EFFECTS OF SHORT-CHAIN NITROCOMPOUNDS AGAINST *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI IN VITRO*

A Thesis

by

SHANE MICHAEL HORROCKS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2006

Major Subject: Nutrition

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Approved by:

Co-Chairs of Committee,	Robin C. Anderson
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ABSTRACT

Effects of Short-chain Nitrocompounds against *Campylobacter jejuni* and *Campylobacter coli in vitro*. (December 2006)

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Campylobacter is an important human pathogen that colonizes the gut of food producing animals. In this study, the effects of 2-nitro-1-propanol, 2-nitroethanol, nitroethane, and 2-nitro-methyl-propionate (0, 10, and 20 mM) on growth of *Campylobacter jejuni* were tested during culture in Bolton Broth adjusted to pH 5.6, 7.0, or 8.2. The effects of the nitrocompounds were also tested against *C. coli* in Bolton Broth but adjusted to pH 8.2 only. Viable cell counts of samples taken at intervals during incubation revealed main effects ($P < 0.05$) of nitroethane, 2-nitro-1-propanol, 2-nitroethanol, and 2-nitro-methyl-propionate as evidence by reduced survivability of *C. jejuni*. A marked effect of pH on the survivability of *C. jejuni* during incubation with all compounds was observed, with greater activity observed at pH 8.2 than at pH 5.6 or 7.0 for nitroethane, 2-nitro-1-propanol, 2-nitroethanol, but not for 2-nitro-methyl-propionate. In the case of 2-nitro-methyl-propionate, survivability of *C. jejuni* was reduced most at pH 5.6. Except for 2-nitro-methyl-propionate, which was ineffective, all nitrocompounds elicited similar effects on *C. coli* when cultured at pH 8.2. The effect of nitroethane and 2-nitro-1-propanol (10 mM) on naturally-occurring *Campylobacter* was further investigated during incubation of a porcine fecal suspension. *Campylobacter*

concentrations decreased more rapidly ($P < 0.05$) during incubation of porcine fecal suspensions supplemented with 2-nitro-1-propanol than unsupplemented control suspensions or suspensions supplemented with nitroethane thus reiterating the superior inhibitory effect of 2-nitro-1-propanol.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

FIGURE Page

INTRODUCTION AND LITERATURE REVIEW

Introduction

Campylobacteria are now considered major foodborne pathogens around the world with an extremely prevalent nature existing in nearly all living animals. These well adapted commensal organisms have evolved to survive within the digestive tracts of warm blooded hosts and are particularly well suited to colonize the gut of avian species, perhaps due to their higher internal resting temperature. Thermophilic *Campylobacter* spp., those species capable of growth at higher temperatures, as well as other *Campylobacter* spp., are able to manifest symptoms such as abdominal cramps, diarrhea, severe bloody stools, headaches, fever, nausea, and acute arthritis. Campylobacteria are small, corkscrew or spherical shaped, flagellated or unflagellated gram negative rods ranging from 0.5 to 5μm in length. *Campylobacter* spp. are capable of surviving on countertops for several days, however, optimal environmental conditions include a microaerophilic (5% O_2, 10% CO_2, and 85% N_2) atmosphere ranging between 37°C - 42°C, with 42°C being optimal for *C. jejuni* and *C. coli* species. For these reasons, campylobacteria are poor competitors with other aerotolerant bacteria such as *Escherichia coli* and *Salmonella* spp. which may effectively survive on household

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products at room temperature. However transmission of *Campylobacter* in kitchens during food preparation has been observed (Luber and others 2006).

Since its emergence as a foodborne pathogen in the 1970's, *Campylobacter* spp. have been estimated to be the most common causative agent of foodborne illnesses, followed by nontyphoidal *Salmonella* and *Shigella* spp. (Mead and others 1999). Of the total estimated 5.2 million bacterial foodborne illnesses, approximately 2.4 million of these cases have been attributed to *Campylobacter jejuni* infections, with 80% being foodborne transmitted and responsible for approximately 7.6% of the total deaths due to bacterial foodborne illnesses (Mead and others 1999). The prevalence of *C. jejuni* has attracted considerable research interest because of the post-infection traumatic risks of acquiring immune-mediated neuropathies such as Guillian Barré Syndrome, or Miller Fisher Syndrome in addition to being associated with acute cases of bacterial diarrhea worldwide (Ang and others 2001; Jacobs and others 1998; Rees and others 1995). More recent studies suggest that *C. jejuni* infections may potentially lead to inflammatory bowel diseases such as Crohn's Disease (Lamhonwah and others 2005).

Campylobacter prevalence has been increasing annually and is now considered as the number one foodborne pathogen. The most widespread and problematic *Campylobacter* species isolated from humans involved as the causative agent in acute gastroenteritis is *C. jejuni*. Ninety-nine percent of *Campylobacter* infections are caused by *C. jejuni*, leaving just 1% of *Campylobacter* infections to all other *Campylobacter* species (CDC 2005). However, *Campylobacter coli* is now accepted as the second most prevalent species and is estimated to have been responsible for approximately 26,000

cases of intestinal inflammatory responses in 2000 (Gillespie and others 2002; Tam and others 2003).

Recently, novel and more sensitive detection methods in microbiology have allowed scientists to more accurately detect, isolate and classify *Campylobacter* spp. as a major foodborne pathogen to humans. Recent advances in surveillance have provided improved information on the prevalence of *Campylobacter* spp. worldwide and now demonstrate that this pathogen can be interspecies specific rather than as once thought to be limited to warm blooded hosts.

Pre-harvest control of *Campylobacter* is severely limited to common cleaning and preparation practices within processing plants. Utilizing acid sprays, irradiation methods, chlorine and hot water rinses, as well as post chilling methods have only been effective in reducing the pathogen. Cross contamination from other infected carcasses promotes the survival of the organism into supermarket retail raw meats. Stronger and more effective on-farm, pre-harvest control methods with aid in the development of new sanitizing techniques in food preparation are an absolute necessity to ensure safer consumer products. Eliminating or significantly reducing *Campylobacter* on the farm, and increasing processing hygiene practices can be effective in decreasing *Campylobacter* prevalence within retail meat and vegetable products as well as reducing *Campylobacter* within environmental sewage and water reservoirs.

Prevalence

Campylobacter spp. naturally colonize the gastrointestinal tracts of domestic and feral animals (Jones, 2001) and are asymptomatic in most food production animals.

Campylobacter prevalence has been reported at 89% in ruminants (Stanley and Jones 2003) and more than 80% in swine (Pearce and others 2003) and poultry (Corry and Atabay 2001; Sahin and others 2002). According to a surveillance by The Foodborne Diseases Active Surveillance Network (FoodNet), the incidence of human *Campylobacter* infections accounted for more than 34% of the total laboratory confirmed cases in 2005. Although there has been an overall 30% decrease in the incidence of *Campylobacte*r infections since reported in 1998 (CDC 2006).

To date, Campylobacteria have been detected nearly everywhere from farm and urban environments to slaughter plants; isolated from humans, wild birds and mammals, companion animals, drinking water, farm production animals (predominantly broiler poultry flocks), common seals (*Phoca vitulina*) and one harbor porpoise (*Phocoena phocoena)* (Foster and others 2004, Broman and others 2002, Peterson and others 2001b, Waldenström and others 2002, Damborg and others 2004, Kemp and others 2005, Alter and others 2005a, Patrick and others 2004, Moser and others 2001, Colles and others 2003, Hald and others 2004a, Engberg and others 2000, Waage and others 1999, Rosef and others 1985).

Humans

The threat of *Campylobacter* infections to cause acute and chronic gastroenteritis in humans and its potential cause of Guillain-Barré Syndrome and Miller Fisher Syndrome has received heightened attention. The majority of *Campylobacter* infections in humans originate from consumption of raw or undercooked meat products, however,

unpasturized milk, environmental water sources, and vegetables are all known as potential *Campylobacter* reservoirs.

A common method of human exposure and acquisition of a *Campylobacter* infection is during traveling. The term "Travelers' Diarrhea" initially given to enteropathogens such as *Escherichia coli* has now been included in diarrheic patients with *Campylobacter* infections. *Campylobacter* infections have been isolated and well documented in individuals with diarrhea who have recently traveled to other countries. Acute symptoms of travelers diarrhea caused by a *Campylobacter* infection have been reported and studied in U.S. military men and women deployed to Thailand in which positive stool samples have been observed in 33-55% of patients with diarrheic symptoms (Sanders and others 2002, Walz and others 2001, Kuschner and others 1995, Murphy and others 1996). Approximately 13,000 cases of travelers' diarrhea in individuals from England and Wales have been reported as caused by *Campylobacter coli* (Tam and others 2003). *Campylobacter* infections have also been reported in non-traveling residents and studies on domestically acquired *Campylobacter* infections in Finnish patients have recently been reported (Vierikko and others 2004). Of the 3,303 cases confirmed in Finland between July 1 and September 30, 1999, 533 isolates were identified estimating a value of 41.2 *C. jejuni* cases per 100,000 individuals residing in Finland for at least two weeks prior to showing symptoms of *Campylobacter* infection. This number is much higher than CDC's report from FoodNet in 2005 (CDC 2006) of 12.72 per 100,000 U.S. individuals which has drastically decreased from 25.2 cases per 100,000 individuals in 1997 (CDC 2000). It has also been suggested that those

individuals native to developing countries with high *Campylobacter* prevalence may acquire natural immunity once exposed to a *Campylobacter* infection. Walz and others (2001) reported individuals with an elevated IgA titer before traveling to Thailand had a decreased risk of acquiring campylobacteriosis than those with IgA titers < 450. Although *Campylobacter* infections have dramatically reduced within the last decade in the United States, *Campylobacter jejuni* is still the number one foodborne pathogen responsible for acute gastrointestinal enteritis in humans.

Poultry

The higher core-body temperatures of poultry species likely enables poultry to be the predominant reservoir for thermotolerant species since *C. jejuni* and *C. coli* grow optimally at environmental temperatures approximately 42ºC, similar to those found in avian species. This preference for increased temperatures may have an effect on metabolic activity since higher temperatures may allow thermophilic species to regulate gene expression that benefit motility and energy regulation based on specific growth requirements within a specific environmental temperature (Stintzi 2003, Alm and others 1993, Bolton and others 1984). Generally, *Campylobacter* colonize throughout the poultry digestive tract with potentially higher concentrations in the crop, ceca and colon and are usually asymptomatic to their poultry host.

Many risk factors can be linked to co-infection and transmission of *Campylobacter* spp. in broiler flocks such as flock size, age of birds, environmental water supplies, insects and even airborne isolates. Adkin and others (2006) identified thirty-seven contributing factors to the infection of *Campylobacter* in broilers. Although

season and hygiene variables were shown to be possible contributors to infection, some of the highest factors for *Campylobacter* infection in birds include the presence and number of contaminated broiler houses on the same farm, and the interaction between birds and of on-site workers. Transmission of *Campylobacter* from infected birds to humans is possible and risk factors often increase as contact between humans and birds increases. Nadeau and others (2001) found genotypic similarity of multiple *Campylobacter* isolates between human and poultry genotypes with the majority of the birds colonized with *C. jejuni*. Evidence has suggested that *Campylobacter* colonization may be host specific, limiting common serotypes between humans and poultry (Broman and others 2002, Petersen and others 2001a,b). *C. jejuni* tends to be the predominant species in most poultry flocks, however according to El-Shibiny and others (2005), *C. coli*, isolated from organic and free-ranging chickens, was reported to be the majority of *Campylobacter* isolates.

Once *Campylobacter* is established within an individual bird, horizontal transmission often rapidly spreads the bacteria through the flock. *Campylobacter* has been isolated from poultry as early as eight days in free ranging chickens (El-Shibiny and others 2005); however, average colonization of a flock takes several weeks (Bull and others 2006, Stern and others 2001b, Berndtson and others 1996). The number of colony forming units (CFU) necessary to initialize colonization within birds may play a key role in horizontal transmission. One-day-old chicks challenged with only 40 CFU of *C. jejuni* strain 81116 recovered from a colonized cecum was successful in colonizing chicks to numbers as high as 10^8-10^9 cfu/g of cecum contents (Cawthraw and others

1996). Although a much higher dose was needed to initially colonize the primary chicken model, the study suggests a much smaller dose is needed for horizontal transmission within flocks. This may also explain the rapid colonization and detection of *C. jejuni* within broiler houses once *Campylobacter* is prevalent. Occasionally, however, some flocks in close proximity to infected flocks are never colonized (Hiett and others 2002 Berndtson and others 1996, Stern and others 2001b). Other studies concerned with vertical and horizontal transmission of *Campylobacter spp.* within poultry flocks have been performed while the actuality of concluding evidence in favor of vertical transmission is still an arguable matter (Cox and others 2002, Petersen and others 2001b, Shanker and others 1986). Studies involved in analyzing vertical transmission have shown that *Campylobacter jejuni* may potentially enter the eggshell under specific conditions (Sahin and others 2003) but the majority of supporting evidence does not suggest that vertical transmission of *Campylobacter* is a significant risk factor for the colonization of newly hatched chicks. Bull and others (2006) were unable to report vertical transmission from parents to progeny in sampled flocks, but the subtypes identified in colonized flocks were comparable to airborne subtypes identified either inside or outside of the broiler house. Transmission of *Campylobacter* through the air may also be another potential mode of horizontal transmission since airborne *Campylobacters* have been isolated down wind of broiler houses (Bull and others 2006).

Although the predominant *Campylobacter* species such as *C. jejuni*, and *C. coli* are often isolated and reported within most poultry flocks, *Campylobacter* diversity has also been extensively reported on poultry farms (Rivoal and others 2005, Hiett and

others 2002, Bull and others 2006, El-Shibiny and others 2005). Initial colonization of *Campylobacter* subtypes has been shown to differ from the dominant subtypes prevalent at the time of slaughter (Bull and others 2006). The shift from one dominant species to another is poorly understood, but may reflect seasonal variations, geographical preferences, environmental sources and potential vectors.

Processing stations throughout the abattoir are constantly seeking innovative methods to reduce *Campylobacter* contamination on raw poultry carcasses. At poultry processing plants, *Campylobacter* is predominantly found on the skin of infected birds mostly due to inevitable contamination from cecal and gut contents during the evisceration process. However, contamination within the muscle has also been reported in retail meat products. Scherer and others (2006) found that nearly half of all retail packaged chicken legs were contaminated on the skin alone, less than 1% of samples were positive within the muscle alone, while the contamination of both skin and muscle together accounted for 27%. Another study reported a *C. jejuni* incidence of more than 71% isolated from retail chicken products in Japan (Saito and others 2005). Transportation coops to and from the processing plants have been shown to amplify cross-contamination between birds while detectable CFU of *Campylobacter* in chill and scald water were also isolated in some plants (Stern and others 2001b). During a three year surveillance study in the United Kingdom, the overall prevalence of *Salmonella* from fresh poultry samples, retail, butchers, and frozen samples declined 41.7% while *Campylobacter* numbers declined only approximately 3% overall (Meldrum and others 2006).

Cattle

According to USDA Economic Research Service reports (2006), U.S. beef consumption increased approximately 600 million pounds (2.2%) from 2003 to 2004 while total U.S. beef production increased over one billion pounds from 2004 to 2005. With increasing beef demands, and limited research on *Campylobacter* transmission in cattle, research has been initiated to evaluate *Campylobacter* prevalence and identify primary sources and modes of *Campylobacter* infection in the beef and dairy industries. *Campylobacter* is generally asymptomatic in most colonized cattle species; however, it has been known to cause diarrhea and gastroenteritis in calves further leading to an increased usage of antibiotics on-farms and in feedlots.

Processing methods may vary among processing plants so it is no surprise that the prevalence of foodborne pathogens can also vary within retail supermarket products. In studies using retail raw beef products, *Campylobacter* has been isolated from 59 supermarket stores (0.5%) in the Greater Washington D.C. area (Zhao and others 2001) to as much as 10% in Tehran, Iran (Taremi and others 2006). More strict sanitary processing methods may eventually help to further reduce foodborne pathogens from retail products, but the key to controlling *Campylobacter* may lie at the source of the infections. *Campylobacter jejuni* has been repeatedly detected primarily in the gastrointestinal tract of cattle (Besser and others 2005, Inglis and others 2006, Inglis and Kalischuk 2003). Garcia and others (1985) sampled multiple internal viscera for *C. jejuni* and *C. coli* and successfully isolated multiple *C. jejuni* serotypes from the gall bladder, large intestine, small intestine, liver and lymph nodes. Motile *Campylobacter*

are capable of migration via blood vessels to other internal viscera. The gallbladder mucosal tissue (Garcia and others 1985) and bile (Saito and others 2005) both have been shown to be an excellent source of infection, 33% and 21.8%, respectively. Because of the repeated detection of *Campylobacter* isolates reported from gallbladders, as well as livers from cattle with positive gallbladder samples, attention must directed to the possibility of consumers acquiring a *Campylobacter* infection from consumption of undercooked beef livers. Studies have reported *Campylobacter*-positive liver samples varying from 12% in beef cows to 54.2% in Japanese Oxen where *C. jejuni* alone comprised of nearly half of the species identified (Kramer and others 2000).

Beach and others (2002) compared the prevalence of campylobacteria in feedlot and pasture cattle. Feedlot cattle were found to be *Campylobacter*-positive through fecal shedding before and after transport to the abattoir at 64% and 68%, respectively. However, adult pasture cattle exhibited much lower *Campylobacter* counts in fecal shedding, with 6.3% and 7.3% *Campylobacter*-positive swabs recovered pre- and post-transport, respectively. Hide swabs from these same cattle also showed an increased prevalence of *Campylobacter* in feedlot cattle as opposed to adult pasture cattle. Without detectable numbers of *Campylobacter* isolated from the transport vehicle it is difficult to conclude that direct transmission by means of hide to hide contact may have occurred in route to the slaughter facility. In feedlots, close confinement, increased stocking density, community feed and water troughs, and constant physical contact with feces from other animals, may all potentially increase the transmission of pathogenic bacteria. Besser and others (2005) reported a 60% increase in fecal shedding of *C. jejuni* in cattle after four

months from their initial arrival to the feedlot. However, assumptions concerning an increased prevalence of *Campylobacter* within feedlots may also be invalid. Confinement of cattle may support horizontal transmission within a herd, however Bae and others (2005) reported that the prevalence of *Campylobacter* in feedlot cattle (46.9%) was only slightly lower than the prevalence of *Campylobacter* on beef cow-calf ranches (49.4%).

Campylobacter prevalence on conventional and antimicrobial free dairy farms in Wisconsin was shown to be similar at 29.1% and 26.7% respectively (Sato and others 2004). The prevalence of *Campylobacter* in calves was found to be higher than in cows and higher in smaller than large farms (Sato and others 2004). Greater concerns of *Campylobacter* prevalence in dairy herds focus within the milk produced by the herd. Campylobacteria may be more prevalent in milk than any other foodborne pathogen. *Campylobacter jejuni* isolates have been isolated from bulk tank milk in eastern South Dakota and western Minnesota from 9.2% of 131 samples while *Salmonella* was prevalent at only 6.1 % of samples (Jayarao and Henning, 2001). Other studies in Ireland and China reported 1 of 62 (1.6 %), and 82 of 300 (27.3%) raw milk samples to be positive for *Campylobacter,* respectively (Whyte and others 2004, Yang and others 2003).

Companion animals

There has been considerable speculation about the possible transmission of *Campylobacter* from household pets to humans via constant direct physical contact. Direct transmission of *C. jejuni* from canine species to human patients has been

observed (Damborg and others 2004, Wolfs and others 2001) and the possibility of *Campylobacte*r transmission from other household pets to humans is still a possible risk factor.

Domesticated pets are known to harbor *Campylobacter* spp. in their digestive tracts ranging from 11% to as much as 92% of stool samples when evaluated by either culture, polymerase chain reaction (PCR), or pulse-field gel electrophoresis (PFGE) (Damborg and others 2004, Shen and others 2001, Hald and others 2004a, Workman and others 2005). Reports demonstrate that most animals are diversely colonized with numerous *Campylobacter* spp. with one study isolating sixteen different species of *Campylobacter* in cats over a six year period (Shen and others 2001). In a two year study with Danish canine species, *C. jejuni* was isolated from 56 of the 278 positive samples while *C. upsaliensis* accounted for the majority (75%) of isolates (Hald and others 2004a). As better research methods for detecting and culturing *Campylobacter* spp. are developed, more researchers are finding *C. upsaliensis* and *C. helveticus* to be the most prevalent species of campylobacteria in dogs and cats, respectively, and commonly out numbering *C. jejuni* (Workmann and others 2005, Hald and others 2004a, Shen and others 2001, Baker and others 1999, Moser and others 2001). Risk factors for acquiring a *C. jejuni* infection from puppies may now be much more prevalent than initially thought since studies have reported a higher prevalence of *C. jejuni* in dogs less than 1 year old (22%) than dogs between 1-2 years of age (4.7%) (Hald and others 2004a).

Swine

Despite being shadowed by the more prevalent *C. jejuni* isolated from nearly all food producing animals, *C. coli's* fastidious nature in swine should not be underestimated. In England and Wales, *C. coli* was reported to be responsible for nearly 26 thousand cases of gastrointestinal inflammations in 2000 (Tam and others 2003). Recent studies have isolated *C. coli* from swine fecal samples at more than 99% (Thakur and Gebreyes 2005, Alter and others 2005b) and with a lower prevalence of *C. jejuni*. Duffy and others (2001) estimated that *C. coli* and *C. jejuni* combined accounted for only 1.3% of *Campylobacter* contamination on retail pork products which was lower than both *Salmonella* (9.6%) and *Listeria* spp. (41.9%). Another study reported a reduction in *Campylobacter* prevalence within a particular Czech Republic slaughterhouse consistently for three years (Steinhauserová et al 2005).

In a one year study conducted in northeastern Italy, 63.5% of porcine rectal swabs tested positive for *C. coli* while only 10 of 175 and 7 of 175 of retail raw meat samples were positive for *C. coli* and *C. jejuni*, respectively (Pezzotti and others 2003). Jensen and others (2006) studied the establishment of *C. coli* and *C. jejuni* in outdoor organically-raised pigs to monitor potential shifts from *C. coli* to *C. jejuni* in intestinal colonization. Their results demonstrated the excessive fluctuations in swine positive for *C. jejuni*, with recoveries ranging from 0 to 78.6% among the three trials. In all three studies, *C. coli* was more prevalent than *C. jejuni*. Although *C. coli* appears to be the predominant *Campylobacter* species in swine, different production systems have exhibited fluctuating *C. coli* numbers in their swine facilities (Thakur and Gebreyes

2005). Young and others (2000) reported an increased prevalence of *C. jejuni* in cecal or rectal contents of guilts, sows, and weaned piglets (76.3, 89, and 82% respectively). *Campylobacter coli* (68.3%) was only more prevalent than *C. jejuni* (31.7%) in neonates within 24 h of birth. Alter and others (2005b) were unable to detect shedding of *C. coli* on one farm within 24 h of birth but most of the organic pigs were colonized (75%) predominantly with *C. coli* by one week of age. Transmission of *Campylobacter* species, primarily *C. jejuni*, from other livestock species to pigs within the same farm could be a possibility; however, this has yet to be proven (Boes 2005). Isolation methods and growth fluctuations, particularly in environmental samples, may underestimate *Campylobacter* prevalence on farms. Alter and others (2005b) reported undetectable amounts of *Campylobacter* in the feed and drinking water used on seven organic pig farms and only 1 of 97 water troughs were positive for *C. coli*. Samples taken throughout the rearing period (0 to 24 wks), demonstrated an incidence of *C. coli* ranging from undetected amounts (neonates) to more than 78% in fattened pigs post transport to the abattoir.

The use of antibiotics may also alter *Campylobacter* prevalence within the host. Thakur and Gebreyes (2005) reported that antibiotic-free swine nurseries had a 50% increase in the number of *C. coli* isolates compared to traditional farms that use antibiotics. At the finishing farm, *C. coli* isolates were found in all pigs at approximately the same concentration regardless of previous antibiotic use (Thakur and Gebreyes 2005). The practical aspect of utilizing antibiotics for the sole purpose of reducing *Campylobacter* numbers prior to finishing must be questioned since pigs may ultimately

become colonized at the finishing farm. Ultimately, the use of antibiotics may not be worth the antimicrobial resistance effects (Thakur and Gebreyes 2005).

In each harvest operation, individual pigs may be subjected to multiple cleaning stations and processing equipment. A study detecting the prevalence of *Campylobacter* in swine through the different processing stations and comparing carcass, colon and rectal samples throughout a slaughter operation was conducted by Pearce and others (2003). Combining the results from four different recovery methods, *C. coli* were found in 151 of 202 isolates with recovery of *C. jejuni* accounting for only 1% of samples tested. Malakauskas and others (2006) reported that *C. coli* was prevalent in 92 of 120 isolates taken from fecal, carcasses and slaughter line surfaces combined while *C. jejuni* isolates accounted for 28 of 120 of positive samples recovered from carcasses and slaughter line surfaces.

Due to potential physiological differences among *Campylobacter* spp., *C. jejuni* may comprise of a greater resistance to selective stresses than *C. coli* (Madden and others 2000). These selective stresses may negatively affect the survivability of *C. coli* through processing methods such as chilling or air exposure. General processing methods such as chilling or rinsing with chlorinated water may be enough to effectively decrease less resilient *C. coli* bacteria, thereby decreasing total *Campylobacter* concentrations in retail pork products.

Sheep

The prevalence of *Campylobacter* in ovine species has not been as extensively studied as in other agriculture based animals. *Campylobacter* spp., predominantly

Campylobacter fetus subsp. *fetus* in pregnant ewes has been known to cause acute septic abortions (DeLisle and others 1987, Mannering and others 2003; 2004, Fenwick 2000) and is the number one cause of abortions in sheep. Vertical transmission of *Campylobacter* in sheep may also play a role in the initial colonization of lambs since studies have reported the detection of *Campylobacter* isolates within gut contents of aborted fetuses (Erganis and others 2002).

Campylobacter spp. have been isolated from ovine liver (Fenwick and others 2000, Kramer and others 2000), gallbladder (Raji and others 2000,), gut contents (Acik and Cetinkaya 2006, Zweifel and others 2004, Raji and others 2000), and feces (Acik and Cetinkaya 2006, Brown and others 2004) and has been isolated from sheep carcasses at slaughter facilities at relatively low numbers (<1%) after chilling (Phillips and others 2006) while 17.5 % of slaughtered sheep in Switzerland were found to harbor *C. jejuni* and *C. coli* isolates in cecal contents (Zweifel and others 2004).

Wildlife and environment

Wildlife *Campylobacter* serotypes have been reported to be significantly different from human or poultry serotypes when compared to the O:2 and O:4 complex types (Petersen and others 2001a) while serotype similarities between humans and poultry have been shown to exist (Petersen and others 2001a, Rosef and others 1985). The species-specific relationship suggests that colonization of *Campylobacter* species may necessitate adaptation for survivability measures. It is also important to note that closely related strains have been identified among humans, poultry, turkey, and canine species, in which all of these animal species have been domesticated and share a

common environment with humans by means of direct contact (Huang and others 2005, Zhang and others 2000, Damborg and others 2004, Wolfs and others 2001). The consistent contact among species may allow some campylobacteria to adapt into a transmissible species among hosts. Divergent genotypes of *Campylobacter* spp. found between humans and non-domesticated wildlife may be unrelated due to infrequent confrontations between these different reservoirs harboring specific species adapted to a particular host.

Transmission of *Campylobacter* among wild animals is still poorly understood. Studies have linked wild birds, flies and other small wildlife animals as possible vectors of transmission (Hald and others 2004b, Meerburg and others 2006, Hiett and others 2002). *Campylobacter coli* has been identified in only 0.7% of 1474 environmental samples including rodents, wild birds and cats (Alter and others 2005b). Workman and others (2005) identified positive thermophilic *Campylobacter* spp. from 35.7% of wild birds but only 6 of 70 vervet monkeys (*Cercopithecus aethiops sabaeus*), while Jensen and others (2006) found *C. jejuni* in 18 of 19 wild birds (crowbirds, jackdaws, magpies and crows). Water may also participate in the transmission of *Campylobacter* in wild animals. *Campylobacter* has been detected in many free environmental streams or stagnant water systems, with 15% to greater than 40% of samples testing positive (Kemp and others 2005, Hörman and others 2004, Brown and others 2004). Rodents and insects have also been identified as possible vectors for *Campylobacter* transmission to agricultural animals. Stern and others (2001b) detected *Campylobacter* from mice and insects 4 weeks prior to a *Campylobacter* infection in poultry flocks however, 50% of

wild bird fecal specimens were positive for *Campylobacter* at the initial time of detection of *Campylobacter* spp. in the poultry feces or cecal contents.

Serotypes and Strain Differentiation

Methods to determine specific thermophilic *Campylobacter* strains have been available for precise identification of genomic DNA. Pulse-field gel electrophoresis (PFGE), flagellin typing (*Fla*A/*Fla*B), and amplified fragment length polymorphism (AFLP) are commonly used to identify and compare distinct genotypes among humans and animals. Zhang and others (2000) identified the specific gene (*cmp* gene) encoding a specific major outer membrane protein (MOMP) commonly shared by thermophilic *Campylobacter* species. The *cmp* gene encoding the MOMP may have an important role in the pathogenesis of human *Campylobacter* enteritis (Moser and others 1997, Bacon and others 1999) and is considered an excellent method of classifying strains due to the ubiquitous nature within all thermophilic *Campylobacter* strains (Zhang and others 2000). The *cmp* gene type B2 has now been identified in *Campylobacter* spp. found in humans and poultry (Huang and others 2005, Zhang and others 2000) as well as type D1 in humans and turkeys (Zhang and others 2000). Common types of *Campylobacter* found in both humans and poultry have been analyzed and detected by AFLP (Duim and others 1999) and PFGE (Michaud and others 2005). Penner serotypes heat-stable (HS) antigens 1, 2, 4, and 21, all reported from *C. jejuni* isolates have been identified as shared serotypes by humans, poultry, cattle and sheep (Steinhauserová and Fojtíková 1999, Hudson and others 1999). Serotype HS50 has been prevalent in chickens (14.4%), lamb liver (17%), ox liver (45.3%), pig liver (5.6%) and humans (18.8%) (Kramer and

others 2000). Guévremont and others (2004) were unable to detect, using PFGE, genotypic similarities between swine and human isolates, with the human isolates recovered from patients experiencing diarrhea and living within close geological proximity to the source of swine isolates. In ovine species, *Fla*A 16 was confirmed present in 45% of *C. jejuni* isolates from the intestine, while *Fla*A 26 was the predominant strain isolated in the feces (Acik and Cetinkaya, 2006). *Campylobacter coli* subjected to strain differentiation were identified as subtype *Fla*A 8 (34%) and *Fla*A 1 (45%) for intestinal or gall bladder isolates respectively, while 41% from feces were restricted to *Fla*A 16 subtype (Acik and Cetinkaya, 2006). Pulsed-field gel electrophoresis type B1 (201/293) has been determined to be the majority of *C. fetus* subsp. *fetus* isolated from sheep abortions followed by type B2 (21/293) (Mannering and others 2004).

Season

Various outbreaks and seasonal peaks of Campylobacteria have been reported in the warmer months; however, other studies have failed to identify specific climatic fluctuations in *Campylobacter* prevalence (Nadeau and others 2001). Stern and others (2001b) measured seasonal fluctuations in *Campylobacter* prevalence in poultry over the course of one year. The majority of positive *Campylobacter* samples from cecal contents occurred in the spring while the majority of positive fecal samples were identified in the summer months (approx. 38% and 46%, respectively). In a New Zealand study during the months of August and February, 64 of 113 raw chicken samples were positive for *Campylobacter* (Hudson and others 1999). From the identified isolates, the study

identified variable outbreaks of different *C. jejuni* serotypes found either in the winter or summer.

Campylobacter isolation from the skin of retail chicken legs in Berlin, Germany was most prevalent in February (100%) while muscle samples showed 70% contamination in September and both muscle and skin showed decreasing *Campylobacter* concentrations from August through December (Scherer and others 2006). Acute seasonal outbreaks of *Campylobacter* on retail raw chicken over a three year period in Wales were found between March and June of 2002, April and June of 2003, and June through August of 2004, with the highest annual peaks falling within the months of June ’02, December ’03 and August ’04 (Meldrum and others 2006). Other studies have compared the seasonal prevalence of *Campylobacter* in humans and broilers using temperature, sunlight, humidity, and precipitation (Patrick and others 2004); however, explanations for peak outbreaks in either humans or animals have been inconclusive. More research is needed to determine the significant risk factors associated with seasonal fluctuations within *Campylobacter* isolates.

Control and Management

To reduce the risk of campylobacterosis, careful management practices focus on innovative methods to avoid cross contamination from raw meat products. Predominantly, the reduction of contamination of raw meats is handled at the processing plants through a post-harvest cleaning process. Pre-chilled carcasses that harbor campylobacteria (Stern and Robach 2003, Izat and others 1988) may lead to contamination of retail consumer products. Product contamination by conventional

evisceration processes fluctuate greatly depending on the processing plant (Rosenquist and others 2006, Oosterom and others 1983, Izat and others 1988). Methods to reduce pathogens before arrival to the abattoir are of interest because pre-harvest interventions may diminish possible retail sources of infection thereby decreasing human illness associated with foodborne pathogens (Vugia and others 2003).

Pre-harvest control

Considering that *Campylobacter* colonization of food-producing animals is most likely the result of direct physical contact among infected and uninfected animals, then interventions that target primary sources of infection may be able to reduce the infectious cycle. While antimicrobial treatments exist for reducing gut concentrations of bacterial pathogens such as *Campylobacter*, their use for pre-harvest control may be undesirable because of potential residue and antimicrobial resistance issues. Consequently, alternative methods are being sought to reduce the carriage of *Campylobacter* in food animals on the farm. Some of these methods involve the application of specific management practices such as bunk cleaning, water trough cleaning, etc., but considering the ubiquitous nature of *Campylobacter* and high probability that animals may be continually exposed to infectious doses, these interventions may have limited utility. Perhaps the most effective interventions are those that are targeted to reduce concentrations just before slaughter (Pearson and others 1993) particularly when applied in conjunction with post-harvest interventions such as enhanced cleaning implementations within processing plants. Wesley and others (2000) reported 14 out of 15 dairy herds to be infected with *C. jejuni* when feed was accessible

to wild birds. This number was reduced nearly 25% when feed access to the wild birds was denied. Simple but effective alterations in management practices implemented for reducing *Campylobacter* carriage on the farm may help reduce the number of organisms reaching the processing plant. Proper cleaning techniques coupled with strict management operation implications, may potentially help to reduce *Campylobacter* infection, therefore techniques for pre-harvest control are sought to reduce *Campylobacter* concentrations without the aid of antibiotics.

Because of the microaerophilic and thermophilic nature of *Campylobacter*, focusing on reducing gut concentrations of the organism may be a superior target to suppress environmental microbial activity. Harvey and others (2000) reduced rectal concentrations in neonatal pigs when removed from their maternal sows and reared within separate nursery facilities compared to those piglets that remained on the sow for a period of 20 days. Novel development of feed additives are being sought to reduce *Campylobacter* colonization in animals, such as short chain nitrocompound supplementation. Recent studies have shown that 2-nitro-1-propanol exhibits broad spectrum antimicrobial activity against *Salmonella* serovar Typhimurium, *Escherichia coli* 0157:H7, and *Enterococcus faecalis in vitro* (Jung and others 2004a) and against *Salmonella* Typhimurium when administered via oral gavage to broilers (Jung and others 2004b). Likewise, this and similar nitrocompounds have been reported to reduce gut concentrations of *Salmonella* and *Campylobacter* in pigs (Jung and others 2003), and to inhibit methane-producing activity in bovine and avian gut contents (Anderson and

others 2004; Saengkerdsub and others 2006), uric acid degrading bacteria (Kim and others 2005), and *Listeria monocytogenes in vitro* (Dimitrijevic and others 2005).

Recently, an experimental feed additive containing chlorate as its active ingredient has been developed for its use in controlling *E. coli* O157:H7 and *Salmonella* in production animals (Anderson and others 2001a, b, Anderson and others 2000b, Byrd and others 2003, Callaway and others, 2002, 2003, Edrington 2003). This technology is attractive in that it specifically targets pathogenic bacteria, such as *E. coli* and *Salmonella*. Bacteria comprising the family *Enterobacteriaceae* express, under anaerobic conditions, a membrane bound respiratory nitrate reductase enzyme (Nar) that coincidentally catalyzes the reduction of chlorate to cytotoxic chlorite while not depopulating the gut of beneficial gut anaerobes, most which lack this enzyme (Anderson and others 2000b). For instance, in vivo results from studies with cattle, sheep, swine, and poultry have shown more than 100-fold reductions in gut concentrations of *E. coli* O157:H7 and *Salmonella* Typhimurium following single day administrations of sodium chlorate (Anderson and others, 2001a,b; Byrd and others 2003; Callaway and others 2002, 2003; Edrington and others 2003). At present, the chlorate technology has not been proven successful against *Campylobacter* spp. which also possess respiratory nitrate reductase activity, presumably because *Campylobacter* express a periplasmic respiratory nitrate reductase enzyme (Nap) which unlike the membrane bound enzyme, is not thought to reduce chlorate to chlorite (Moreno-Vivián and others 1999). The chlorate technology has not yet been approved for commercial use.

Other possible methods to reduce gut pathogens involve the oral administration of select intestinal microflora commonly referred to as competitive exclusion (Hakkinen and Schneitz 1999, Stern and others 2001a). For agricultural purposes, competitive exclusion is achieved by orally administrating mixed strains of beneficial bacterial cultures to selectively colonize ecological niches within the gut while displacing pathogenic bacteria. However, most competitive exclusion techniques have been marginally successful in reducing intestinal and cecal concentrations of pathogens. While competitive exclusion studies have been used mainly in neonates as to prevent colonization of undesirable microflora, it may be limited to its effects upon displacement of an already established species. Heres and others (2004) have examined the use of organic acids as feed supplements to reduce fecal concentrations of *Campylobacter* in broilers. While the use of organic acids was found to be effective, growth performance was substantially reduced. Avian species have fewer taste receptors in the oral cavity compared to those of bovine, porcine and ovine species. The addition of lactic and acetic acids to feeds intended for animal species other than poultry may decrease palatability and may potentially reduce feed intake. Also, hindgut fermentation results in the production of copious amounts of volatile fatty acids which exhibit direct inhibitory activity against some bacteria as well as affecting indirect inhibition via alteration of colonic pH. Supplementation of acid-rich feeds may intensify the amount of water needed to normalize the colonic pH leading to acute cases of diarrhea.

Post harvest control

A predominant issue in the control of foodborne pathogens is cross contamination of carcasses within the processing facilities. Numerous treatments on carcasses have been implemented to reduce pathogens before final distribution to retail outlets. *Campylobacter jejuni* activity has been inactivated (99%) with only 1 ppm of free chlorine (pH 6-8 and 25°C) after 15 min (Blaser and others 1986), however *C. jejuni* and other bacterial pathogens have been shown to increase resistance (> 50 fold) and survivability to chlorinated water by infecting and utilizing protozoa species as a temporary reservoir (Axelsson-Olsson and others 2005, King and others 1988). A pre-harvest method such as supplemented chlorine to the drinking water was ineffective in controlling *Campylobacter* prevalence in feedlot cattle water troughs (Besser et al 2005). The use of chlorine additives have also been ineffective in one poultry processing plant, reporting 40.9% of samples were positive for *Campylobacter* after chilling in water treated with as much as 50 ppm chlorine (Stern and others 2001b). Gaseous ozone treatments of chicken meats challenged with *Salmonella infantis* or *Pseudomonas aeruginosa* have been unsuccessful in reducing pathogen concentrations (Al-Haddad and others 2005) and may not be a tool for controlling *Campylobacter.* Electron irradiation (Todoriki and others 2002) and hot water immersion techniques (Corry and others 2006) have been reported to decrease bacterial counts on products intended for human consumption. Treatment of chicken carcasses with lactic acid or alkaline chemicals may also be a valuable tool for the reduction of select foodborne pathogens on the carcass surface (Okolocha and Ellerbroek 2005). Blast and conventional chilling had been

shown to reduce *E. coli*, *L. monocytogenes*, *S.* Typhimurium, and *C. coli* from swine skin samples (Chang and others 2003, Pearce and others 2003).

Conclusions

Campylobacter is a widespread and common bacterial foodborne pathogen capable of extremely negative impacts on human health. Due to the exceptionally prevalent nature of the organism, *Campylobacter* is proficient in effectively invading the synergistic environments of humans and animals worldwide. The control of *Campylobacter* is a serious and problematic issue for the poultry, pork, dairy and beef industries in the U.S. Presently, the approach towards reduction and elimination of *Campylobacter* from retail consumer products can not be accomplished by a single technique. Rather, a combination of pre- and post-harvest methods will likely be needed to more effectively control this foodborne pathogen. Decontamination measures should be an implemented practice in all harvesting facilities while novel pre-harvest and post-harvest techniques should be thoroughly researched.

MATERIALS AND METHODS

Bacterial Strains

Campylobacter jejuni strain CC326 and *C. coli* strain CAA-39 used in this study originated from Holstein cattle (Harvey and others 2004; 2005). Isolated colonies of either *C. jejuni* or *C. coli* strains were incubated for 48 h on Campy-Cefex agar (Stern and others 1992) then harvested and stored in a 20% glycerol solution at -70 ^{o}C when not in use. Inocula for pure test cultures were incubated overnight in Bolton Broth without antibiotics prepared with 50 mL lysed horse red blood cells/1000 mL (Lampire Biological Laboratories, Pipersville, Pa., U.S.A.).

Test Conditions and Incubations

Tests with pure cultures were performed using Bolton Broth adjusted to pH 5.6, 7.0, or 8.2 for *C. jejuni* isolates and adjusted to pH 8.2 only for *C. coli* isolates via additions of 37% HCl or 5 N NaOH. 2-Nitro-1-propanol, 2-nitroethanol, nitroethane, and reagents used in the synthesis of 2-nitro-methyl-propionate were purchased from Sigma Aldrich Inc. (St. Louis, Mo, USA). 2-Nitro-methyl-propionate was synthesized by the method of Kornblum and Blackwood (1962) from methyl-bromopropionate, sodium nitrite, and phloroglucinol using dimethyl sulfoxide as the solvent. The product was distilled under vacuum from the reaction mixture as a clear liquid with a purity of 98% as determined by ^{1}H-NMR ($CDCl_3$); δ5.23 (q, 1 H, J = 7.2 Hz), 3.85 (s, 3 H), 1.81 (d, H, J = 7.2 Hz); MS, *m/e* (relative abundance) 102.0 (8), 87.1 (13), 59.0 (100), 56.0 (15), and 55.0 (13). Nitrocompounds were supplemented to 9 mL of pH adjusted Bolton broth

to achieve 0, 10, or 20 mM by adding small volumes from filter sterilized (0.2 μm Acrodisc Syringe Filter, Pall Life Sciences, Ann Arbor, Mi., U.S.A.) 150 mM stock solutions prepared in distilled water. All tubes were inoculated with 10^{-2} mL of overnight grown inoculum and then brought to a final volume (10 mL) via additions of appropriately pH adjusted Bolton Broth followed by a 48 h incubation time at 42 ^{o}C under a microaerophilic gas phase (10% CO_2, 5% O_2, and 85% N_2). The effect of 0 or 20 mM 2-nitro-1-propanol or nitroethane on wildtype *Campylobacter* during mixed culture was accomplished by incubating (37 ^{o}C) suspensions (10 mL) of freshly collected porcine fecal material that had been mixed 1:5 with anaerobic 0.1 *M* sodium phosphate buffer (pH 6.8) for 48 h under an anaerobic gas phase (90% N_2: 5% CO_2: 5%H_2).

Enumeration and Analytical Methods

Samples (1 mL) from all test incubations were collected at 0, 6, 24, and 48 h for enumeration of *Campylobacter*, via plating of serial 10-fold dilutions (in 0.1 *M* phosphate buffer, pH 6.5) to Campy-Cefex agar. Colonies exhibiting typical *Campylobacter* morphology were counted after 48 h incubation. Portions of the 1:10 dilutions from the mixed culture study were also analyzed for volatile fatty acid concentrations by gas chromatography (Hinton and others 1990).

Ten representative 48 h old colonies from the mixed culture study were randomly selected for PCR differentiation, based on the amplification and detection of the *ceuE* gene at either 793-bp or 894-bp, of *C. jejuni* or *C. coli*, respectively (Gonzalez and others 1997). Cells from each colony were added to 500 μL PCR grade H_2O in 1.5 mL microcentrifuge tubes. Samples were boiled for 10 min then centrifuged at 8500 rpm for

15 min to isolate DNA. A master mix for amplification of each isolated colony was prepared by the addition of 25 µl Jumpstart REDTaq polymerase (Sigma-Aldrich), 1 µL of each DNA primer for *C. coli* or *C. jejuni* (Integrated DNA Technologies Inc., Coralville, Ia., U.S.A.), and 16 µL of PCR grade H_2O (Sigma-Aldrich). Template DNA (5 µL) from each isolate was supplemented to 45 µL of the master mix to achieve a total volume of 50 µL. Electrophoresis was performed using a 2% Agarose E-gel from Invitrogen (Carlsbad, Ca., U.S.A.).

Statistical Analysis

All incubations were conducted in triplicate. Effects of nitrocompound (0, 10, or 20 mM) on log transformations of *Campylobacter* concentrations, $\log_{10}$ colony forming units (CFU)/mL, during the incubations were determined by a repeated measures analysis of variance (Statistix®8 Analytical Software, Tallahassee, Fl., U.S.A.). Effects of pH on *C. jejuni* during incubation with each nitrocompound was determined by a general analysis of variance (Statistix®8 Analytical Software) with pH (5.6, 7.0, or 8.2), level of each nitrocompound (0, 10, or 20 mM) and their interaction included in the model statement. Volatile fatty acid concentrations in fluid samples collected after 24 h of the mixed culture incubations were tested for treatment effects by general analysis of variance. Means were further separated by LSD separation of means.

RESULTS AND DISCUSSION

Numerous effective post-harvest processing strategies have been employed to reduce microbial contamination of poultry and red meat carcasses (Castell-Perez and Moreira 2004; Keeton and Eddy 2004; SCVPH 1998). However, considerable interest exists for the development of pre-harvest strategies that can reduce the carriage of foodborne pathogens in animals prior to entering the processing plant (Callaway and others 2004). In the present study, the inhibitory activity of 2-nitro-1-propanol, 2-nitroethanol, nitroethane, and 2-nitro-methyl-propionate on the survivability of *C. jejuni* during incubation in Bolton broth is evident (Figures 1 through 4), although the nitro-alcohols were more effective than the other nitroalkanes in decreasing the survivability of *C. jejuni*. The activity of the nitrocompounds, especially at the higher concentrations, appears to be bactericidal as recovery of *C. jejuni* on Campy-Cefex agar plates was markedly reduced. We can not rule out, however, that the nitrocompounds may have induced the *Campylobacter* cells to enter into a viable but non-culturable state (Ziprin 2004).

Effects of pH were observed on the inhibitory activity of the nitrocompounds against *C. jejuni*, (Table 2). For cultures incubated with 10 mM 2-nitro-1-propanol, *C. jejuni* concentrations decreased more ($P < 0.05$) after 24 h incubation at pH 8.2 than at pH 7.0, with the net decrease of 3.45 $\log_{10}$ CFU observed for cultures incubated at pH 5.6 being intermediate ($P < 0.05$). For cultures incubated with 10 mM 2-nitroethanol, *C. jejuni* concentrations had decreased more ($P < 0.05$) after 24 h at pH 8.2 than at either

pH 5.6 or 7.0. A pH effect was not observed ($P < 0.05$) in incubations with 20 mM 2-nitro-1-propanol or 2-nitroethanol. In the case of nitroethane, inhibitory activity at either 10 or 20 mM addition level was greatest ($P < 0.05$) at pH 8.2 and least ($P < 0.05$) at pH 5.6. Incubations with 20 mM 2-nitro-methyl-propionate showed greatest inhibition ($P < 0.05$) at pH 5.6 but the significant lower activity observed in incubations with 10 mM 2-nitro-methyl-propionate did not differ among the different pH conditions.

Tests of the nitrocompounds against *C. coli* yielded similar results, as inhibitory activity of 2-nitro-1-propanol, 2-nitroethanol, and nitroethane was observed, with activity being greatest ($P < 0.05$) at the higher addition level (Figure 5). Incubations containing 2-nitro-methyl-propionate showed little to no detectable activity on growth inhibition with *C. coli* species (data not shown). Based on our previous results demonstrating that a higher pH had greater inhibitory effect with all of the tested nitrocompounds except 2-nitro-methyl-propionate, we conducted our tests with *C. coli* in medium adjusted to pH 8.2 only which may explain the absence of activity by 2-nitro-methyl-propionate. Alternatively, the inability of 2-nitro-methyl-propionate to produce inhibitory effects may be due to the insoluble nature of the compound when added to *in vitro* aqueous solutions.

When fresh porcine fecal suspensions were incubated 24 h anaerobically with or without 20 mM 2-nitro-1-propanol or nitroethane, wildtype *Campylobacter* concentrations were reduced ($P < 0.05$) 1.16 $\log_{10}$ and 3.92 $\log_{10}$ CFU units from initial microbial concentrations, respectively (Figure 6). Control values also decreased 2.83 $\log_{10}$ CFU units ($P < 0.05$) from their initial concentration after 24 h incubation and this

decrease was greater ($P < 0.05$) than that observed in cultures containing 20 mM nitroethane. Of the 10 representative colonies tested for speciation by PCR, all yielded amplicons of the *ceuE* gene indicative of *C. coli* (Gonzalez and others 1997). The observed decrease in *Campylobacter* concentrations during mixed culture incubation without added nitrocompound may be due to an accumulation of volatile fatty acids. For instance, analysis of 24 h incubation samples revealed less ($P < 0.05$) accumulation of volatile fatty acids in cultures incubated with 20 mM 2-nitro-1-propanol and nitroethane than in control cultures (Table 1). This suggested that at these concentrations the nitrocompounds may have inhibited fermentation of endogenous substrates by the anaerobic population. Decreased acetate and propionate have been associated with increased concentrations of *C. jejuni* in the swine gut (Harvey and others 2001) while increased concentrations of volatile fatty acids have been associated with decreased multiplication of *C. jejuni* in the mouse gut (Jesudason and others 1989).

Results presented here show that 2-nitro-1-propanol and 2-nitroethanol were more effective against *C. jejuni* and *C. coli* than nitroethane or 2-nitro-methyl-propionate and that except for the latter, all nitrocompounds appeared to exhibit greater activity at non-neutral pH, with greatest activity observed at pH 8.2. The nitrocompounds possess labile protons next to the nitro group and thus may be expected to be more reactive at a higher pH. These findings have practical implications considering that ileal, cecal, and colonic contents of weaned pigs are typically pH 7.0 or less (Harvey and others 2001; Mathew and others 1993; Prohászka and Lukács 1984) although the pH of cecal contents in fasted pigs was more alkaline at pH 7.5 (Harvey and others 2001).

Presently, aliphatic nitrocompounds such as these are used as propellants, solvents, and intermediates for organic synthesis. Secondary nitroalkanes such as 2-nitropropane and 2-nitrobutane have been shown to cause damage to rat liver DNA and RNA and to be mutagenic in their ionized form when tested by the Ames Salmonella assay but primary nitroalkanes and nitrocarbinols such as 2-nitro-1-propanol were not found to be carcinogenic or mutagenic when tested similarly (Conaway and others 1991a; b). Furthermore, toxic effects were not observed in rats following a 2 year chronic inhalation exposure to 100 or 200 ppm nitroethane (Griffin and others 1988). The oral LD_{50} of 2-nitro-1-propanol to chicks was found to be >1,300 mg/kg body weight (Jung and others 2004). Whether the nitrocompounds can be developed for use as feed additives to control foodborne pathogens such as *Campylobacter*, *Listeria,* and *Salmonella* will undoubtedly depend on further studies examining their potential toxicity and metabolism. Precedence exists, however, for the experimental feeding of 2-nitro-1-propanol and(or) nitroethane to ruminants without any apparent adverse effects (Anderson and others 2004; Majak 1992). Additionally, earlier studies have shown that oral administration of 2-nitro-1-propanol results in significant reductions in gut *Salmonella* Typhimurium and wildtype *Campylobacter* concentrations, thus demonstrating that this compound may have application in reducing foodborne pathogens in animals (Jung and others 2003; 2004b). In ruminants, and presumably other gut habitats, the various nitrocompounds would be expected to be reduced to their respective amines by *Denitrobacterium detoxificans,* a ruminal bacterium known to use the nitrocompounds tested here as well as 3-nitro-1-propanol and 3-nitro-1-propionic

acid as terminal electron acceptors during anaerobic respiration (Anderson and others 2000a).

CONCLUSIONS

Results presented here confirm the bactericidal activity of select nitrocompounds against *C. jejuni* and *C. coli in vitro*. For *C. jejuni*, inhibitory effects of all nitrocompounds, with the exception of 2-nitro-methyl-propionate, were greatest at a higher pH. For C. *coli*, which was tested only at the higher pH (Bolton Broth adjusted to pH 8.2), the greatest inhibitory effects were seen when grown with 20 mM nitrocompound concentration. Concentrations of wildtype *Campylobacter,* shown by PCR analysis to be *C. coli,* decreased more rapidly during incubation of mixed fecal bacteria with 20 mM 2-nitro-1-propanol, than without added nitrocompound or with 20 mM nitroethane, thus demonstrating the superior bactericidal activity of the nitro-alcohol. Although these nitrocompounds have shown significant inhibitory effect, their mechanism of action has yet to be determined. Results from this study demonstrate that growth inhibition of *C. jejuni* and *C. coli* by the nitrocompounds tested here are pH and dose dependent. Research is underway with these and other nitrocompounds to determine if these exhibit inhibitory activity against other foodborne pathogens and to better understand the limits of their activity.

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APPENDIX

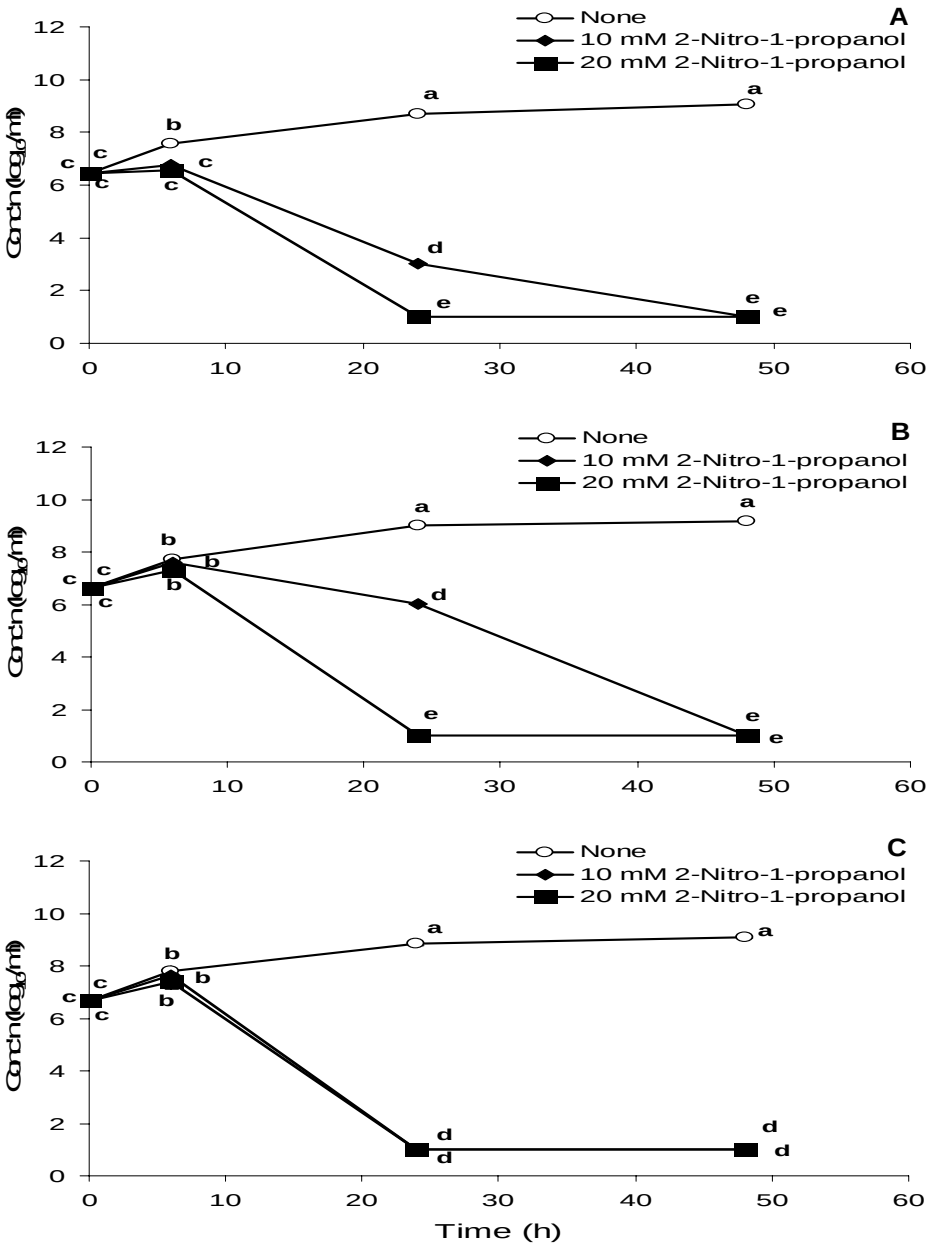

Figure 1 - Effects of 0, 10, or 20 mM 2-nitro-1-propanol on growth or survivability of *Campylobacter jejuni* during incubation in Bolton's broth adjusted to pH 5.6 (A), 7.0 (B), or 8.2 (C). Means (n = 3) with unlike letters differ ($P < 0.05$); SEM = 0.10, 0.07, and 0.08 for at pH 5.6, 7.0, and 8.2, respectively

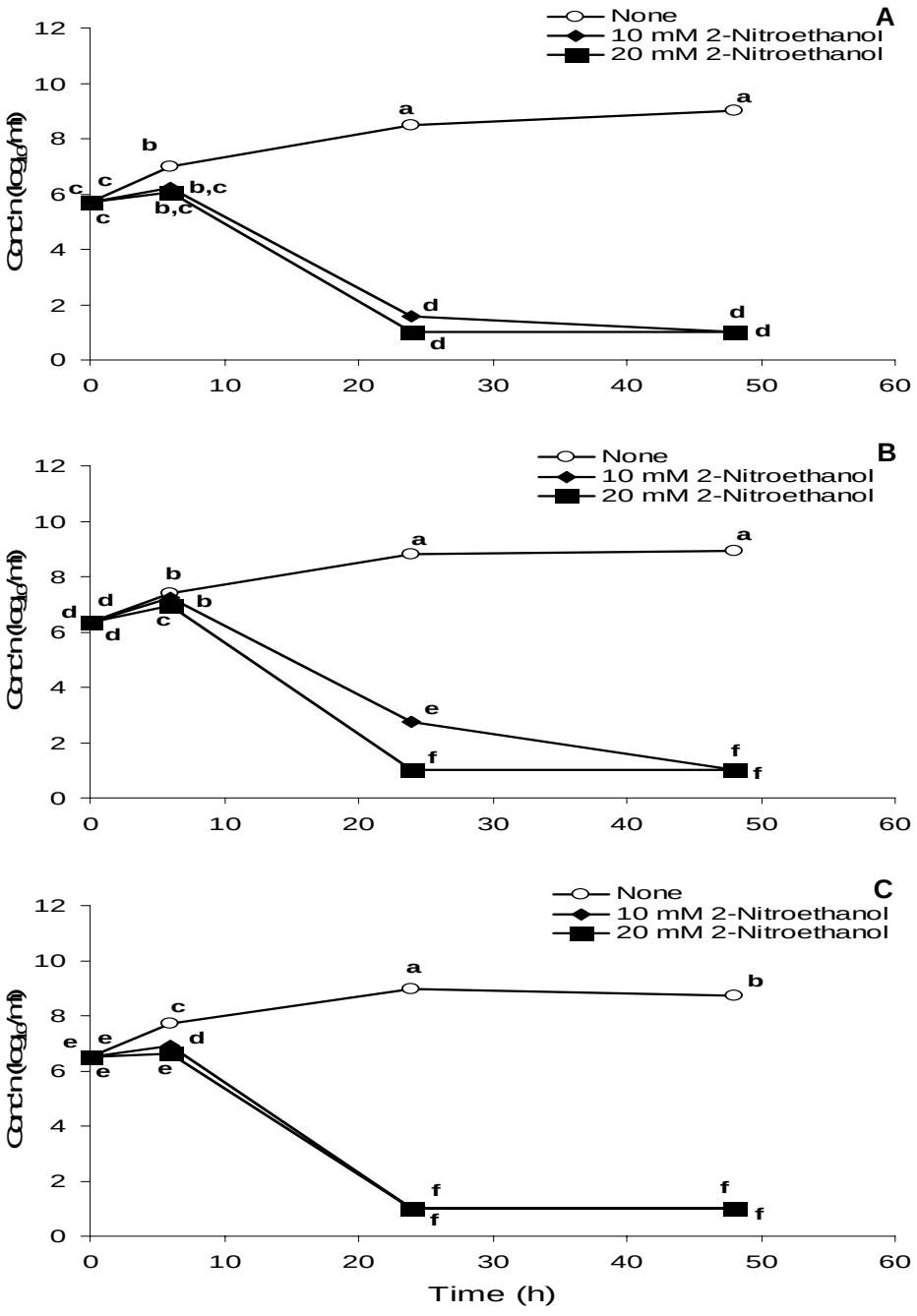

Figure 2 - Effects of 0, 10, or 20 mM 2-nitroethanol on growth or survivability of *Campylobacter jejuni* during incubation in Bolton's broth adjusted to pH 5.6 (A), 7.0 (B), or 8.2 (C). Means (n = 3) with unlike letters differ ($P < 0.05$); SEM = 0.35, 0.10, and 0.05 for at pH 5.6, 7.0, and 8.2, respectively

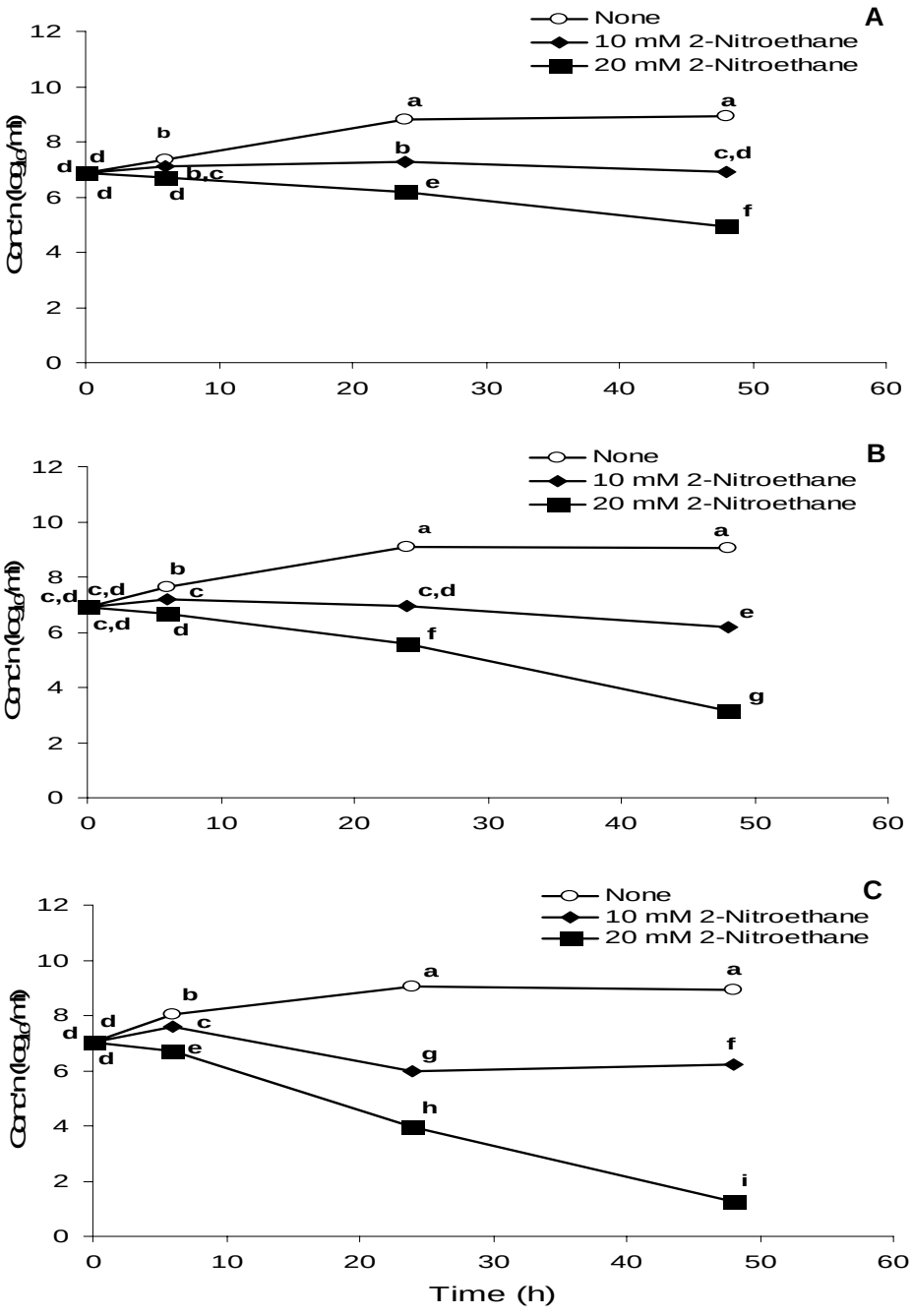

Figure 3 - Effects of 0, 10, or 20 mM nitroethane on growth or survivability of *Campylobacter jejuni* during incubation in Bolton's broth adjusted to pH 5.6 (A), 7.0 (B), or 8.2 (C). Means (n = 3) with unlike letters differ ($P < 0.05$); SEM = 0.08, 0.10, and 0.08 for at pH 5.6, 7.0, and 8.2, respectively

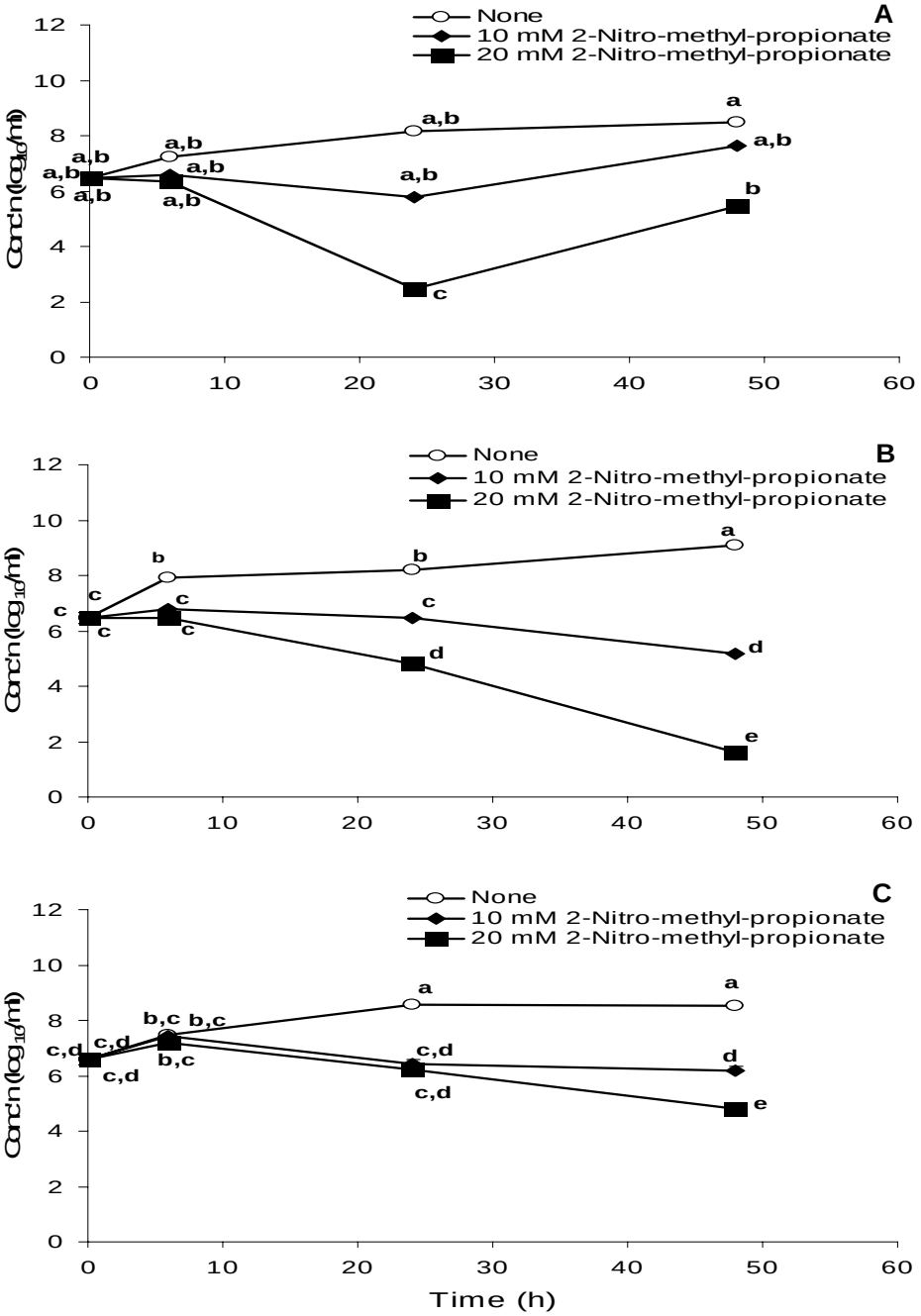

Figure 4 - Effects of 0, 10, or 20 mM 2-nitro-methyl-propionate on growth or survivability of *Campylobacter jejuni* during incubation in Bolton's broth adjusted to pH 5.6 (A), 7.0 (B), or 8.2 (C). Means (n = 3) with unlike letters differ ($P < 0.05$); SEM = 0.79, 0.22, and 0.27 for at pH 5.6, 7.0, and 8.2, respectively

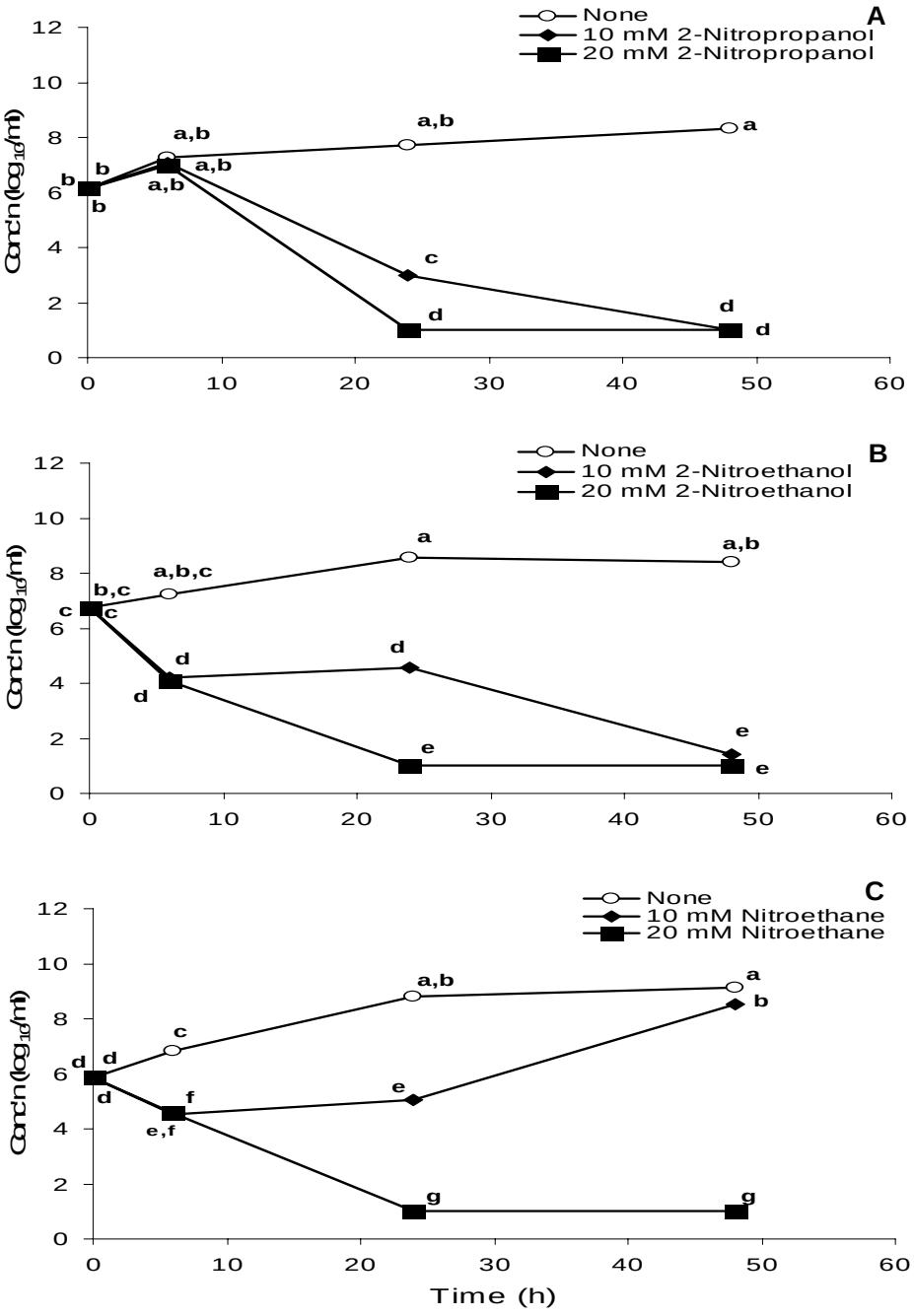

Figure 5 - Effects of 0, 10, or 20 mM 2-nitro-1-propanol (A), 2-nitroethanol (B), or nitroethane (C) on growth or survivability of *Campylobacter coli* during incubation in Bolton's broth adjusted to pH 8.2. Means (n = 3) with unlike letters differ (P < 0.05); SEM = 0.58, 0.57, and 0.15 for 2-nitro-1-propanol, 2-nitroethanol, and nitroethane, respectively

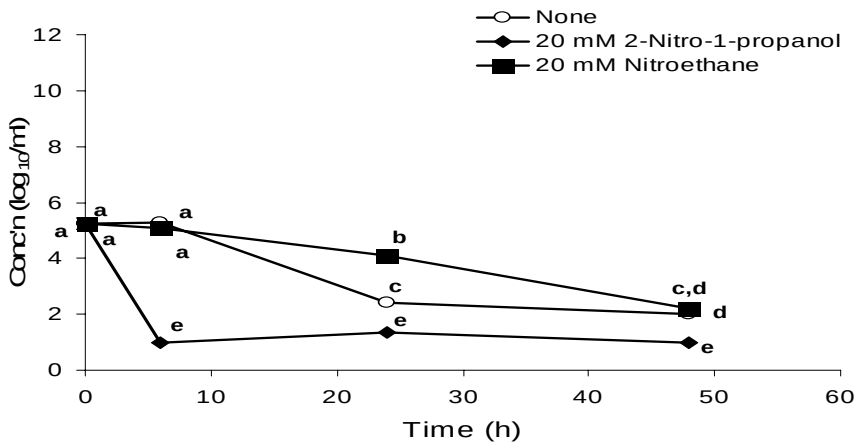

Figure 6 – Effects of 0 or 20 mM 2-nitro-1-propanol or nitroethane on survivability of naturally occurring *Campylobacter* during anaerobic incubation of porcine fecal suspensions. Means (n = 3) with unlike letters differ ($P < 0.05$); SEM = 0.14

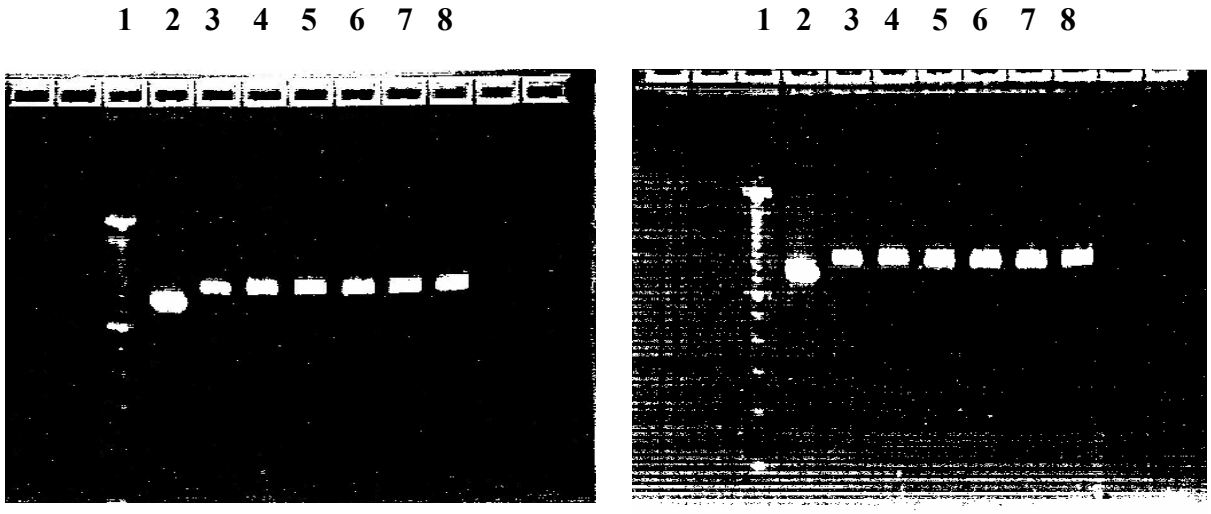

Figure 7 - PCR profiles of naturally occurring *Campylobacter* spp. (a) Isolates 1-5. Lane 1, 100 bp ladder (Sigma-Aldrich), lane 2, *C. jejuni* control template; lane 3, *C. coli* control template; lanes 4-8, naturally occurring *C. coli* template (b) Isolates 6-10. Lane 1, 100 bp ladder; lane 2, *C. jejuni* control template; lane 3, *C. coli* control template, lanes 4-8, naturally occurring *C. coli* template

Table 1. Effects of nitrocompound on volatile fatty acid accumulation after 24 h incubation of freshly collected porcine feces at 37 ^{o}C.

Treatment	Acetate (µmol.mL	Propionate (µmol.mL	Butyrate (µmol.mL	Total (µmol.mL
None	15.56[a]	5.27[a]	3.21[a]	24.02[a]
20 mM 2-Nitro-1-propanol	9,03[b]	3.71[b]	2.07[b]	14.82[b]
20 mM Nitroethane	10.24[b]	3.97[b]	2.53[b]	16.75[b]
Nitro-effect	$P = 0.02$	$P = 0.04$	$P = 0.004$	$P = 0.01$
SEM	1.15	0.34	0.14	1.47

[a, b]Values within columns with unlike superscripts differ ($P < 0.05$), Tests for treatment effects were accomplished by general analysis of variance and a LSD separation of means.

Table 2. Main effects of nitrocompound, pH, or their interaction on net change in *C. jejuni* concentrations (Δ $\log_{10}$ colony forming units) determined after 24 h incubation in Bolton's broth at 42°C[a].

	2-nitro-1-propanol (mM)			2-Nitroethanol (mM)			Nitroethane (mM)			2-Nitro-methyl-propionate (mM)		
pH	0	10	20	0	10	20	0	10	20	0	10	20
5.6	2.23[b]	-3.45[d]	-5.46[e]	2.79[f]	-4.09[g]	-4.68[g, h]	1.97[i]	0.44[j]	-0.68[l]	1.67[o, p]	-0.72[q]	-4.03[r]
7.0	2.38[b]	-0.60[c]	-5.64[e]	2.46[f]	-3.61[g]	-5.34[h]	2.17[i]	0.07[k]	-1.31[m]	1.73[o, p]	0.10[p, q]	-1.65[q]
8.2	2.18[b]	-5.67[e]	-5.67[e]	2.46[f]	-5.51[h]	-5.51[h]	2.03[i]	-1.07[m]	-3.08[n]	1.97[o]	-0.14[p, q]	-0.36[q]
Nitro-effect	$P < 0.0001$			$P < 0.0001$			$P < 0.0001$			$P < 0.0001$		
pH effect	$P < 0.0001$			$P = 0.03$			$P < 0.0001$			$P = 0.03$		
Interaction	$P < 0.0001$			$P = 0.14$			$P < 0.0001$			$P = 0.12$		
SEM	0.14			0.38			0.09			0.66		

[a]Tests for main effects of nitrocompound, pH, or their interaction on net change in *C. jejuni* concentrations were accomplished by general analysis of variance and a LSD separation of means.
[b, c, d, e]Values with unlike superscripts differ ($P < 0.05$).
[f, g, h]Values with unlike superscripts differ ($P < .05$).
[i, j, k, l, m,n]Values with unlike superscripts differ ($P < .05$).
[o, p, q, r]Values with unlike superscripts differ ($P < .05$).

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PUBLICATIONS

Horrocks SM, Jung YS, Huwe JK, Harvey RB, Ricke SC, Carstens GE, Callaway TR, Anderson RC, Ramlachan N, Nisbet DJ. Effects of Short-chain Nitrocompounds Against *Campylobacter jejuni* and *Campylobacter coli In Vitro* (Submitted)

Anderson RC, Jung YS, Oliver CE, Horrocks SM, Genovese KJ, Harvey RB, Callaway TR, Edrington TS and Nisbet DJ. 2006. Effects of nitrate or nitro-supplementation, with or without added chlorate, on *Salmonella enterica* serovar *Typhimurium* and Escherichia coli in swine feces. J. Food Prot. (Accepted)

Gutierrez-Bañuelos H, Slay LJ, Carstens GE, Ramlachan N, Horrocks SM, Callaway TR, Edrington TS, Anderson RC, Nisbet DJ. Zoonotic bacterial populations, gut fermentation characteristics and methane production in feedlot steers during oral nitroethane treatment and after the feeding of an experimental chlorate product. (Submitted)

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