05$) the prevalence for the initial 8 h of marination at 4 and 25 °C.

Whereas, at 10^3 and 10^4 CFU/ gm level of inoculation of *S.* Heidelberg on chicken skin marination with lemon pepper did not result in any significant ($p>0.05$) changes in the prevalence of this strain over the 32 h at 4 and 25 °C. At all levels of inoculation, teriyaki marinade significantly ($p>0.05$) reduced the prevalence of *S.* Heidelberg over the 32 h period at 4 and 25 °C. Prevalence assays for *S.* Senftenberg showed similar results as *S.* Heidelberg (Figures 5 and 6) at all levels of inoculation, while *S.* Typhimurium results were only different at 10^3 CFU/ gm level of inoculation on the chicken skin. The prevalence of *S.* Typhimurium on chicken skin marinated with teriyaki was significantly reduced ($p<0.05$) as compared to lemon pepper marinade over 32 h of marination at all levels of inoculation irrespective of storage temperature (Figures 3 and 4). During the initial 8 h of marination with lemon pepper, no differences ($p>0.05$) were observed in the prevalence of *S.* Typhimurium (10^3 CFU/ gm inoculation level) at 4 and 25 °C, while significantly lower ($p<0.05$) prevalence was observed for the 16, 24, and 32 h period of marination at 4 °C as compared to 25 °C (Figures 3 and 4).

Results from our study show that lemon pepper marinade decreased the prevalence of *Salmonella* spp. at low levels of contamination for up to 16 h of marination. This result is of significance as marination for 4 to 8 h is a common household practice prior to cooking that can further lead to reduction of *Salmonella* thereby enhancing the safety of marinated meat and poultry products. While studies have reported that a single ecological factor such as pH can reduce survival populations of bacteria such as *Campylobacter jejuni* in marinades, its effect is limited on marinated chicken products (Perko-Makela *et al.*, 2000) which is contrary to observations in our study where a significant reduction in the populations of *Salmonella* were observed on chicken skin following marination with teriyaki. The antimicrobial activity of teriyaki marinade can be attributed to the acidic pH and natural antimicrobial agents of spices

such as garlic, soy sauce, phosphates and salt as the dominant ingredients. Shelef and Seiter (2005) reported the suppression of microbial growth by polyphosphates and suggested that their antimicrobial activity can be attributed to their ability to chelate cations essential for microbial growth. The bactericidal activity of garlic extract in reducing 93% of *Staphylococcus epidermis* and *S.* Typhi populations within 3 h of exposure in nutrient broth have been reported by Arora and Kaur (1999) while, soy sauce has been shown to have antimicrobial activity against *Salmonella*, *Shigella*, *Staphylococcus* and *Proteus* (Kataoka, 2005). Salt has an inhibitory effect on microorganisms primarily due to its ability to cause cellular plasmolysis. Marinade sauce containing paprika, garlic, coriander, NaCl, and sodium polyphosphates has been effective in reducing 2.4 CFU/ml of *Campylobacter* cells after 24 h at 4 °C and no cells after 48 h of storage (Perko-Makela *et al*., 2000).

The dominant ingredients in lemon pepper marinade are ground black pepper, lemon peel granules, oils of lemon and some extracts of spices. The pH of the marinade was less acidic as compared to teriyaki marinade and presence of lemon oils can potentially hinder its efficacy as an antimicrobial agent. Although, not compared in our study, it has been reported that Gram positive bacteria are more sensitive to the citrus essential oils *in vitro* than Gram negative bacteria (Fisher and Phillips, 2006). Oils and fats in foods may have a protective effect on the bacterial cells by forming a ‘micelles’ and studies show that the lipid fractions in meat products absorb the spice extracts and thus reduce their concentration in the aqueous phase which also decreases their bactericidal effects (Carlos and Harrison, 1999). Oils of lemon that were a key ingredient in the lemon pepper marinade may prevent the spices in the marinade from being absorbed into the bacterial cells, hence reducing its antimicrobial properties.

Marinades provide excellent alternatives as natural antimicrobial systems for the chicken and meat products. Food processors have successfully used adventurous and spicy flavors of marinades such as, Teriyaki in different types of meats like bacon, turkey as the demand for specialty products is increasing. The global food industry is demanding 'natural' ingredients with increasing resistance at regulatory and consumer levels against chemical food preservatives; indicating a potential for marinades as antimicrobial additives to enhance safety and shelf life of poultry products. Marinades increase the safety, juiciness, tenderness and yield of meat and there is value-addition of the product which is beneficial to the processor as well. Our study revealed that marinating at refrigeration and room temperature did not affect the prevalence of *Salmonella* significantly ($p \leq 0.05$). This is an advantage for the consumers who can easily marinate on bench-top and still maintain the microbiological safety of food. Treatment with marinades can be combined with other thermal procedures like cooking, baking and microwave which will further reduce the any bacteria present in the marinated product to a harmless number. There is also potential of research with non-thermal methods like irradiation and ultra-violet radiation along with marination. Food application studies can be conducted on the sensory aspects as well as the antimicrobial activity of various spice blends in different marinade formulations.

4.5 - References

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F Tables and Figures

Table 1: pH of Teriyaki and lemon pepper marinade at 4 and 25 °C over 32 h of storage

Time (h)	Teriyaki		Lemon Pepper	
	4 °C	25 °C	4 °C	25 °C
0	3.78	3.78	5.15	5.15
4	3.73	3.71	5.08	5.19
8	3.72	3.78	5.10	5.04
16	3.74	3.73	5.07	5.94
24	3.75	3.75	5.04	5.88
32	3.71	3.72	5.02	5.74

Table 2: Survival populations (log_{10} CFU/gm) of 10^8 CFU/ml of *Salmonella* on chicken skin marinated with commercial marinades stored at refrigerated (4 °C) and room temperature (25 °C)

Time	Lemon Pepper Marinade		Teriyaki Marinade	
(h)	4 °C	25 °C	4 °C	25 °C
0	5.23(0.41) [a,A,x]	5.32(0.41) [a,A,X]	5.32(0.41) [a,A,x]	4.47(0.41) [b,A,X]
4	4.47(0.41) [a,A,x]	5.46(0.41) [a,A,X]	5.43(0.41) [a,A,x]	4.45(0.41) [b,A,X]
8	4.64(0.41) [a,A,x]	5.32(0.41) [a,A,X]	4.93(0.41) [a,A,x]	4.91(0.41) [b,A,X]
16	4.32(0.41) [a,A,x]	6.17(0.41) [a,B,Y]	4.03(0.41) [a,A,x]	4.23(0.41) [b,A,X]
24	4.57(0.41) [a,A,x]	6.44(0.41) [a,B,Y]	3.89(0.41) [a,A,x]	3.84(0.41) [b,A,X]
32	5.08(0.41) [a,A,x]	6.27(0.41) [a,B,Y]	4.00(0.41) [a,B,x]	2.67(0.41) [a,A,X]

[@]Least square means (standard error)

ab -Means with different superscripts indicate significant difference ($p<0.05$) within a column

AB - Means with different superscripts indicate significant difference ($p<0.05$) at 4 and 25 °C within a marinade

xy - Means with different superscripts indicate significant difference ($p<0.05$) between the two marinades at 4 °C for each time interval

XY - Means with different superscripts indicate significant difference ($p<0.05$) between the two marinades at 25 °C for each time interval

Table 3: Survival populations (log_{10} CFU/gm) of 10^4 CFU/ml of *Salmonella* on chicken skin marinated with commercial marinades stored at refrigerated (4 °C) and room temperature (25 °C)

Time	Lemon Pepper Marinade		Teriyaki Marinade	
(h)	4 °C	25 °C	4 °C	25 °C
0	1.74(0.51) ^{a,A,x}	2.31(0.51) ^{a,A,X}	2.11(0.51) ^{a,A,x}	1.12(0.51) ^{a,A,X}
4	2.39(0.51) ^{a,A,x}	2.60(0.51) ^{a,A,Y}	1.92(0.51) ^{a,A,x}	1.17(0.51) ^{a,A,X}
8	2.61(0.51) ^{a,A,x}	3.03(0.51) ^{a,A,Y}	1.63(0.51) ^{a,A,x}	1.61(0.51) ^{a,A,X}
16	3.02(0.51) ^{a,A,x}	3.77(0.51) ^{a,A,Y}	1.78(0.51) ^{a,A,x}	1.50(0.51) ^{a,A,X}
24	1.70(0.51) ^{a,A,x}	3.18(0.51) ^{a,B,Y}	1.80(0.51) ^{a,A,x}	1.60(0.51) ^{a,A,X}
32	2.20(0.51) ^{a,A,x}	3.71(0.51) ^{a,B,Y}	1.41(0.51) ^{a,A,x}	1.67(0.51) ^{a,A,X}

$^{@}$Least square means (standard error)

ab -Means with different superscripts indicate significant difference ($p<0.05$) within a column

AB - Means with different superscripts indicate significant difference ($p<0.05$) at 4 and 25 °C within a marinade

xy - Means with different superscripts indicate significant difference ($p<0.05$) between the two marinades at 4 °C for each time interval

XY - Means with different superscripts indicate significant difference ($p<0.05$) between the two marinades at 25 °C for each time interval

Figure Legends

Figure 1A: % Prevalence of *S.* Heidelberg 10^1 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 4 °C ■-Lemon Pepper □Teriyaki Marinade

Figure 1B: % Prevalence of *S.* Heidelberg 10^2 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 4 °C ■- Lemon Pepper □Teriyaki Marinade

Figure 1C: % Prevalence of *S.* Heidelberg 10^3 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 4 °C ■- Lemon Pepper □Teriyaki Marinade

Figure 1D: % Prevalence of *S.* Heidelberg 10^4 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 4 °C ■ - Lemon Pepper □ - Teriyaki Marinade

Figure 2A: % Prevalence of *S.* Heidelberg 10^1 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 25 °C ■- Lemon Pepper □- Teriyaki Marinade

Figure 2B: % Prevalence of *S.* Heidelberg 10^2 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 25 °C ■-Lemon Pepper □Teriyaki Marinade

Figure 2C: % Prevalence of *S.* Heidelberg 10^3 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 25 °C ■- Lemon Pepper □- Teriyaki Marinade

Figure 2D: % Prevalence of *S.* Heidelberg 10^4 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 25 °C ■ - Lemon Pepper □ -Teriyaki Marinade

Figure 3A: % Prevalence of *S.* Typhimurium 10^1 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 4 °C ■- Lemon Pepper □- Teriyaki Marinade

Figure 3B: % Prevalence of *S.* Typhimurium 10^2 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 4 °C ■- Lemon Pepper □ Teriyaki Marinade

Figure 3C: % Prevalence of *S.* Typhimurium 10^3 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 4 °C ■- Lemon Pepper □ - Teriyaki Marinade

Figure 3D: % Prevalence of *S.* Typhimurium 10^4 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 4 °C ■ - Lemon Pepper □ -Teriyaki Marinade

Figure 4A: % Prevalence of *S.* Typhimurium 10^1 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 25 °C ■ - Lemon Pepper □ - Teriyaki Marinade

Figure 4B: % Prevalence of *S.* Typhimurium 10^2 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 25 °C ■ - Lemon Pepper □ - Teriyaki Marinade

Figure 4C: % Prevalence of *S.* Typhimurium 10^3 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 25 °C ■ - Lemon Pepper □ - Teriyaki Marinade

Figure 4D: % Prevalence of *S.* Typhimurium 10^4 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 25 °C ■ - Lemon Pepper □ - Teriyaki Marinade

Figure 5A: % Prevalence of *S.* Senftenberg 10^1 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 4 °C ■-Lemon Pepper □-Teriyaki Marinade

Figure 5B: % Prevalence of *S.* Senftenberg 10^2 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 4 °C ■-Lemon Pepper □-Teriyaki Marinade

Figure 5C: % Prevalence of *S.* Senftenberg 10^3 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 4 °C ■- Lemon Pepper □Teriyaki Marinade

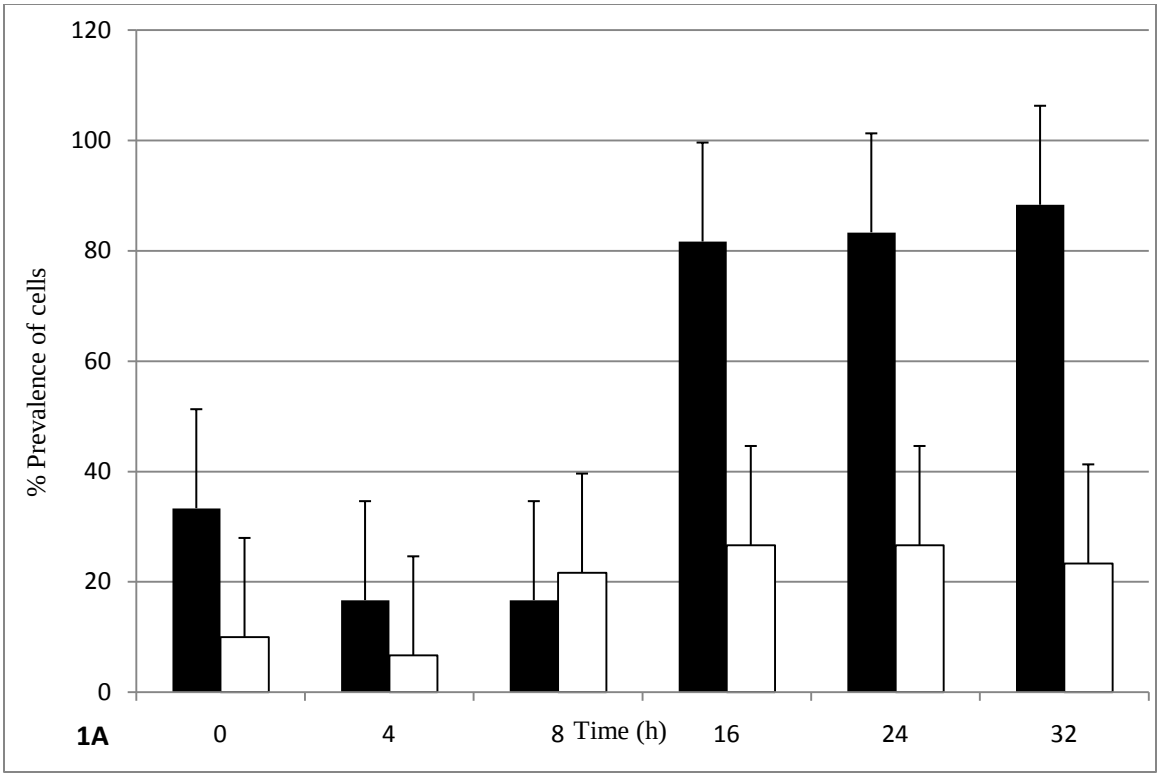
Figure 5D: % Prevalence of *S.* Senftenberg 10^4 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 4 °C ■ Lemon Pepper □Teriyaki Marinade

Figure 6A: % Prevalence of *S.* Senftenberg 10^1 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 25 °C ■ - Lemon Pepper □ -Teriyaki Marinade

Figure 6B: % Prevalence of *S.* Senftenberg 10^2 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 25 °C ■ - Lemon Pepper □ - Teriyaki Marinade

Figure 6C: % Prevalence of *S.* Senftenberg 10^3 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 25 °C ■ - Lemon Pepper □ - Teriyaki Marinade

Figure 6D: % Prevalence of *S.* Senftenberg 10^4 CFU/gm on chicken skin marinated in Lemon pepper and Teriyaki marinades at 25 °C ■ - Lemon Pepper □-Teriyaki Marinade



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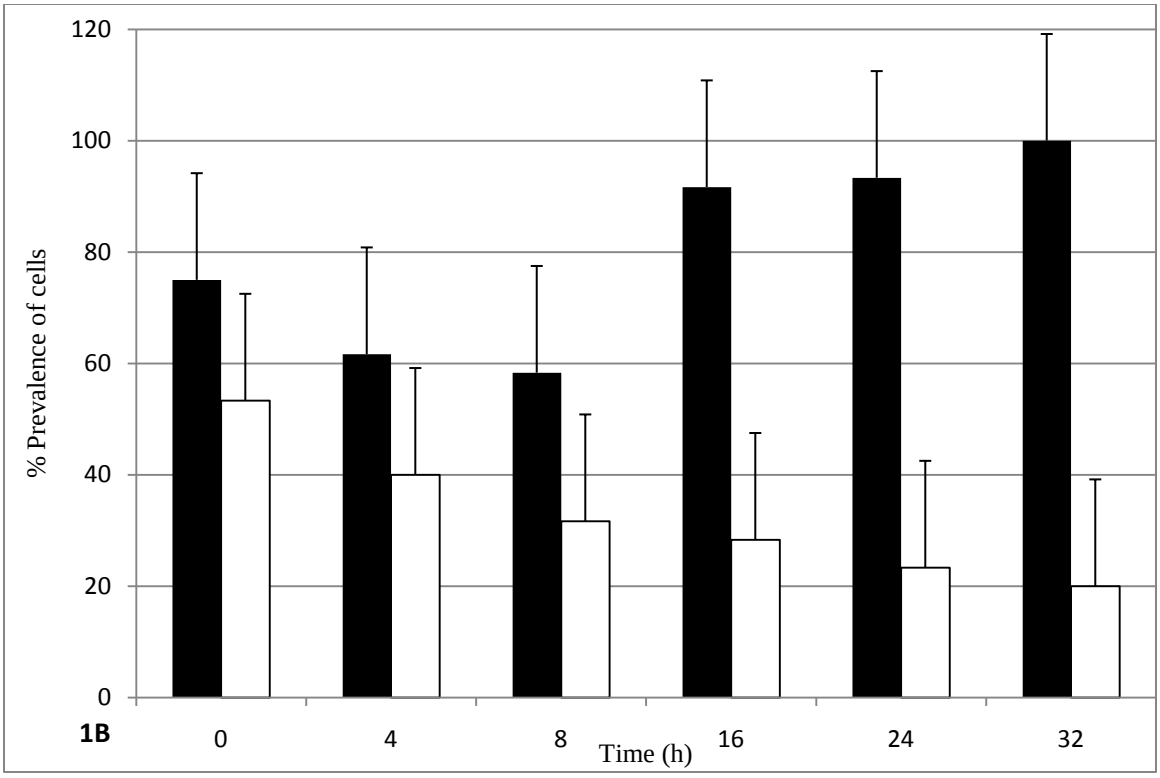

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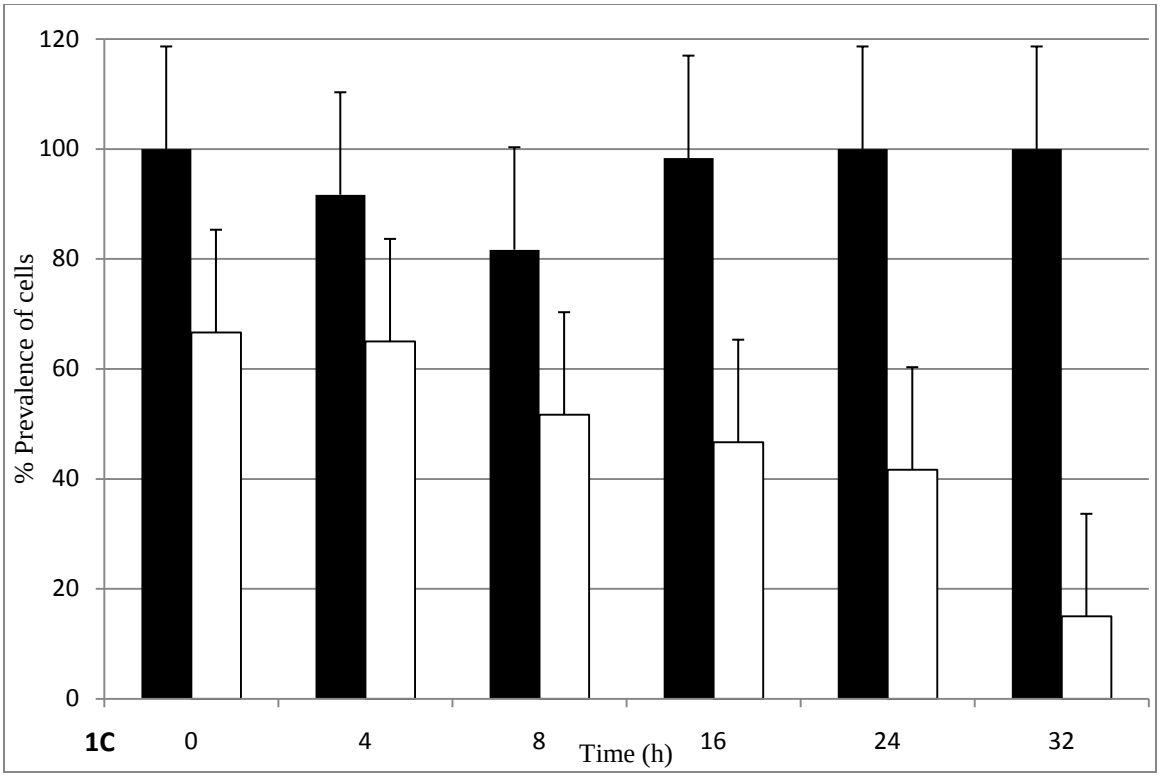
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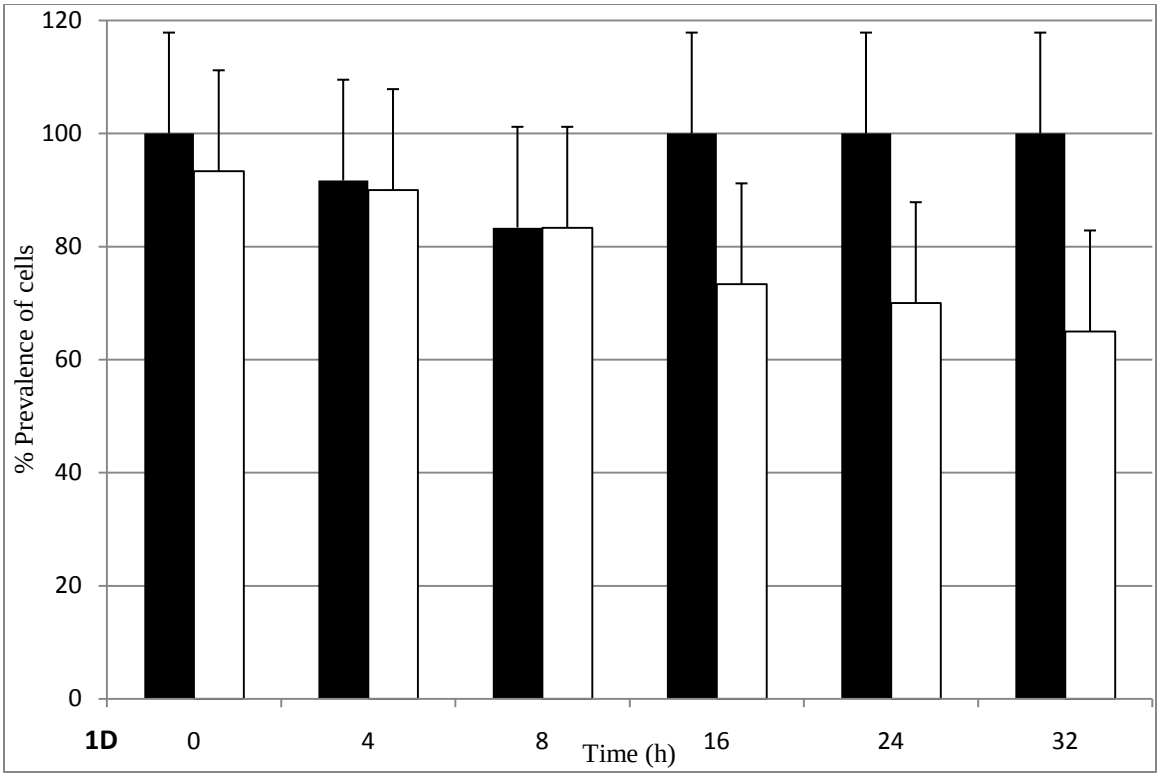
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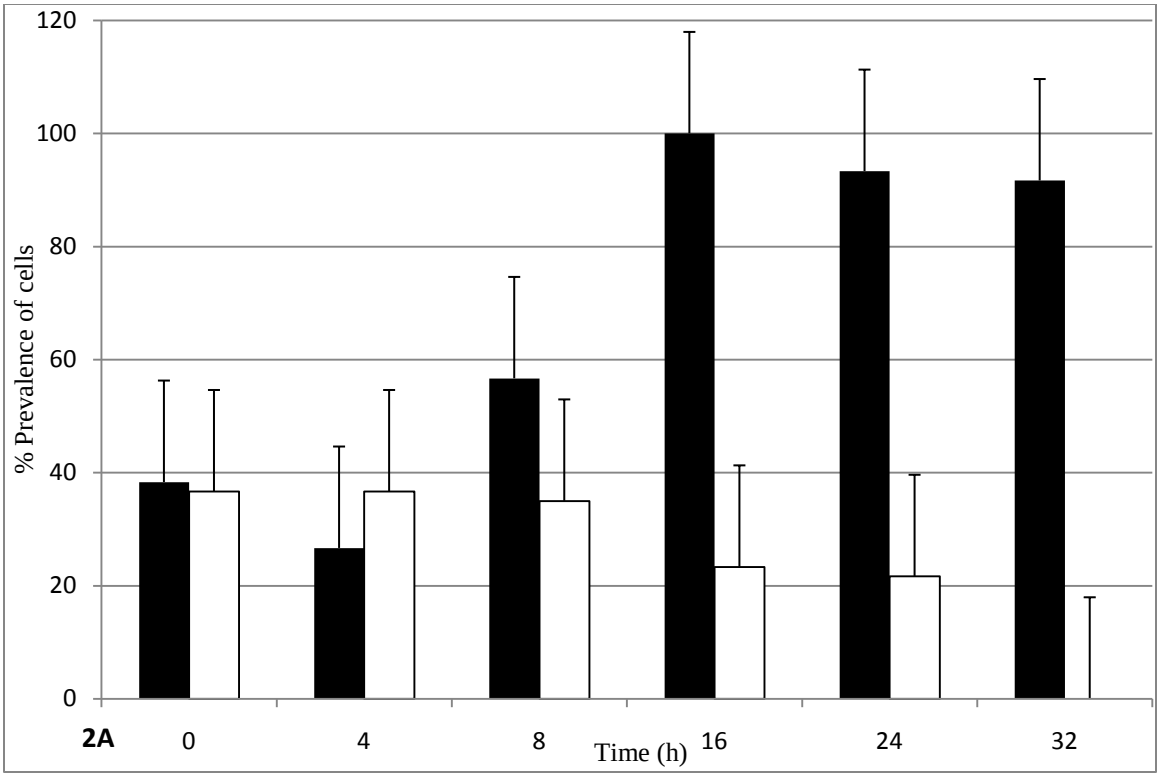
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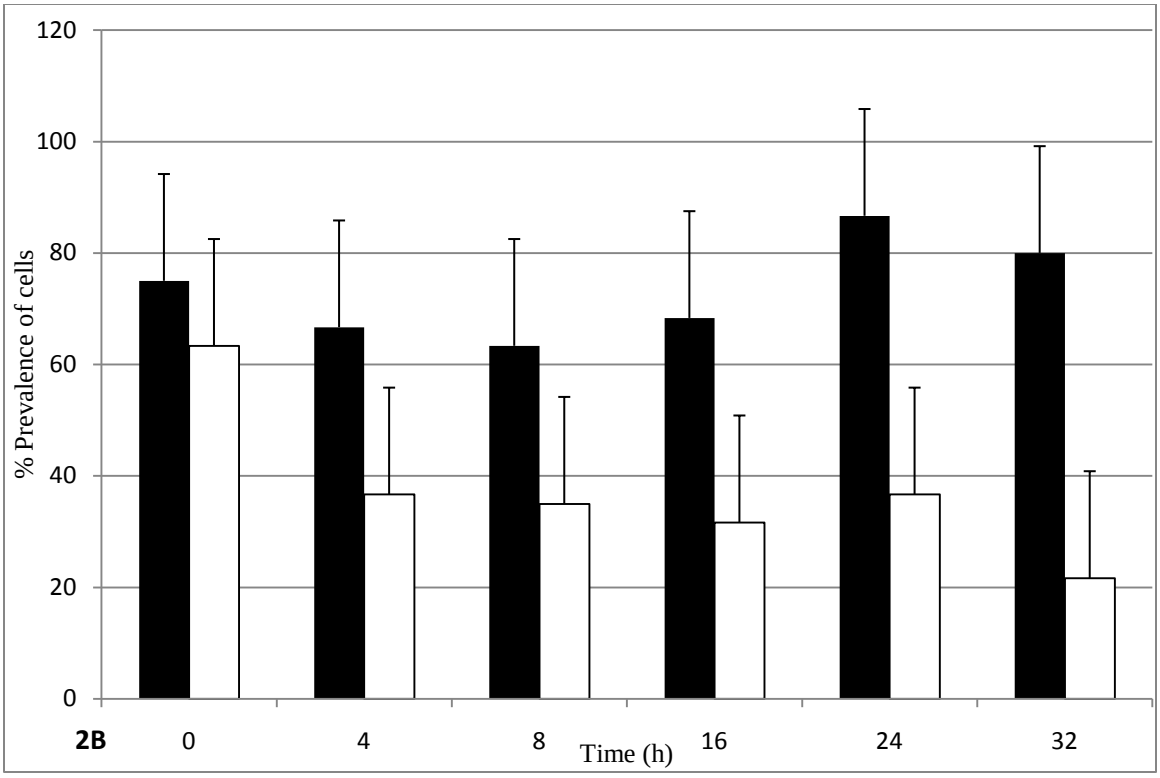
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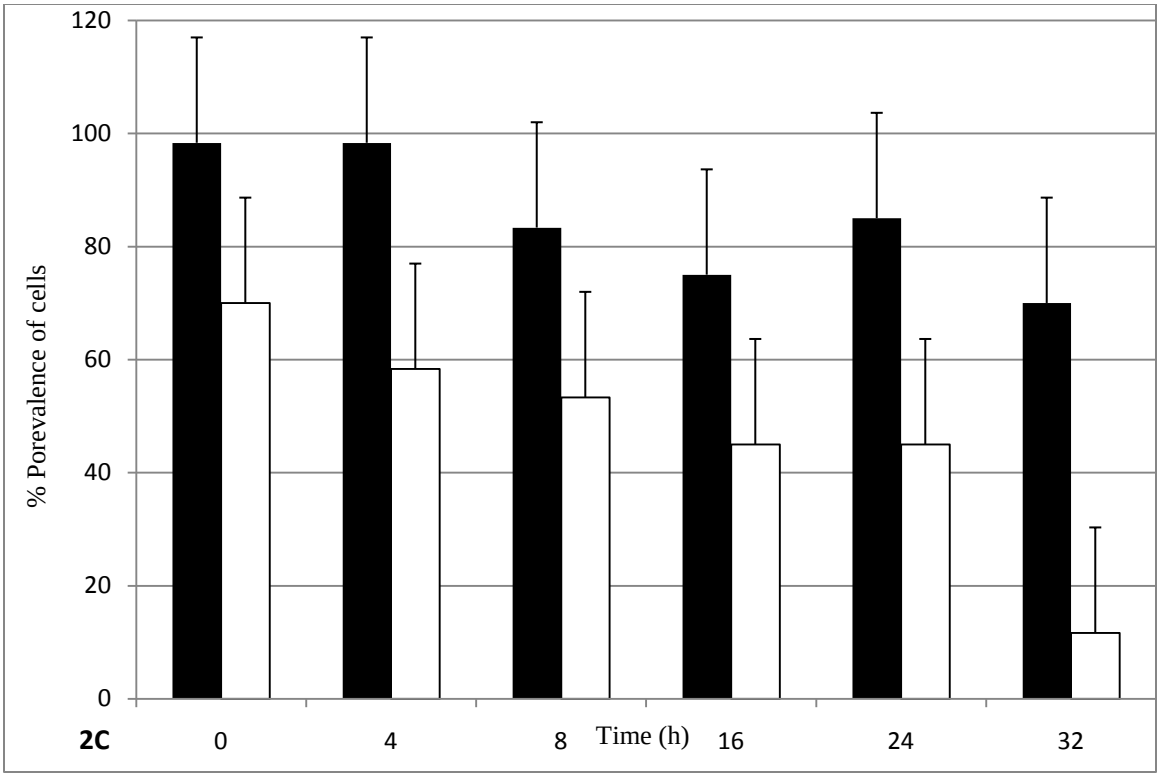
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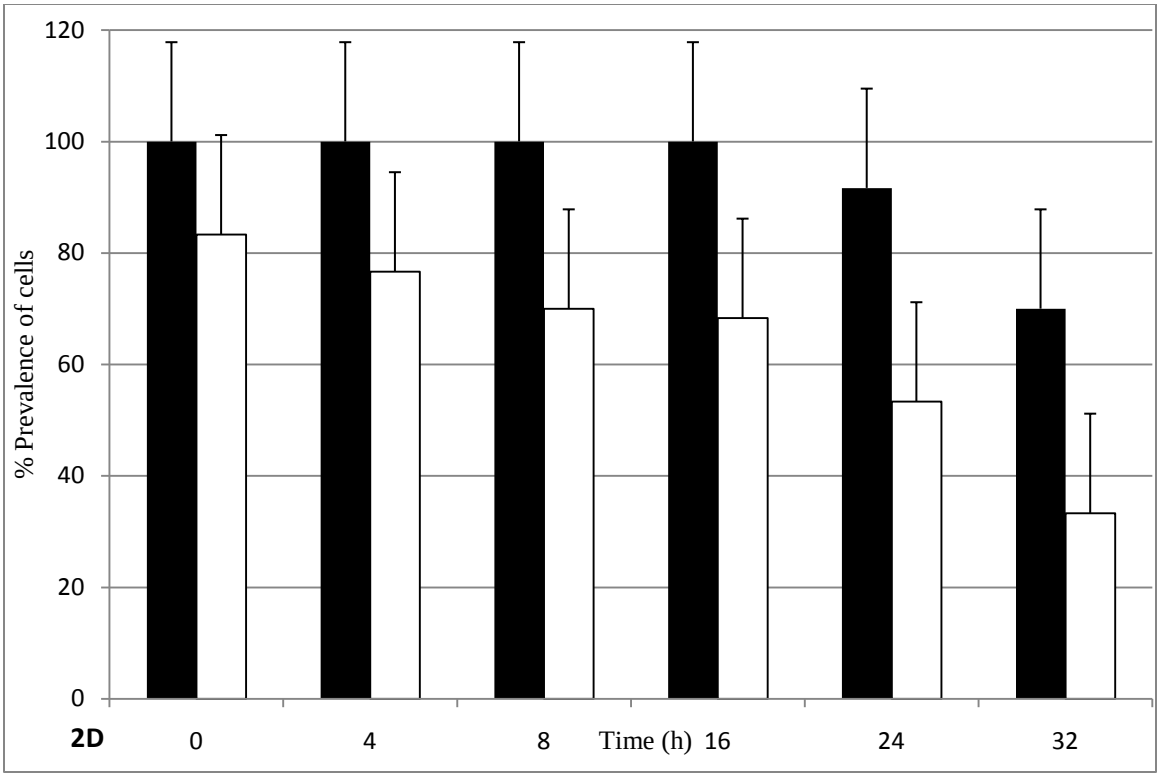
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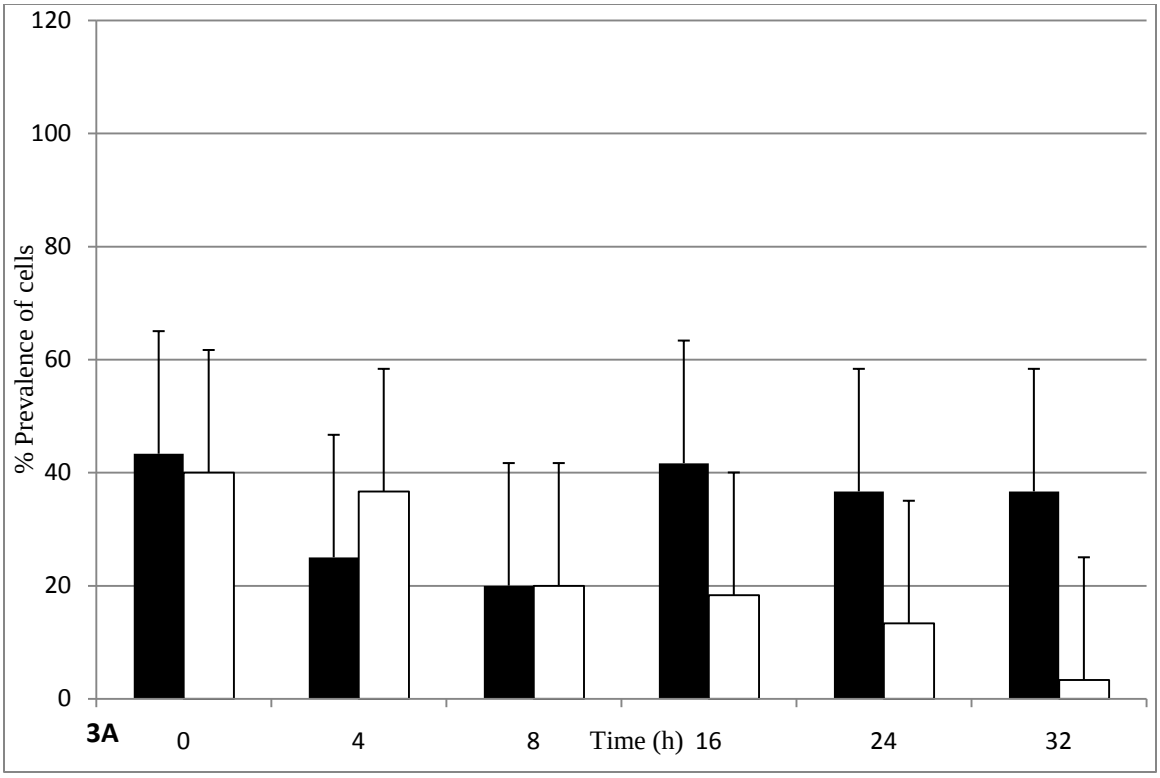
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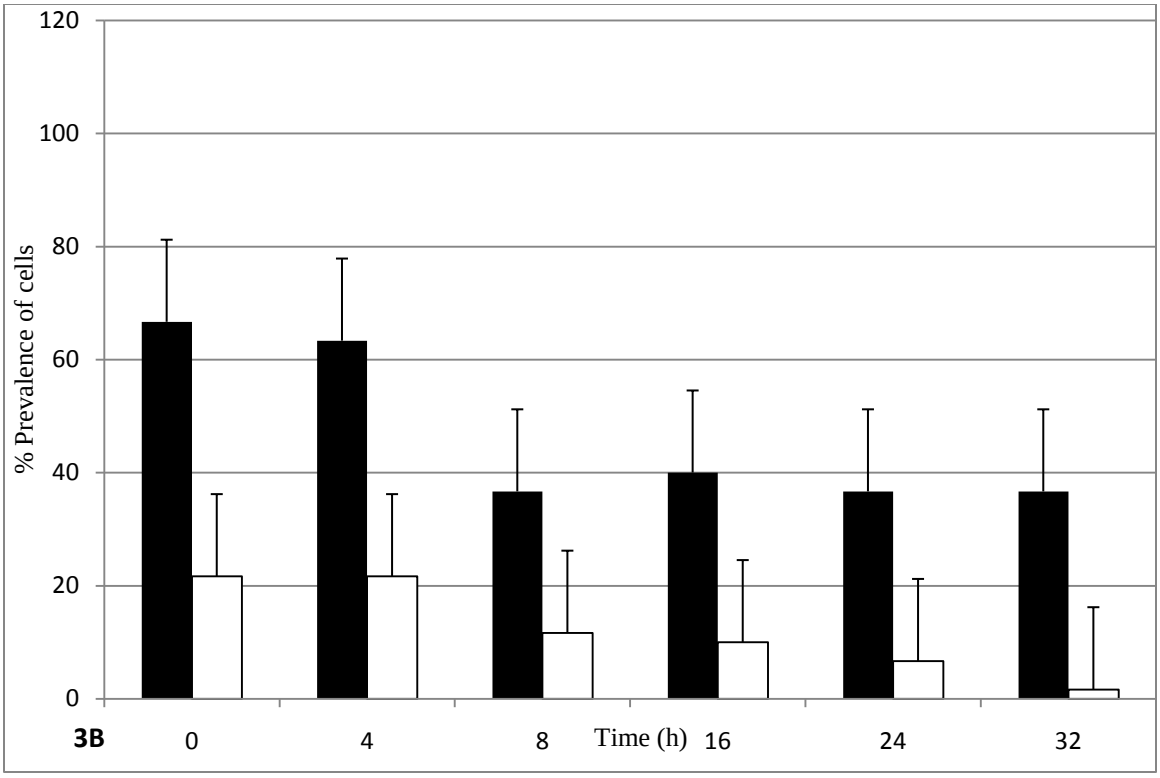
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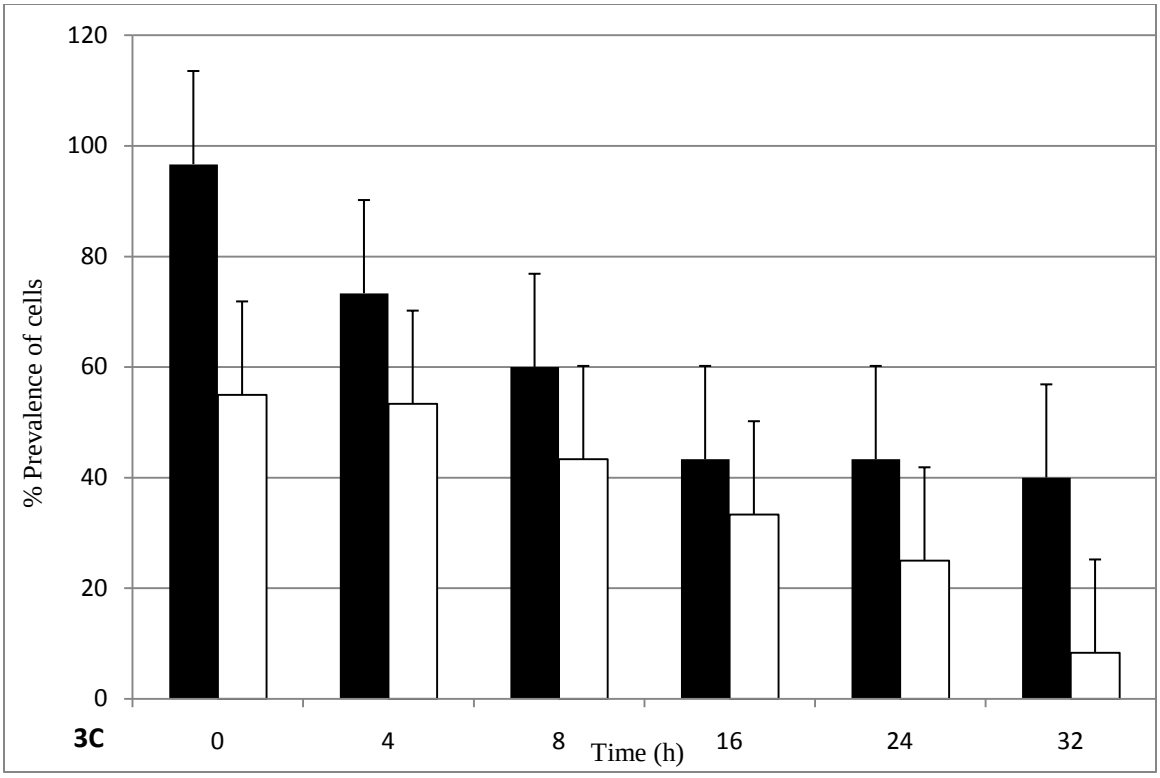
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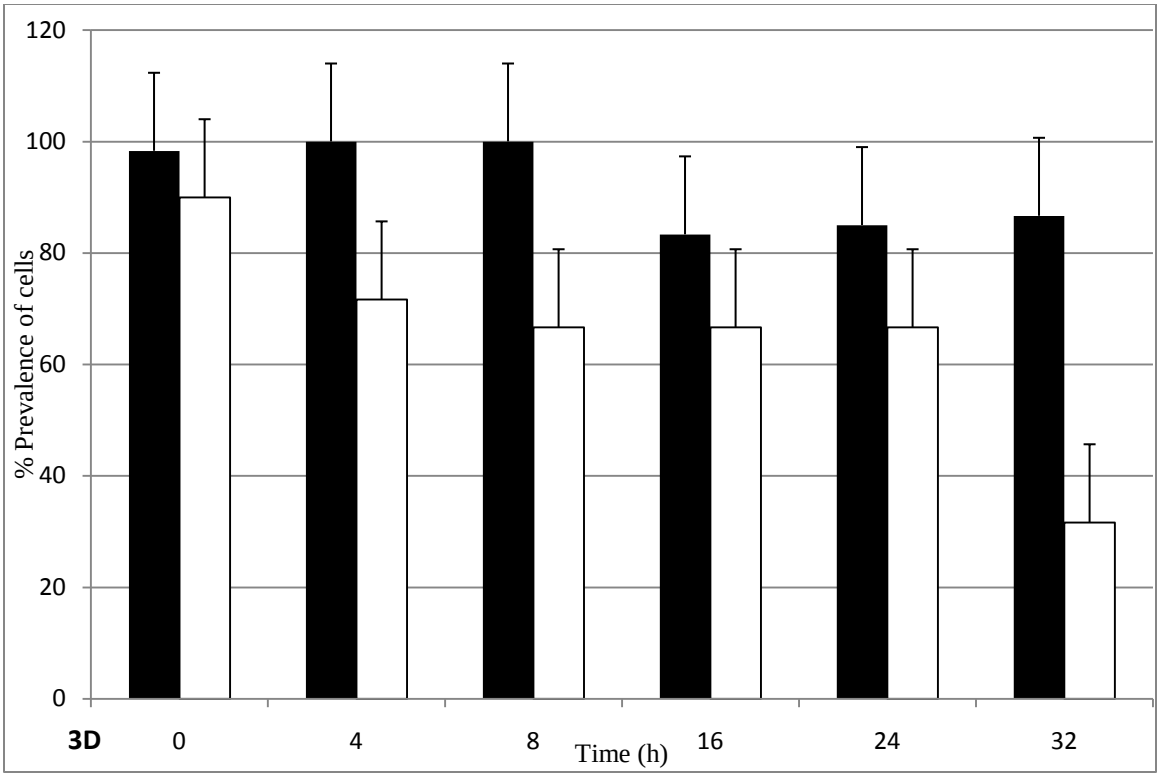
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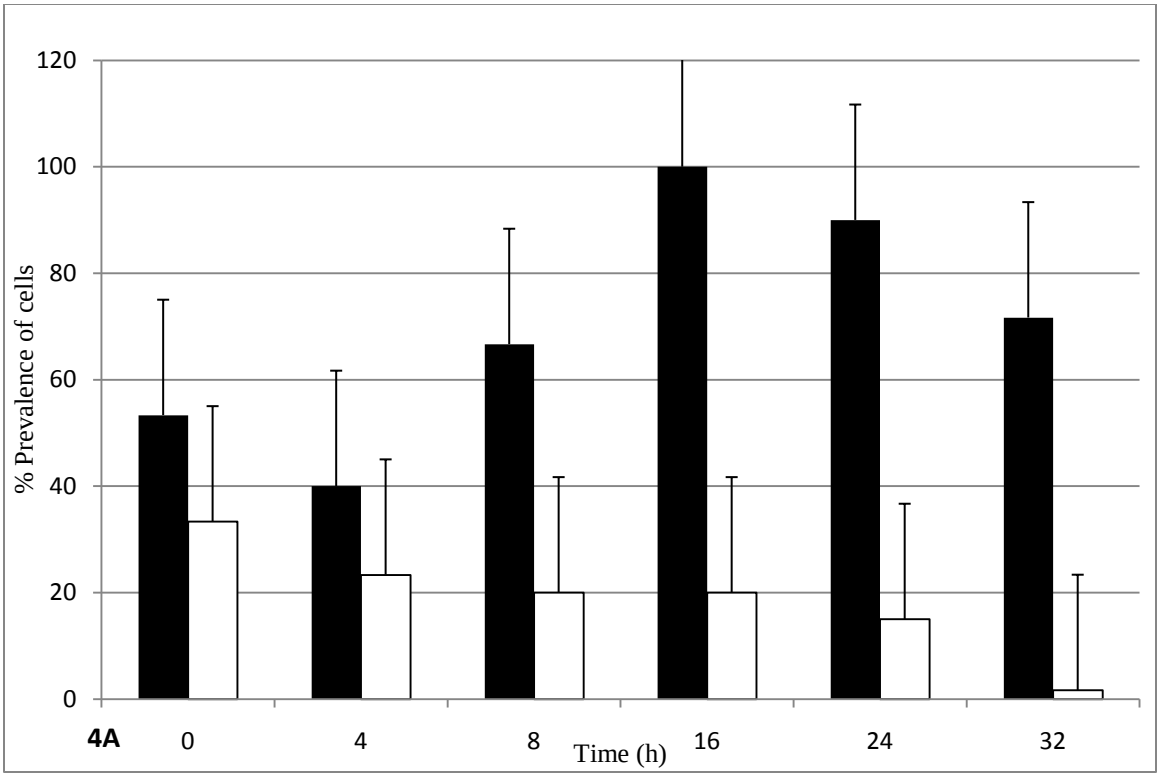
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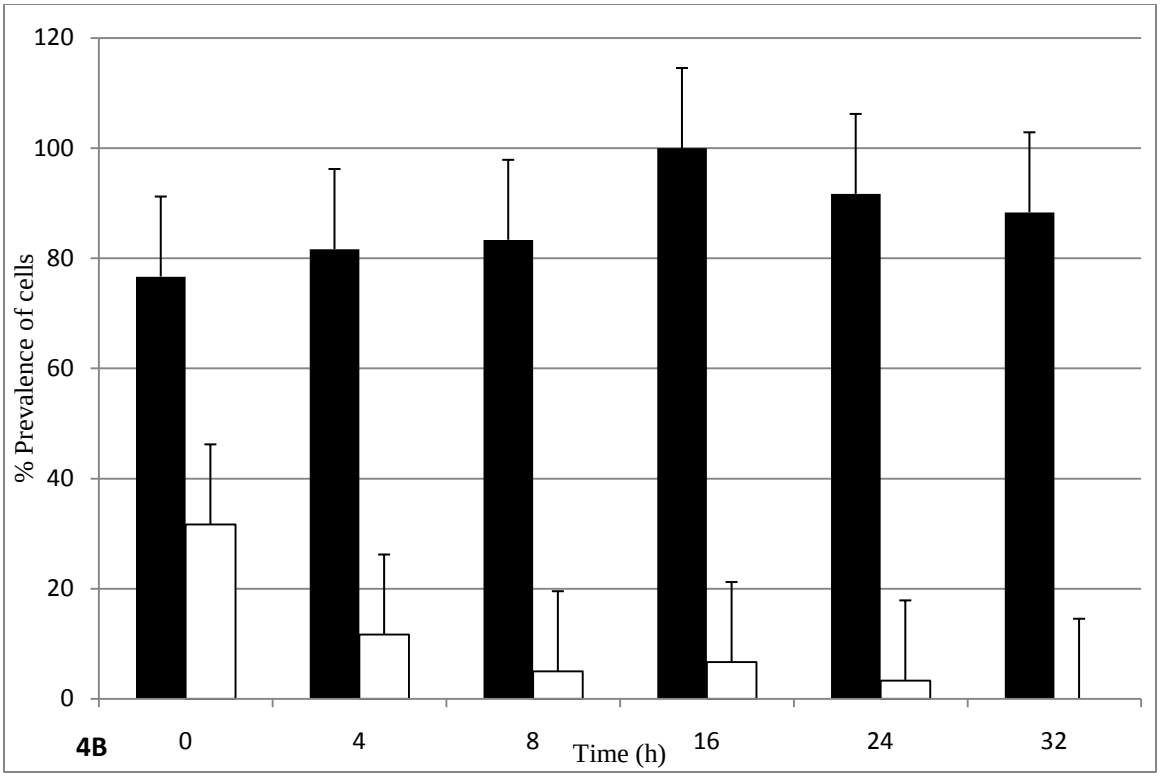

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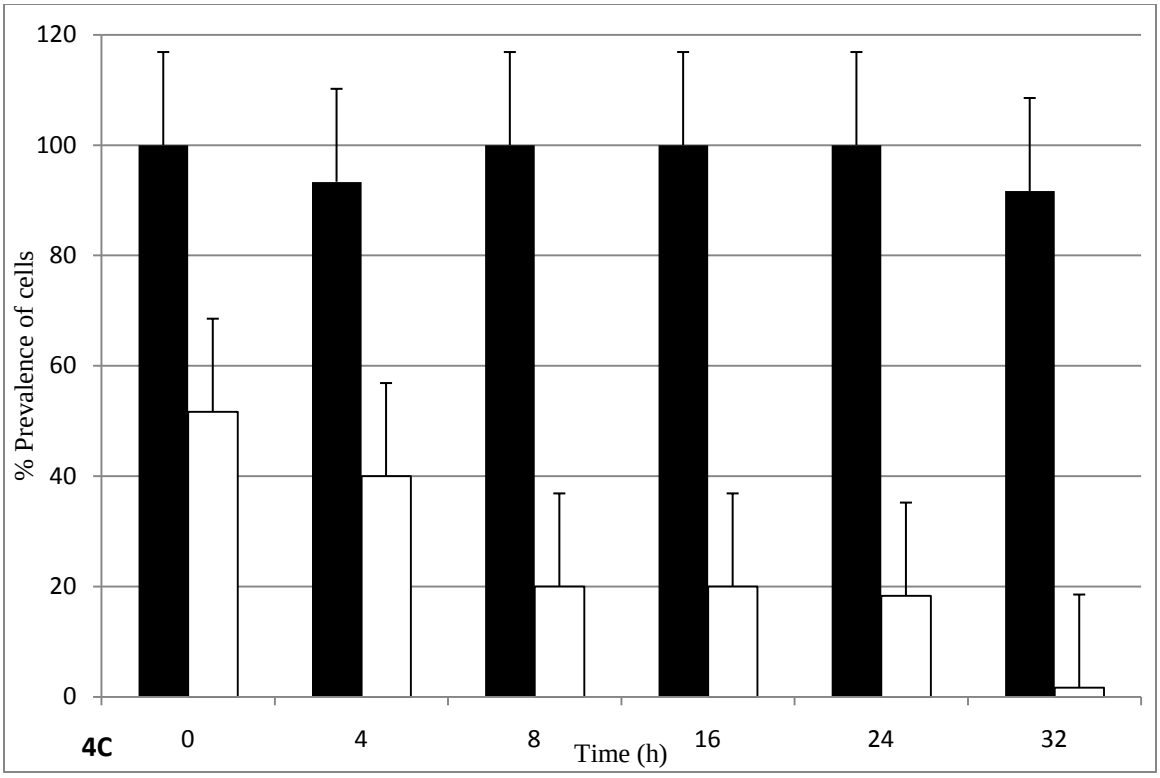
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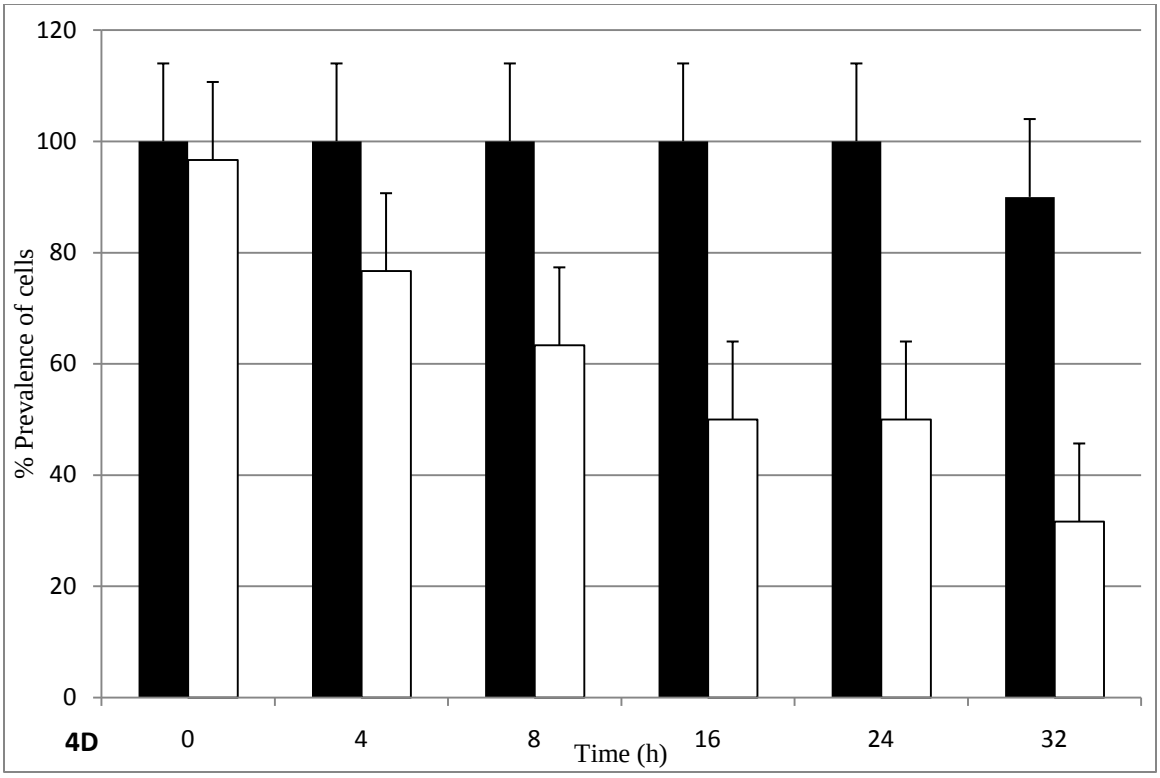
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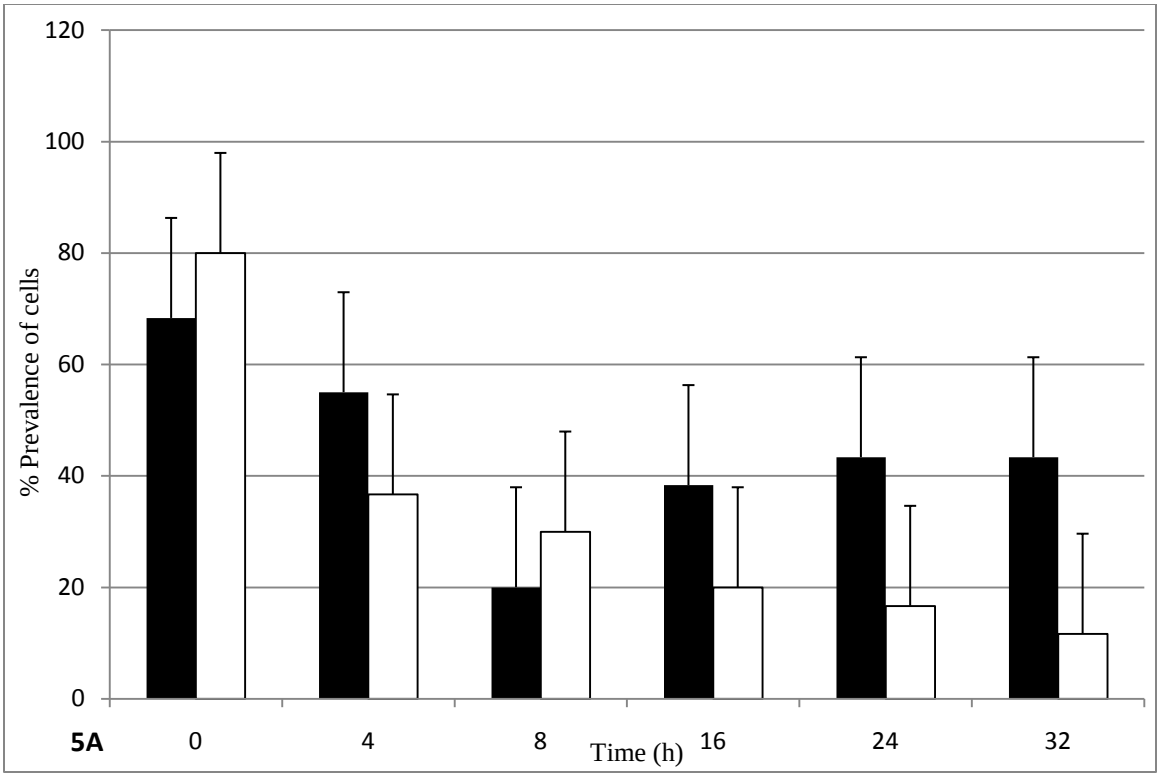

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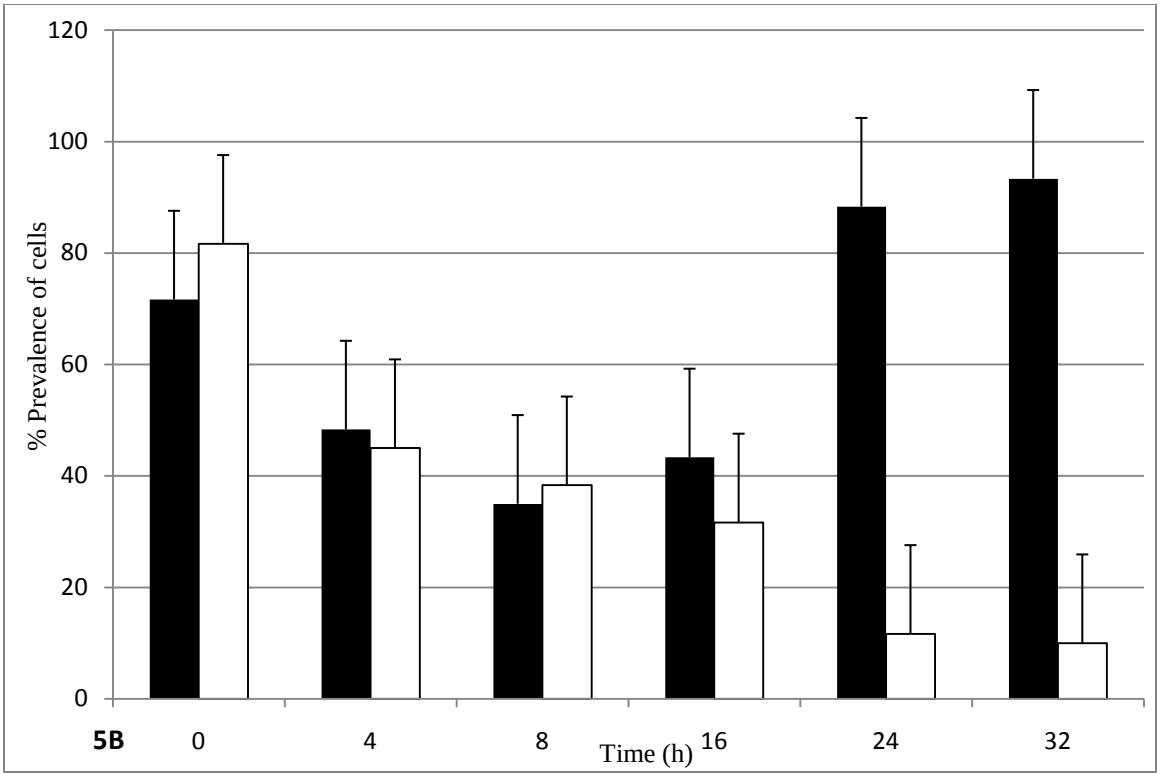
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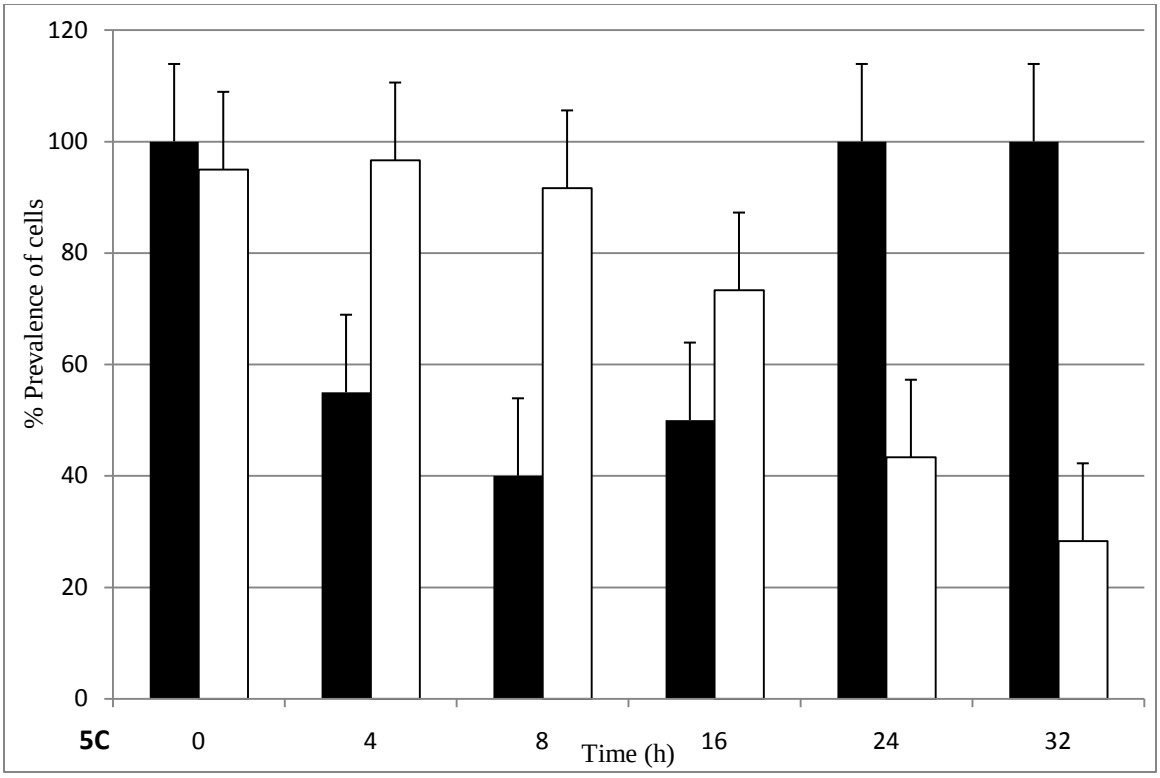
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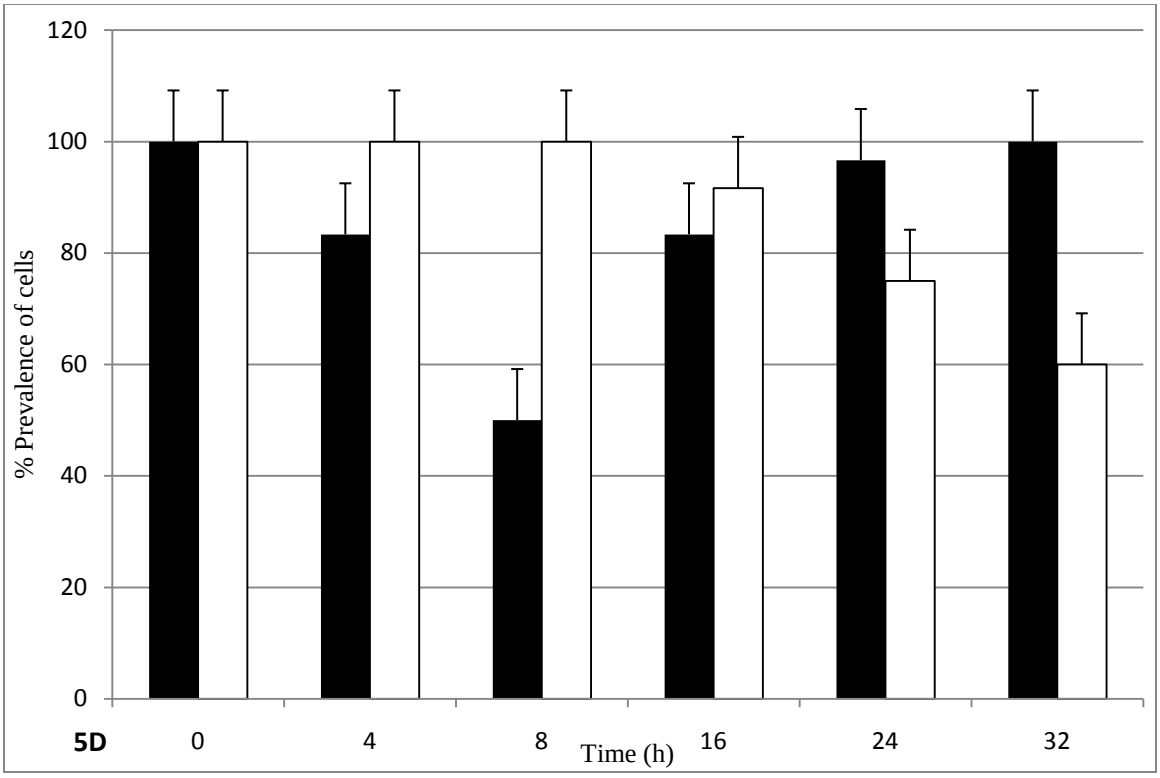
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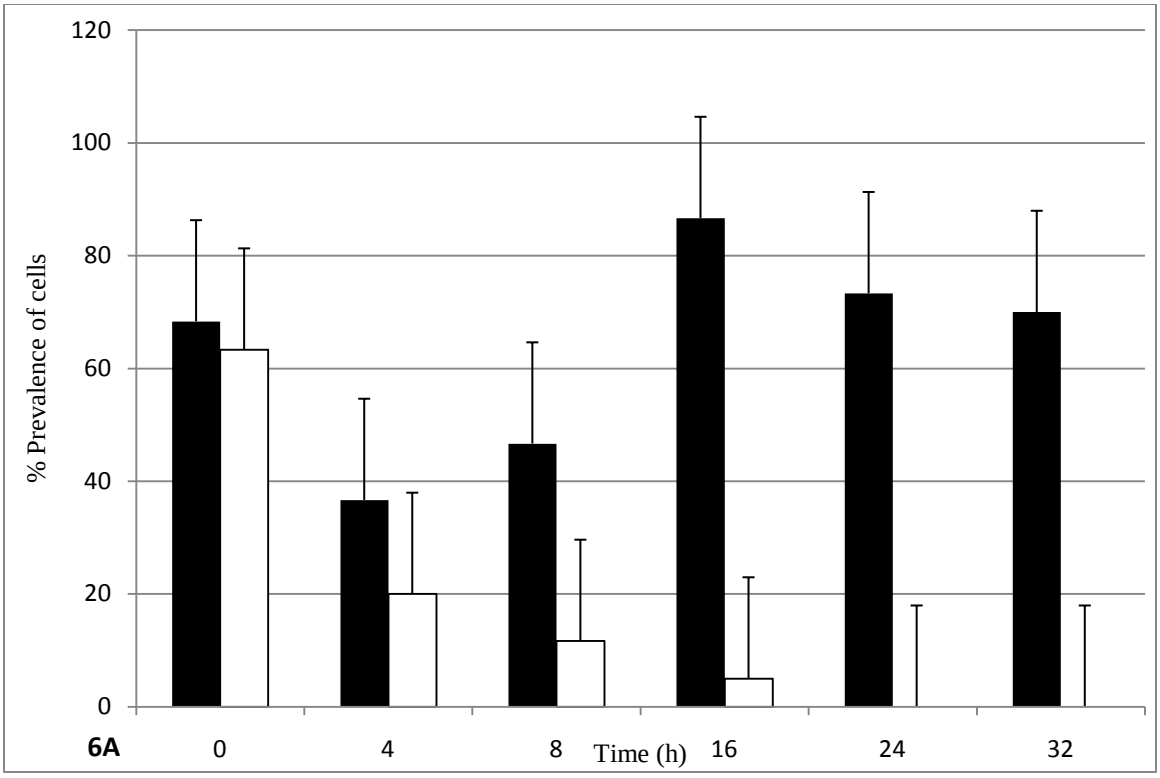

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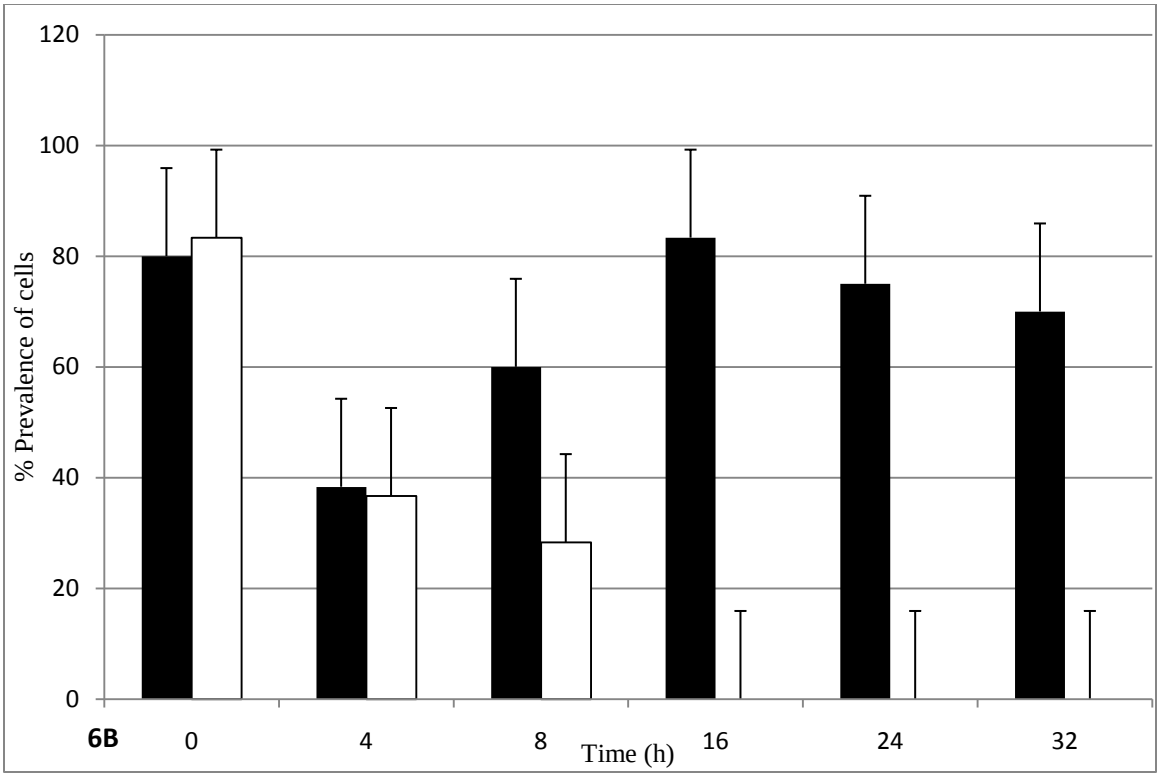
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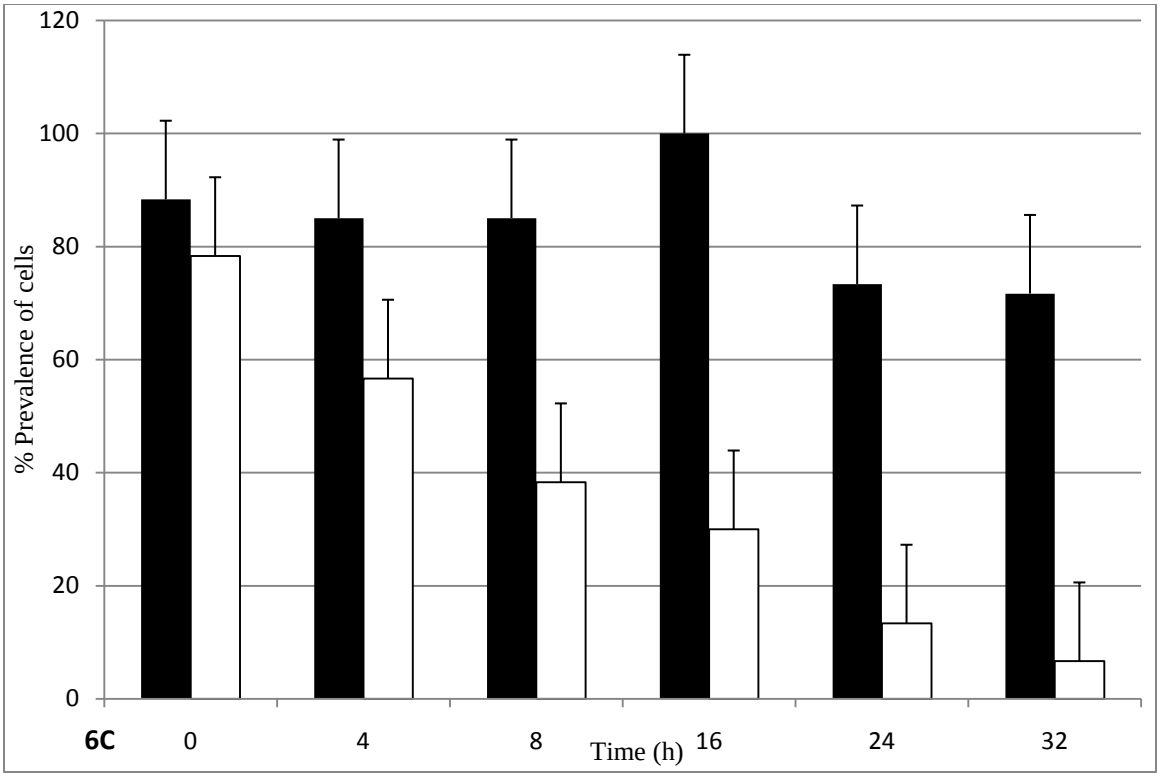
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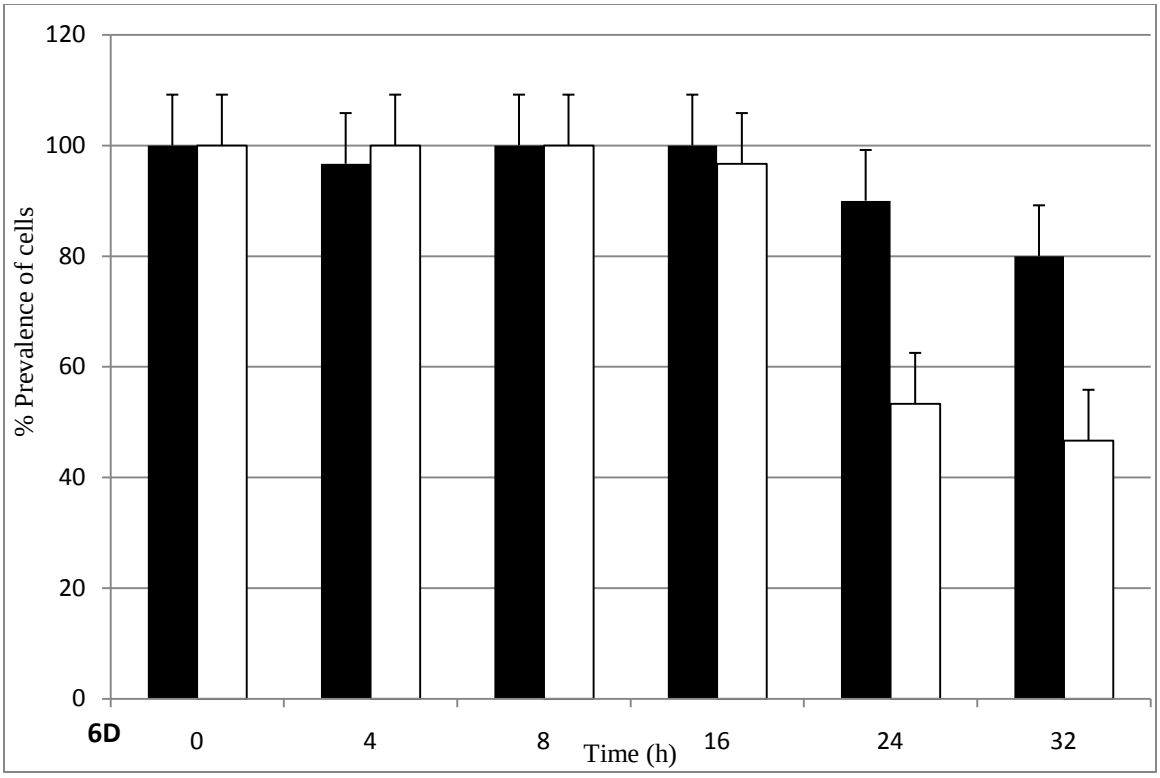
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5 - CONCLUSION

Significant advances have been made in food preservation techniques but microbial contamination during handling, storage, distribution and preparation of poultry products cannot be totally eliminated. The food industry is continuously making efforts to research for newer methods of food preservation which are natural, economical and a consumer acceptable. One such technique that aims to maintain food safety, extend shelf-life of poultry products and is consumer-friendly is marination. Marinades add flavor, maintain tenderness and juiciness of meats during cooking and also have antimicrobial properties.

The goal of the experiments performed was to determine the efficacy of commercial Teriyaki and Lemon pepper marinade in reducing the prevalence of three different *Salmonella* species on chicken skin after immersion marination for 32 h. The marinades proved to be effective antimicrobial systems in reducing the viable numbers of bacteria. Teriyaki marinade had significant ($p<0.05$) bactericidal effects and Lemon pepper marinade had bacteristatic effects in reducing the microorganism tested in the research. There was no significant ($p>0.05$) difference observed in the efficacy of the marinades at refrigeration and room temperature. This is an important observation as products marinated at abuse temperature will still be microbiologically safe. Further, heat treatment of the marinated product during cooking will further inactivate the bacterial cells and reduce the risk of foodborne illness.

The results indicate that these marinades can be used as natural antimicrobial additives in food processing for value-addition, enhancing food safety and quality of poultry products and also consumer acceptability. The microbial contamination of raw chicken can be reduced with good manufacturing practices and further reduction in numbers can be achieved by marinade treatments which also enhance the sensory attributes of the product.

6 – SCOPE FOR FURTHER STUDIES

Industrial injection marination has become popular and marinated meat and poultry products are being increasingly consumed. It is of interest to quantify the antibacterial properties of spices and herbs in marinades and study their synergistic effects with thermal (cooking) and non-thermal (irradiation, UV rays, ultrasound) processes. Cooking the meat products to an internal temperature of 165 °F remains the primary means of eliminating foodborne pathogens from poultry and other meat and ground muscle foods. Significant reduction in pathogens can be achieved by combining marination along with cooking. The spices and herbs have bacteristatic or bactericidal effects on the bacterial cells which can be inactivated to harmless numbers by cooking the product further. Studies have been done to determine the efficacy of herbal extracts added to chiller water on the microorganisms on broiler carcasses (Dickens. et al, 2000). This can prevent the discoloration of carcass skin on exposure to the chlorine in the chillers.

Marinated meat and poultry products can be contaminated due to im-proper handling during processing. Marination can be used in synergism with irradiation and ultra-violet radiation to reduce the contamination. High power ultrasound waves with low frequencies of 20 to 100 kHz are capable of disrupting living cells (Jayasooriya et al, 2004). Application of ultrasound treatment to pork medallions marinated in red wine has shown antibacterial effects on *Listeria* and *Campylobacter* (Birk and Knochel, 2009).

Numerous *in vitro* studies have been conducted on the antimicrobial activities of essential oils, herbs, spices and their extracts. Food application studies are needed to determine their

effective concentrations as concentrations effective in culture media may not be sufficient enough to cause the same activity in the foods systems. Sensory evaluation studies of the marinated products can be conducted to evaluate the flavor attributes of the marinades. The only limitation of using marinades for food preservation may be that higher concentrations are needed to inhibit the spoilage bacteria which can result in off flavors or unacceptable flavors. Care should be taken in preparing marinade solutions with strict hygienic measures to prevent cross-contamination and risk of food-borne illness. Exploiting antibacterial characteristics of marination with ingredients commonly used could help to optimize consumer friendly pathogen reduction strategies.

6.1 - References

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