

FATE OF *ARCOBACTER* SPP. UPON EXPOSURE TO ENVIRONMENTAL
STRESSES AND PREDICTIVE MODEL DEVELOPMENT

by

ELAINE M. D'SA

(Under the direction of Dr. Mark A. Harrison)

ABSTRACT

Growth and survival characteristics of two species of the 'emerging' pathogenic genus *Arcobacter* were determined. The optimal pH growth range of most *A. butzleri* (4 strains) and *A. cryaerophilus* (2 strains) was 6.0-7.0 and 7.0-7.5 respectively. The optimal NaCl growth range was 0.09-0.5 % (*A. butzleri*) and 0.5-1.0% (*A. cryaerophilus*), while growth limits were 0.09-3.5% and 0.09-3.0% for *A. butzleri* and *A. cryaerophilus*, respectively. *A. butzleri* 3556, 3539 and *A. cryaerophilus* 1B were able to survive at NaCl concentrations of up to 5% for 48 h at 25°C, but the survival limit dropped to 3.5-4.0% NaCl after 96 h. Thermal tolerance studies on three strains of *A. butzleri* determined that the D-values at pH 7.3 had a range of 0.07-0.12 min (60°C), 0.38-0.76 min (55°C) and 5.12-5.81 min (50°C). At pH 5.5, thermotolerance decreased under the synergistic effects of heat and acidity. D-values decreased for strains 3556 and 3257 by 26-50% and 21-66%, respectively, while the reduction for strain 3494 was lower: 0-28%. Actual D-values of the three strains at pH 5.5 had a range of 0.03-0.11 (60°C), 0.30-0.42 (55°C) and 1.97-4.42 (50°C). z-values for the three strains ranged from 5.20-6.11°C and 5.55-6.28 °C at pHs of 7.3 and 5.5, respectively. D-values for a cocktail of the three strains in raw ground pork were 18.51 min at 50°C and 2.18 min at 55°C. The one strain tested (*A. butzleri* 3556) was highly sensitive to the combined effects of mild heat (50°C) followed by cold shock, exhibiting a 3.2-4.0 log₁₀ decrease in cell numbers at a cold shock temperature of 4°C, as compared to a 0.33-1.12 log₁₀ decrease at a temperature of 16°C. Predictive mathematical models (quadratic and cubic) were constructed from a dataset of growth curves obtained for a cocktail of three *A. butzleri* strains. These models described the main and interactive effects of incubation temperature (12-37°C), pH (6.0-7.5), sodium chloride (0.09-3.5%), sodium nitrite (0-180 ppm), and sodium tripolyphosphate (0-0.012%) levels on the growth characteristics of the cells. Predictions for generation time (GT), lag phase duration (LPD) and Gompertz parameters B and M were analyzed from natural logarithm transformed quadratic models.

INDEX WORDS: *Arcobacter*, *A. butzleri*, *A. cryaerophilus*, D-values, z-values, pH, sodium chloride, sodium nitrite, sodium tripolyphosphate, predictive growth model

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DEDICATION

This work is dedicated to my parents, to my brother, and to my spiritual family here in Athens, Georgia.

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CHAPTER 1

INTRODUCTION

The history of organisms belonging to the genus *Arcobacter* (from the Latin word for ‘arc-shaped’ bacterium) began long before they were officially designated as a separate genus in 1991 (Vandamme et al., 1991a). The close relatedness of *Arcobacter* species (four have been identified to date: *A. butzleri*, *A. cryaerophilus* hybridization groups 1A and 1B, *A. skirrowii* and *A. nitrofigilis*) to members of the genus *Campylobacter*, allows them to share a relatively common background of sources, illness manifestations in humans and animals, and history, dating from the early 1900’s, when the vibrio-like campylobacters were first studied. No discussion on arcobacters would therefore be complete without providing a short summary of the epidemiological role of campylobacters as pathogenic species.

About 15 species and six subspecies of *Campylobacter* have been identified (Atabay and Corry, 1997). Thermophilic campylobacters notably *C. jejuni* and *C. coli* are thought to be the leading cause of foodborne human diarrheal illness in many developed countries including the U.S. (Atabay and Corry, 1998; CDC-MMWR, 2001). These campylobacters are present as a reservoir in the intestinal tract of food animals and have been identified in up to 100% of carcasses and raw poultry products, sometimes at levels of $10^6 - 10^7$ CFU/g of poultry caecal contents. Cross-contamination of carcasses may occur during processing, although other studies have demonstrated the ease with which experimental infections with campylobacters can be spread through a poultry house flock.

Transmission of infection in cases of human campylobacteriosis is thought to occur from raw products (especially poultry) via unsafe food handling practices including unwashed hands, kitchen utensils and implements, insufficient cooking or direct hand to mouth transfer (Atabay and Corry, 1997).

Arcobacter spp. have been known to mainly cause gastritis in humans, and abortions and gastritis in livestock (Wesley, 1994). Estimates of the occurrence of *Arcobacter* spp. in the environment and in raw or cooked food products, may be possible as the result of an educated guess or an extrapolation from *Campylobacter*-related data, since limited studies have been conducted globally to date, on the actual prevalence of these organisms. Furthermore, most classical laboratory tests are geared towards the detection of the thermophilic campylobacters (Bolton et al., 1992) and include the use of antibiotic-containing media, incubation at 42°C and microaerobic growth; thus, other campylobacters or arcobacters could go undetected. Additionally, biochemical tests that accurately identify and distinguish *Arcobacter* genus and species are extremely limited and somewhat unreliable. These tests include catalase activity (*A. cryaerophilus* exhibits a strong catalase reaction and *A. butzleri* a weak one), nitrate reduction, fatty acid analysis, sensitivity to cadmium chloride, growth in the presence of glycine or sodium chloride, and growth on MacConkey agar (Wesley, 1994). However, glycine tolerance, which has been noted in 25-78% of *Arcobacter* isolates is also a major identifying characteristic of *Campylobacter fetus* subsp. *fetus* strains that have been associated with animal abortions. A further confounding feature is the ability of *C. fetus* subsp. *fetus* strains (Wesley, 1994), and the recently discovered capability of *C. jejuni* strains (Engvall et al., 2002) to develop aerotolerance (a distinguishing characteristic of

Arcobacter spp.) on subculture. Cross-reactivity with *C. jejuni* and *C. fetus* antigens in complement fixation tests was also noted in patients suffering from *A. butzleri* illness, where *A. butzleri* was the only enteric pathogen isolated from the patients (Vandamme et al., 1992a). Thus serological tests too, when used as medical laboratory identification techniques, may not completely distinguish between *Campylobacter* and *Arcobacter* spp.

Initial studies on arcobacters, carried out in the 1970s and 1980s led to the recommendation that they be included in the genus *Campylobacter* based on morphology, observed motility, inability to metabolize common carbohydrates in biochemical tests and initially calculated %G + C content. However, their aerotolerant nature, ability to grow below 37°C and inability to react with antisera to *C. fetus* heat-stable A, B and C antigens suggested that they are distinct from other campylobacters, and in particular from *C. fetus*, the *Campylobacter* spp. most commonly linked to abortions in cattle and pigs (Wesley, 1994). *Campylobacter (Arcobacter) cryaerophila* and *Campylobacter (Arcobacter) nitrofigilis* were the first two species of this genus to be recognized for their greater homology (87%) to each other than to other *Campylobacter* spp. (67%) based on partial 16S rRNA sequence analysis (Wesley, 1994). Thus a new genus was proposed (Vandamme et al., 1991a) based on immunotyping, SDS-PAGE of cellular proteins, fatty acid analysis and various nucleic acid hybridization studies. Further studies revealed that aminoglycosides, fluoroquinolones and miocycline were the most effective antibiotics against *A. butzleri* and *A. cryaerophilus*; more specifically ampicillin, carbenicillin, chloramphenicol, kanamycin, metronidazole, nalidixic acid, polymyxin B and streptomycin were determined to be inhibitory to *Arcobacter* spp. (Wesley, 1994). Most isolates were resistant to macrolides including erythromycin. *C. jejuni* and *C. coli* on the

other hand, were noted to be sensitive to erythromycin and clindamycin, while increasing resistance to fluoroquinolones including nalidixic acid is being observed globally (Nachamkin and Blaser, 2000). Though the information put forward in the preceding sections underscores the necessity of achieving a clear clinical distinction between these closely related genera, this goal has not always been historically realized.

Attempts have been made to resolve the confusion in differentiating *Campylobacter* and *Arcobacter* spp. by the use of several molecular and nucleic acid-based techniques. One of the most accurate and useful of these appears to be the construction of genus-specific and species-specific DNA probes complementary to the highly conserved 16S rRNA molecule in *Arcobacter*. A genus-specific *Arcobacter* probe did not react with any member of the taxonomically related *Campylobacter*, *Helicobacter*, *Wolinella* or *Bacteroides* spp. tested. Individual species-specific probes have been designed for *A. butzleri*, *A. cryaerophilus* (each subgroup) and *A. skirrowii* (Wesley et al., 1995). More recently, species-specific oligonucleotide probes were developed based on the highly conserved *glyA* gene, that were used in a PCR-oligonucleotide hybridization scheme to accurately differentiate between *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *A. butzleri* and an *A. butzleri*-like strain. The high sensitivity of this technique would help identify these related type strains in a diagnostic laboratory environment and suggest treatment schemes, since earlier assays were often unable to distinguish between *C. jejuni* and *C. coli* (Al Rashid et al., 2000).

Although studies on *Arcobacter* spp. have spanned only a short period, infections with these organisms cause economic losses in livestock herds and compromise human health. Given the current status of *Campylobacter* as the leading cause of foodborne

gastrointestinal illness in the U.S., and possibly globally, and the possibility that some of the so-called *Campylobacter* infections may be misidentified *Arcobacter*-related infections, as detailed above and mentioned in literature (Engvall et al., 2002), it was deemed necessary to study *Arcobacter* in greater depth. The differences between the two genera noted, to date, are sufficient to presume differences in growth and survival characteristics of the infectious species. In this series of studies, we have attempted to identify hitherto unknown growth and survival characteristics of *Arcobacter* spp. with a view to identifying intervention techniques that would reduce their presence in food products and environments with which they have been associated. Additionally, we have compounded the studies to develop mathematical microbiological models that will predict the growth of *Arcobacter butzleri* under various environmental conditions (temperature, pH, sodium chloride, sodium nitrite and sodium tripolyphosphate levels).

Predictive modeling techniques have been used for a few decades. They have assumed a particular attractiveness recently, with growth that has occurred in the food manufacturing industry, providing consumers with an ever-widening choice of raw, partially processed and read-to-eat grocery store offerings. Wider choices warrant larger manufacturing facilities, higher throughput of materials processed and more modifications in a basic food product recipe. These changes can possibly alter the microenvironment available for growth of food-associated pathogens. Recognition of the impossibility to ‘test-case’ each new food product for microbiological safety has led to an increasing reliance on predictive micromodels, one of the best known of which is the Pathogen Modeling Program (PMP) developed and updated regularly by the USDA. The computer program, now available as an online user-friendly version 6.0.0, has the ability

to predict the growth behavior of several foodborne pathogens (*Aeromonas hydrophila*, *Bacillus cereus*, *Clostridium perfringens*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Shigella flexneri*, *Staphylococcus aureus* and *Yersinia enterocolitica*) and the inactivation patterns of some pathogens, under a series of variable environmental combinations (USDA, 2001). Our study will make available a model similar to the PMP growth models, for *Arcobacter butzleri* in liquid media.

CHAPTER 2

LITERATURE REVIEW

History

John McFadyean first isolated campylobacters in pure culture in 1913, from an aborted sheep fetus. For several years, the organisms were referred to as *Vibrio* (e.g., *Vibrio fetus*) and were thought to mainly cause abortions and infertility in animals. Ewes, goats, horses, dogs, mink, monkeys and rodents were some of the species in which the organism was detected. Almost six decades later, the role of *Campylobacter coli* and *Campylobacter jejuni* (*Campylobacter jejuni* subsp. *jejuni*) in human disease was established, as was the role of the closely related *Helicobacter pylori* (formerly *Campylobacter pylori*) in the incidence of gastritis, peptic ulcers and gastric cancer. In 1963, the genus *Campylobacter* was proposed and includes 16 species and 6 subspecies of fastidious microaerophilic vibrio-like organisms (On, 2001). It is now established that *Arcobacter*, *Campylobacter*, *Helicobacter* and *Wolinella* species make up the rRNA Superfamily VI of the ϵ -class of Proteobacteria and are described as Gram-negative, nonsporing, spiral or curved, motile rods. This Superfamily comprises three rRNA homology groups, the first containing the genus *Campylobacter* and two misnamed *Bacteroides* species, the second, species of *Arcobacter* and the third being more diverse, comprises the genera *Helicobacter*, *Wolinella*, *Gastrospirillum*, *Flexispira*, and a number of other ‘*Campylobacter*-like’ organisms (On and Holmes, 1995). Vandamme and DeLey (1991) further proposed that *Arcobacter* and *Campylobacter* be included in a separate

family (*Campylobacteraceae*) based on their shared features. Ursing et al., (1994) proposed minimal phenotypic, biochemical and molecular standards to describe new species of the family *Campylobacteraceae*. The most studied, medically significant species of *Campylobacter* (other than *C. fetus*) are the ‘thermophilic’ campylobacters (i.e., *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*), so named due to their high optimal growth temperature around 42°C. *C. jejuni* is the most common species in cases of human campylobacter enteritis, followed by *C. coli* that can usually cause between 5-10% of such infections (Skirrow, 1994), though some suggest that this distinction is the result of the higher antibiotic sensitivity and low-temperature (4°C) sensitivity of *C. coli*, thus preventing their competitive survival in antibiotic-containing selective media. *C. jejuni* and *C. coli* do not grow below 30°C and thus probably do not grow in appropriately processed and stored foods. Infection with the genus would therefore be dependent on their survival mechanisms in the food environment.

Disease-causing campylobacters are ubiquitous in warm-blooded animals (where they are sometimes non-symptomatically ‘carried’) and in the environment, and have been commonly isolated from poultry and wild birds. Some campylobacters like *C. jejuni* cause abortions in livestock, commonly in sheep and goats. A significant feature is their ability to exist for long periods of time (several months) as viable, but non-culturable forms (VNC), and thus serve as reservoirs of infection for various animals, and thence, to humans (Colwell and Grimes, 2000). Documented human infection is both foodborne, through contaminated meat, milk and water, or acquired directly from pets and farm animals (Skirrow, 1994) and may take the form of diarrhea, or of bacteremia, in immunologically compromised individuals. A rare complication of *Campylobacter*

infection which occurs in young adults is the Guillain-Barré syndrome (GBS), a complicated paralyzing polyneuritis. Though the mortality rate for GBS is low, patient recovery is slow and the economic costs involved are extremely high (Koenraad et al., 1997). Recent concerns have addressed the role of *Campylobacter*-related organisms (including *A. butzleri*) in HIV patients in developing countries, where the associated 'morbidity and mortality' is 'substantial' (Coker et al., 2002).

The genus *Arcobacter*, proposed in 1991 based mainly on DNA-DNA hybridization and immunotyping, is comprised of four species belonging to rRNA cluster II: *A. nitrofigilis*, *A. cryaerophilus*, *A. butzleri* and *A. skirrowii* (Vandamme et al., 1991a). The type species *A. nitrofigilis* is the only member that has not been isolated from humans or animals. This species is found in a nitrogen-fixing commensalistic association with the roots of the marsh grass *Spartina alterniflora*. *A. cryaerophilus* species are further characterized into two hybridization groups 1A and 1B, indistinguishable by morphological or biochemical tests (Schroeder-Tucker et al., 1996). These organisms (*Campylobacter cryaerophila*, now *Arcobacter cryaerophilus*) were initially described in 1977 in cases of bovine abortions in Northern Ireland (Ellis et al., 1977). The *Spirillum*-like organisms were grown in *Leptospira* isolation media incubated at 30°C for up to 8 weeks. Growth was detectable as a cloudy zone 2-5 mm below the surface of the media 2-3 days after inoculation. Typically, arcobacters resemble campylobacters in morphology, but are able to grow in air, and at lower temperatures (15°C) than most campylobacters. They are 1-3 µm by 0.2-0.9 µm in size, motile, possessing a single, unsheathed polar flagellum. Using dark-field microscopic techniques, long cells (20 µ or more) with a rapid, darting motility are observed. Swarming of cells has been noted on

agar media, and colonies are 1 mm in diameter, convex and nonpigmented (Wesley, 1994). α -hemolysis is a common feature in *A. skirrowii* and occurs in some *A. butzleri* strains (Vandamme et al., 1992b).

Due to the reclassification and renaming of members of the group, studies have been conducted that were aimed at confirming the taxonomic status of preexisting isolates within the defined scheme, or attempting to obtain an accurate technique of classifying newly obtained isolates. A polyphasic taxonomic study was conducted (Vandamme et al., 1991c; Vandamme et al., 1992b) on 77 *Arcobacter* strains originally classified as *C. cryaerophila*. Forty-nine of these were confirmed as *A. cryaerophilus*, whereas 10 were *A. butzleri* and 18 belonged to a then unknown *Arcobacter* species. A detailed description of the characteristics of the emended genus *Arcobacter* has been made available by these authors. Guidelines followed thus far have maintained that two strains exhibiting less than 97% 16S rRNA gene sequence similarity represent different species. However, it has recently been suggested for the ϵ -class of Proteobacteria, that this threshold be lowered to 94% similarity (On, 2001). Kiehlbauch et al., (1991) studied 78 aerotolerant *Campylobacter* isolates, both phenotypically and by DNA hybridization, and determined the existence of 2 DNA relatedness groups: 64 strains fell into *Campylobacter* DNA hybridization group 2 (the proposed name was *C. butzleri* sp. nov.). The majority of the *C. butzleri* strains were isolated from patients with diarrheal illness. The type strain of *C. cryaerophila* and 13 other animal or human strains made up the genetically diverse DNA hybridization group 1. Five strains that were more closely related to the type *C. cryaerophila* were termed DNA hybridization group 1A and the eight that were more closely related to each other were termed DNA hybridization group

1B. *A. butzleri* shares just 40% homology with *A. cryaerophilus*; its closest relative being *A. skirrowii*. Characteristics of these three groups are noted in Table 2.1 (On et al., 1996; Vandamme et al., 1992b; Wesley, 1994; Atabay and Aydin, 2001).

Phenotypic and biochemical tests used in identification of campylobacters and arcobacters

Many of the tests used are described in detail in a report on the methods used for identification and biotyping of campylobacters by Bolton et al. (1992). Tests that help distinguish 'aerotolerant campylobacters' from other *Campylobacter* spp. include hydrolysis of indoxyl acetate, growth temperature, the inability to hydrolyze hippurate and cadmium chloride (CdCl_2) susceptibility. *A. butzleri* is resistant to CdCl_2 while *A. cryaerophilus* is sensitive or variable to CdCl_2 at concentrations tested. As a subclass, ϵ -Proteobacteria are relatively metabolically inert; organic acids, TCA intermediates and amino acids are probably used as carbon sources. The usefulness of the indoxyl acetate test for rapid differentiation of ϵ -Proteobacteria was studied in depth by Popovic-Uroic et al. (1990). They reported that all 34 strains of *C. cryaerophila* tested yielded positive results, but the one strain of *C. nitrofigilis* tested negative. The test can be performed in 5-30 min and the authors strongly promoted this test in comparison with the trimethylamine N-oxide test used by other workers.

Burnens and Nicolet (1993) suggested the inclusion of arylsulfatase and pyrazinamidase activities and susceptibility to polymyxin B in the battery of tests to distinguish *Campylobacter* and related organisms; *Arcobacter* strains tested were negative for all three tests. On and Holmes (1992) analyzed the reproducibility of 9 enzyme tests used in the identification of campylobacters, four (catalase, oxidase,

Table 2.1: Some characteristics of the clinically important *Arcobacter* spp.

Characteristic	<i>Campylobacter (now Arcobacter) butzleri</i>	<i>Campylobacter (now Arcobacter) cryaerophila</i>
% G + C content	30 ± 1 mol%	29-32 mol% ^a
Colony characteristics	Produces larger white colonies on HIA ^b with/without sheep's blood	Small, watery yellowish colonies on HIA
Catalase	Negative or weakly positive	+
Oxidase	+	+
Growth in 1.5% NaCl	+	-
Growth in 3.5% NaCl	V ^c	-
Growth in 1% Glycine	+	-
Effect of temperature	Growth at 15, 25, 30, 36 and 40°C, not at 5°C	Growth at 15, 25, 36°C, not at 5 or 42°C
Nitrate reduction	+	V
H ₂ S production	-	-
Indoxyl acetate hydrolysis	+	+
Urease	-	-
Hippurate hydrolysis	-	-
Growth on MacConkey agar	+	-
Cadmium chloride, 2.5µg	Resistant	Sensitive/V
Antibiotics resistant to (some examples)	Cephalosporins, trimethoprim, macrolides, sulfamethoxazole, amphotericin B	Cephalothin
Antibiotics sensitive to (some examples)	Aminoglycosides, quinolones, colistin, polymyxin B, rifampin, nalidixic acid	Nalidixic acid, aminoglycosides, miocycline

^a DNA hybridization group 1A

^b Heart infusion agar

^c Variable reaction

urease production, hippurate hydrolysis) were found to be fully reproducible for all 21 strains studied. Interestingly, *Arcobacter* spp. are some of the few organisms known to exclusively produce respiratory menaquinones and monomethylmenaquinones (i.e., MK-6 and MMK-6) (Finster et al., 1997), detection of which could be used as an identification test.

Susceptibility to antimicrobial agents has also been used to characterize this genus. Kiehlbauch et al. (1992) studied the susceptibility of 78 human and animal strains of *A. butzleri* and *A. cryaerophilus* (1A and 1B) to 22 antimicrobial agents. All *Arcobacter* spp. studied were susceptible to colistin, polymyxin B and rifampin. Also, the antimicrobial resistance patterns of *Arcobacter* spp. were different from *C. jejuni* and might therefore necessitate a modification of the existing isolation media and methods that are more suitable for the isolation of thermophilic campylobacters. A recent study conducted by Atabay and Aydin (2001) on the susceptibility of thirty-nine *A. butzleri* poultry isolates to 23 antibiotics yielded some interesting results. All isolates were resistant to aztreonam, cefuroxime sodium, cephalothin, orbenin, oxacillin, penicillin G and trimethoprim/sulphamethoxazole. All except one were resistant to Cephalosporins (commonly used in selective media) and Mezlocillin. Twenty-six isolates were also resistant to amoxicillin, amoxicillin/clavulanic acid and ampicillin. All were susceptible to amikacin, chloramphenicol, danofloxacin, enrofloxacin, nitrofurantoin, nalidixic acid, tetracyclines and tobramycin. The authors note that the majority of the isolates were thus found to be resistant to antibiotics commonly used to treat humans and animals. The susceptibility in terms of Minimum Inhibitory Concentrations (MICs) of 111 isolates of the three pathogenic *Arcobacter* spp. to 12 antimicrobial agents, was studied by Houf et

al. (2001b). They concluded that *A. skirrowii* was overall more sensitive to these agents than *A. butzleri* or *A. cryaerophilus*. All isolates tested were highly resistant to amphotericin B, cycloheximide and 5-fluorouracil. Most strains were resistant to novobiocin. Between the two cephalosporins, cefotaxime was more active than cefoperazone. The authors suggest that the environmental isolation patterns observed for *Arcobacter* spp. heretofore, may have been strongly influenced by the type of selective agent incorporated in the medium used, the levels of some of which were only slightly below the inhibitory concentrations observed for *Arcobacter* spp. For example, some *A. cryaerophilus* and *A. skirrowii* strains are susceptible to piperacillin at 64 µg/ml, whilst de Boer et al. (1996) added 75 µg/ml of this agent into their selective medium, and that was perhaps the reason that this group isolated only *A. butzleri* from meats. Also, the inclusion of 32 µg/ml cefoperazone in this medium may be disadvantageous to the growth of stressed cells of *A. cryaerophilus* and *A. skirrowii* since the cefoperazone MIC levels are only slightly above this concentration. They concluded that the use of some currently popular *Arcobacter* medium supplements may not be suitable for isolation of all three species. The most suitable medium that supported all three strains was that of Atabay, [containing (CAT) cefoperazone (8 µg/ml), amphotericin B (10 µg/ml) and teicoplanin (4 µg/ml)] and EMJH (containing 5-fluorouracil). However, neither of these media completely eliminate the incidence of background flora. Hence, a balance has to be struck between medium support for the desired strains and its selectivity. Additionally, an abortifacient strain of *A. cryaerophilus* studied by Fernández et al. (1995) was found to be resistant to colistin, cephalothin, polymyxin B and ampicillin, which are some of the antibiotics used routinely in *Campylobacter* isolation media.

Sources

Arcobacter spp. have been isolated from water, raw foods, poultry, humans and animals with enteritis, and aborted animal fetuses (Wesley, 1996) with reported isolation rates of up to 43% in porcine abortions (Schroeder-Tucker et al., 1996). *Arcobacter* spp. were detected in both healthy and diseased pigs, 40% of fecal samples from healthy swine testing positive (Wesley, 1997). Thus the organisms may be part of the indigenous flora of healthy animals, causing opportunistic infections under suitable conditions. In another study, a higher incidence of *Arcobacter* was observed in aborted porcine fetuses than in porcine fetuses obtained from a slaughterhouse; however, the organism was not significantly associated with rectal, preputial or vaginal swabs taken from the herd that had known reproductive problems. However, Ellis et al., (1977) reported several recoveries of arcobacters from aborted litters and the genital tracts of swine, with higher recoveries from infertile sows than from normal animals. Strains from both diseased and normal animals, however, were not antigenically different, underscoring, again, the probable opportunistic role of the arcobacters involved. In South America, *A. cryaerophilus* was first isolated in 1995 from a case of bovine abortion, in Chile. The organisms were later also found to be associated with porcine abortions in Brazil. de Oliveria (1999) studied the incidence of *Arcobacter* spp. in hog farms with noted reproductive problems and obtained 18 isolates that were mainly *A. cryaerophilus* 1A and 1B. Suarez et al. (1997) reported *Arcobacter* spp. in 33/86 of swine stomachs studied.

The first probable *Arcobacter*-associated swine illness in the U.S. was noted in 1992 in North Carolina, and included late-term abortions, repeat breedings and a higher than usual rate of stillbirths. Arcobacters were recovered from the herd, from fetal kidney

and liver tissue and were identified as *A. cryaerophilus* 1B (69%), *A. butzleri* (7%), and *A. cryaerophilus* 1A (5%). Antibiotics did not provide much relief, but an autogenous vaccine proved efficacious (Wesley, 1994). Another study carried out at an 'integrated swine operation' in Texas (Harvey et al., 1999) reported a low prevalence of *Arcobacter* (5%), but significant contamination with *Campylobacter* and *Salmonella* in cecal and ileocolic lymph node analyses of market age pigs. Hume et al. (2001) reported isolation of *Arcobacter* spp. in growing pigs and nursing sows on three farms, with increased reporting of *Arcobacter*-positive samples as the swine aged. Genotypic variability observed in isolates (mainly *A. butzleri*, with few *A. cryaerophilus*) led to the speculation that the animals were colonized by multiple *Arcobacter* genotypes and that they probably additionally underwent genomic rearrangement in animal passages. The authors therefore stress the importance of genotypic phenotyping as a means of isolate identification and monitoring during outbreaks.

Surveys of cattle have detected *Arcobacter* spp. in 10.52 % dairy cattle fecal samples (Wesley, 1997). The organism was found in both healthy animals and in feces of those with diarrhea or mastitis. Wesley et al., (2000) studied the shedding of *Campylobacter* and *Arcobacter* spp. in the feces of dairy cattle in various U.S. states, using a rapid multiplex-PCR assay and detected *Arcobacter* spp. in animals from 22 of 31 farms, with 14.3% fecal samples being positive. The organism was detected most frequently in cull market cows. Analysis of farm management practices revealed that feeding of alfalfa and use of individual waterers protected the cattle from infection with *Arcobacter*, and the authors suggest that feed and dietary supplements may alter gut homeostasis, thus influencing microbial colonization. However, use of chlorinated

drinking water had no such protective effect. With respect to individual animals, cows from the Southern U.S. were more likely to harbor *Arcobacter* (18.6%) than cows from the North (12.4%). Also, alley flushing as the method of cleaning premises resulted in higher incidence of *Arcobacter* and infection was lower in lactating cows fed brewer's by-products. A comparison of factors influencing colonization with *C. jejuni* with those affecting *Arcobacter* spp. was attempted, but no correlation was observed. In a separate study, Canadian researchers reported the incidence of *A. skirrowii* among other arcobacters recovered in livestock abortions and in bovine enteritis, a hitherto rare occurrence (Wesley, 1994).

Epidemiological characteristics and pathogenicity

Though *A. cryaerophilus*, *A. butzleri* and *A. skirrowii* have each been associated with infections, *A. butzleri* is thought to be the primary human pathogen (Wesley, 1996). Atabay and Corry (1998) have suggested a zoonotic basis for human disease, which is mainly self-limiting enteritis, and rarely bacteremia or appendicitis. It has also been postulated that the main modes of transmission of *Arcobacter* spp. include food and water. The disease is often acquired through travel in developing areas/countries or areas with unhygienic food preparation practices, cross-contamination, and person-to-person contact. *Arcobacter* spp. are not routinely found in human feces. In a Belgian study in the early 1990s, less than 0.1% stool specimens were positive for *Arcobacter*. In addition, no arcobacters were found in 879 samples from 468 elderly patients during a one-year study in Belgium (Phillips, 2001).

The true infectious and invasive nature of this genus has yet to be defined since infection-induced abortions and experimental infection of laboratory animals have not

been successful. Researchers were able, however, to induce mastitis experimentally, using ‘aerotolerant’ campylobacters (Wesley, 1994). Fernández et al. (1995a) studied two strains of *A. cryaerophilus* by the rat ileal loop test and by culture in Hep-2 lines for an invasion assay. Positive results for the ileal loop test were confirmed by loop distension, fluid accumulation and enhanced electrolyte concentration, while internalization of cells in cultured monolayers confirmed the invasive capacity of both strains. An earlier study involving primates that were naturally infected with *A. butzleri* observed that the animals developed colitis; no *A. butzleri* were isolated from normal feces. It was suggested that this infection was endemic in the primate population studied, and probably perpetuated through infected food or water. These observations may help determine the pathogenesis of infection in humans (Anderson et al., 1993). Higgins et al. (1999) reported the fatal infection of a 20-yr old female rhesus macaque with diarrheal illness over a 10-month time period, in Montreal, Canada. Fecal examination yielded an *Arcobacter*-like isolate that was identified as *A. butzleri*. The illness did not respond to chemotherapy.

Wesley et al. (1996) studied the colonization of newborn piglets with the three pathogenic *Arcobacter* spp., indicating their probable invasiveness. *Arcobacter* spp. were recovered from lung, kidney, ileum, liver and brain tissues, but no gross pathological lesions were observed. Musmanno et al. (1997) studied the potential virulence features, biotype, serotype, toxin profile, adhesivity and invasivity of 18 *A. butzleri* isolates obtained from river water samples in Italy. Six biotype and five serotype patterns were observed, all strains but one were cytotoxic *in vitro*. This one strain however produced an adherent and a cytotoxic-like effect. No evidence of invasiveness or cytolethal activity was seen. The authors believe that these results support the theory of potential virulence

in *A. butzleri*. Tsang et al. (1996) characterized the *Arcobacter* haemagglutinin to be an immunogenic protein ~ 20 kDa, possibly with a lectin-type structure which binds to RBCs via a glycan receptor containing D-Gal as part of its structure. Finally, Harrass et al. (1998) detected five kbp plasmids in 24% of *A. butzleri* poultry isolates. No isolate contained more than one plasmid and there was no correlation between antimicrobial resistance pattern and incidence of a plasmid. Thus, the significance of plasmid presence in arcobacters has yet to be elucidated.

Identification methodology

Arcobacter spp. are considered 'emerging' pathogens based on the characteristics they share with campylobacters, potentially extending from morphological similarities to infectious capabilities and transmission routes (Wesley, 1996). Miller et al. (1998) discuss characteristics of *A. butzleri* that contribute to its consideration as an 'emerging' pathogen, and suggest that factors involved in the emergence of *Escherichia coli* O157:H7 may be shared by *A. butzleri*. A topic of some discussion has been the possible misidentification of *Arcobacter* spp. as campylobacters, leading to an underestimation of the role of arcobacters as pathogens (Wesley, 1996).

Molecular techniques are increasingly being used for definitive identification of this usually hard-to-identify genus. Many recently constructed molecular identification methods have been based on bacterial 16S rRNA sequences, as these molecules have 'crucial structural constraints' thereby making certain regions in the molecule common to all bacteria. The intervening variable regions can thus be targeted for amplification, conferring on them diagnostic potential. Newer PCR-based DNA fingerprinting methods using *Campylobacter flaA/flab* and 16S rRNA *A. butzleri*-specific genes have

successfully helped distinguish between *Arcobacter* and *Campylobacter* strains. Conventional biotyping (e.g., API Campy[®]) methods would have identified the arcobacters as atypical campylobacters. One study that focused on achieving this distinction, involved testing of strains from various drinking water treatment plants and surface water sources in Germany (Jacob, 1996). Wesley et al. (1995) devised *Arcobacter*-specific and *A. butzleri*-specific 16S rRNA-based probes which indicated interspecies levels of similarity of 94% or more, within the bacterial group, whereas the similarity between arcobacters and campylobacters was ~ 86%. Bastyns et al. (1995) described a 23S rRNA based PCR assay for detection of *Arcobacter* spp. and each of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. Cardarelli-Leite et al. (1996) devised an RFLP method for analysis of PCR-amplified DNA from the 16S rRNA gene and used it to type 148 strains from the rRNA Superfamily VI. A 283-bp fragment was generated from all species and the simple, rapid method was able to distinguish between *Campylobacter* spp. and differentiate between closely related genera of the Superfamily. *A. butzleri* was found to give a restriction pattern that was unlike the other *Arcobacter* species.

Harmon and Wesley (1997) devised a multiplex PCR assay that both identifies *Arcobacter* isolates and distinguishes between species. Two primer sets, the first targeting the 16S rRNA genes of *Arcobacter* spp. and the second an area of the 23S rRNA gene unique to *A. butzleri*, were used. This assay was used to rapidly screen 410 swine fecal samples and determined that an isolate previously classified as *A. cryaerophilus* was *A. butzleri*, based on the PCR products obtained. Subsequently, de Oliveira et al. (1997) studied 17 porcine isolates of *Arcobacter* spp. from Brazil. These

isolates, which were phenotypically presumed to be *A. cryaerophilus*, were confirmed by PCR and RFLP as *A. cryaerophilus* 1A (24%), 1B (71%) and *A. butzleri* (6%). The observations were in agreement with the reported U.S. levels of *Arcobacter* in porcine abortions (i.e., *A. cryaerophilus* 1A, 16%; *A. cryaerophilus* 1B, 60%; *A. butzleri*, 8%). Hurtado and Owen (1997) devised a PCR technique targeted at 23S rRNA genes from 118 strains of *Campylobacter* and *Arcobacter* and used the restriction patterns as an identification scheme for this bacterial family. *A. cryaerophilus* and *A. skirrowii* had identical restriction patterns, while *A. butzleri* and *A. nitrofigilis* had unique specific combinations of patterns. These investigators believe that use of the 23S rRNA region is more discriminating as it is larger and has more variable residues.

Marshall et al. (1999) devised a two-step rapid method based on PCR-RFLP of the 16S rRNA gene to differentiate *Campylobacter*, *Arcobacter* and *Helicobacter* isolates. The method can use either purified DNA or crude cell lysates and has potential for use as a clinical laboratory diagnostic tool. Al Rashid et al. (2000) devised a PCR assay targeting degenerate primers in the *glyA* gene to identify various *Campylobacter* and *Arcobacter butzleri* strains. González et al. (2000) tested a PCR technique which amplifies a 181-bp 16SrRNA fragment, on 96 retail chickens, 53% of which were positive for *Arcobacter* spp. Samples were enriched in *Arcobacter* broth (Oxoid) and plated on mCIN (cefsulodin-irgasan-novobiocin) agar with added CAT (cefoperazone-amphotericin-teicoplanin) to reduce competing flora. A 16 h enrichment was found to be optimum for PCR amplification and results were available within 2 working days.

Winters and Slavik (2000) developed a multiplex PCR assay to simultaneously detect and distinguish *A. butzleri* and *C. jejuni* in the same reaction tube, and the method

was tested by inoculating various types of fresh and ready-to-eat foods at a level of 20 CFU/g of each organism, or in combinations of strains, at the same level. An 8-h detection time is necessary after a 24-h enrichment period. The authors postulate that this rapid, specific technique would decrease the chances of misidentification between these common human pathogens. Houf et al. (2000) devised a sensitive, specific, multiplex-PCR assay based on 16S and 23S rRNA sequences with 5 primers, for the simultaneous detection and identification of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. The method was tested with the successful detection of *A. butzleri* and *A. cryaerophilus* from 34 neck skin and 14 poultry meat samples, after a 24-h enrichment in *Arcobacter* broth (Oxoid) with CAT supplement. An alternate enrichment-mPCR method, using *Arcobacter* Enrichment broth of de Boer et al. yielded *Arcobacter* spp. in 39% fewer samples. Most recently Antolin et al. (2001) successfully enumerated *Arcobacter* spp. at levels of 10^1 - 10^4 CFU/g from inoculated raw chicken portions using a combined PCR-ELISA assay preceded by a short selective enrichment step.

Polyphasic identification methods

Since observable phenotypic characteristics are often still the most common method for identification, use of a probability matrix for identification of an unknown strain based on a study of 344 strains and using 67 tests, has been investigated (On et al., 1996). Another study analyzed 347 strains and examined for 67 phenotypic characteristics by computerized numerical analysis, to confirm groupings and gene-based relationships in these 'taxonomically complex and economically important' bacteria (On and Holmes, 1995). Vandamme et al., (1991b) reported the use of numerical analysis of

cellular protein patterns of *Campylobacter*, *Arcobacter* and *Helicobacter* as a useful identification tool since all these species form separate electrophoretic clusters.

Harrass et al. (1998) analyzed the biochemical capabilities, antimicrobial resistance patterns and plasmid content of 89 *A. butzleri* isolates from 170 broiler chickens in Germany. They suggest that biochemical tests (API Campy[®]) and antimicrobial resistance typing are the most 'discriminatory' tests, which might be used along with plasmid analysis for epidemiological identification purposes. Nineteen API Campy[®] profiles, 13 antimicrobial resistance patterns and 4 plasmid profiles were identified in the 89 isolates. Wesley and Franklin (1996) studied plasmid profiles in 80 field isolates of *Arcobacter* spp. Plasmids ranging in size from 3.1- to > 16.2 kb were detected in 6%, 36% and 54% of *A. skirrowii*, *A. butzleri* and *A. cryaerophilus*, respectively. The overall *Arcobacter* plasmid detection rate was 33% and was similar to the rate of detection in *H. pylori* (39%) but lower than that in *C. jejuni* (55-100%). In comparison, a previously mentioned study by Harrass et al., (1998) detected plasmids in 24% of *A. butzleri* poultry isolates.

Isolation from foods and comparison of isolation methods

Jacob et al. (1996) isolated *Campylobacter*-like organisms (CLOs) that were identified as *A. butzleri*, from German surface waters and drinking water treatment plants. Researchers studying the incidence of *Arcobacter* spp. in meats in the Netherlands isolated the organisms from 24.1% retail poultry products and from 1.5 % and 0.5 % minced beef and pork products, respectively, using a method that included enrichment in *Arcobacter* Selective Broth (ASB) followed by isolation on semisolid *Arcobacter* Selective Medium (ASM). These were mainly identified as *A. butzleri* serogroup 1, the

most common serogroup of human isolates, suggesting a significant transmission link from poultry to humans (de Boer et al., 1996). Meanwhile, in France, Squinazi et al. obtained 162 *A. butzleri* isolates from 201 raw poultry carcasses (de Boer et al., 1996). In an interesting study in the U.K., arcobacters were isolated from all ten ready-to-eat supermarket and fifteen post-eviscerated broiler chicken carcasses after enrichment. The ready-to-eat samples were from four different processing plants, while the raw samples were obtained directly from a poultry slaughter facility, from four different flocks. It was also noted in this study that campylobacters were only isolated from these carcass samples after direct plating of original samples, but not after the common enrichment step that had yielded the *Arcobacter* isolates. On the other hand, caecal content samples of raw poultry were both directly plated and enriched and yielded predominantly *Campylobacter* isolates and just one *Arcobacter* isolate. It was thus concluded that unlike campylobacters, arcobacters were most likely not normal flora of chicken intestines but are probably environmental contaminants, and that a combination of direct plating steps and enrichment be employed in routine analysis of samples for CLOs (Atabay and Corry, 1997).

An analysis of raw ground pork samples from Iowa pork-processing facilities over a 9-month period revealed the association of the organisms with unhygienic conditions and underscored the fact that cross-contaminating processes probably perpetuated their survival in the environment. While the isolation rate was 5% in four out of the five plants studied, the remaining plant demonstrated consistently high isolation rates (89-90%) for *Arcobacter* spp. Though commodity selection may have been the primary factor in this study, reports on the detection of *Arcobacter* spp. in poultry

outnumber those studying red meats (Collins et al., 1996b). These results are comparative when examining available literature noting the higher frequency of detection of *Campylobacter* spp. in poultry than in red meats (de Boer et al., 1996; Wesley, 1997).

A. butzleri was recovered from 81% poultry carcasses examined in one French study, while in Canada, the organism was recovered from 97% of poultry carcasses from 5 processing plants (Wesley, 1997). In a study involving turkey samples obtained from a single Iowa processing plant, 80% were found to be positive for *A. butzleri* (Manke et al. 1998). The authors later surveyed mechanically separated turkey samples in three Iowa processing plants and determined the presence of *Arcobacter* spp. (77%) and *A. butzleri* (74%) in the samples. Analysis of ERIC (Enterobacterial Repetitive Intergenic Consensus) sequences in 121 *A. butzleri* strains tested suggested that multiple sources of contamination existed. Wesley and Baetz (1999) detected *Arcobacter* spp. in 15% of chickens from three flocks, while *A. butzleri* was found in 1% cloacal samples. On attempting to experimentally infect poultry with *A. butzleri*, no *Arcobacter* were detected in chickens 5 days post-infection, whereas *A. butzleri* was recovered in 6% of outbred turkey poults and 65% of Beltsville White turkey poults. Recovery rates in the same study for infection with *C. jejuni* were higher than for *Arcobacter* spp.

Atabay et al., (1998) isolated 99 strains of *Arcobacter* from 10 supermarket and 15 abattoir chicken carcasses and attempted to differentiate them by several methods, including the API Campy[®] system and Preston's 16-test scheme. Fifty of these strains were further analyzed by SDS-PAGE of proteins and a probabilistic phenotypic identification scheme (partially based on the scheme of On et al., 1996). *A. butzleri* was detected on all carcasses. Smaller numbers of carcasses yielded *A. cryaerophilus* and *A.*

skirrowii. Using the API Campy[®] scheme only 20/99 strains were correctly identified as *A. cryaerophilus*, since this was the only organism included in the database when the testing scheme was constructed. Preston's scheme identified all *Arcobacter* strains as *A. cryaerophilus* because of the same limitations. The computer-assisted probabilistic scheme correctly identified all but one strain. The authors report that the comparatively weak catalase reaction (10 s after addition of 6% H₂O₂) observed in *A. butzleri* strains, as well as its abundant growth on blood agar after 2-3 d incubation, could be a useful diagnostic tool in *Arcobacter* species differentiation. As opposed to this, both *A. cryaerophilus* and *A. skirrowii* strains gave an immediate strong positive catalase reaction and grew as small colonies even after 3 d of incubation. Additionally, mCCDA modified charcoal cefoperazone deoxycholate agar) was not recommended as an isolation medium, as it failed to support the growth of *Arcobacter* spp. present. This was attributed to the possible synergistic sensitivity of the genus to sodium deoxycholate and cefoperazone. The findings of this study underscore the widespread prevalence of arcobacters, especially of *A. cryaerophilus* and *A. skirrowii*, hitherto unassociated with poultry.

A study conducted on four flocks of commercially reared ducks detected the three pathogenic *Arcobacter* spp. on carcasses but not in cecal contents, suggesting that members of this genus are not part of the normal flora of ducks, but are probably introduced through the processing environment (Ridsdale et al., 1999). Interestingly, detection of arcobacters generally necessitated selective enrichment and was almost never obtained from direct plating of samples. An earlier study (Ridsdale, 1998) detected the same three *Arcobacter* spp. on 50 d-old Pekin duck carcasses. The most effective isolation methods proved to be selective enrichment in *Campylobacter* enrichment broth

containing CAT supplement followed by plating onto mCCDA (or blood agar) after filtration.

Johnson and Murano (1999b) studied the effectiveness of three methods aimed at isolating *Arcobacter* spp. from mixed poultry microflora. Two of these were the previously published methods using EMJH P-80 and CVA-B (cefoperazone-vancomycin-amphotericin B), and the method of de Boer et al. using *Arcobacter* Selective broth and medium (ASM). The third method was one devised by the authors, using JM (Johnson-Murano) broth (special peptone number 2, 0.05% pyruvate, 0.05% thioglycollate, 3% activated charcoal, 0.25% bile salts number 3, 32 mg/L cefoperazone, 200 mg/L 5-fluorouracil and 0.2% agar) followed by plating on JM medium composed of special peptone number 2, 0.05% pyruvate, 0.05% thioglycollate, 32 mg/L cefoperazone and 5% defibrinated sheep's blood. The JM method was reported to be the most efficient and the fastest, detecting *Arcobacter* in 42/50 samples, as compared to 24 and 15 positive samples detected by methods 1 and 2, respectively. More recently Houf et al. (2001a) developed a selective supplement that is composed of amphotericin B (10 mg/L), cefoperazone (16 mg/L), 5-fluorouracil (100 mg/L), novobiocin (32 mg/L) and trimethoprim (64 mg/L). When used with *Arcobacter* broth (Oxoid), the supplement allows growth of all three pathogenic *Arcobacter* species, at the same time suppressing background flora in poultry and poultry products. No enrichment procedure was used. Interestingly, the authors determined that the contamination levels of *Arcobacter* in Belgian poultry products examined (broiler neck skin, skinless chicken breast meat) were higher than the levels of thermophilic *Campylobacter* spp.

The presence of *Arcobacter* spp. in seafood and raw milk remains unknown. Eggs do not seem to be a source of arcobacters as reported in an Italian study (Phillips, 2001; Zanetti et al., 1996b). No arcobacters were detected in 57 manually shelled egg samples and in the same study, only one pork sample of 130 meat samples yielded an *A. butzleri* isolate.

***Arcobacter* spp. in non-food environments**

Unusual occurrences of unnamed members of the genus include the detection of naturally-occurring *Arcobacter* spp. with two other genera in ground water. Outgrowth of the cells resulted in the clogging of a simulated fracture in a limestone aquifer (Ross et al., 2001). The organisms created a biobarrier through production of extracellular polymers, similar to the process of biofilm formation. Telang et al. (1999) isolated a nitrate-reducing sulfide-oxidizing *Arcobacter* strain (FWKO B) from oil fields in Western Canada, together with a *Thiomicrospira* strain. FWKO B survived at higher sulfide concentrations as compared to the *Thiomicrospira* spp. and was thought to derive its energy in absence of acetate, not by oxidation of sulfide by nitrate, but by the reduction of sulfur to sulfide with hydrogen as the electron donor. Use of this organism in bioremediation of sulfide – containing oil water mixtures was suggested. Teske et al. (1996) identified an *Arcobacter* strain in a co-culture that was capable of conserving sulfate reduction after oxygen exposure. The other member was a strain of *Desulfovibrio*.

Maugeri et al. (2000) isolated *Arcobacter* from mussels (4 isolates) and from the surrounding brackish water (16 isolates) at 35.8 ‰ salinity and a pH of 9.1. Of these, 14 were identified as *A. butzleri* and two as *A. nitrofigilis*. Stampi et al. (1999) reported the presence of *A. butzleri* in all 4 stages of sewage sludge processing (primary, activated,

thickened, anaerobically digested) in an Italian sewage treatment plant studied, with a frequency of 80% in activated and thickened sludge and 41% in digested sludge. In general, the primary sludges were more contaminated than the secondary sludges. Numbers were higher in Spring/Summer ($\sim 5.1 \times 10^4$ cells/g dry matter), peaking in April, May, June and September. The uniformly higher numbers of *Arcobacter* across the processes was attributed to the ability of the organism to survive and grow under aerobic conditions, while the seasonal variation observed was the same as that for *Campylobacter*. Zanetti et al., (1996a) compared the efficacy of halogenated disinfectants on the inactivation of *A. butzleri* in secondary sewage effluent in Bologna, Italy, after \sim 20 min contact time. Overall, *A. butzleri* though not completely destroyed, was more sensitive to the disinfectants than other bacteria tested. Chlorine dioxide (at 2, 4 ppm) was more effective than bromine chloride (4, 8, 12 ppm). Thus at the doses considered economically viable, BrCl was not a good choice as a disinfectant. In an earlier study, Stampi et al. (1993) monitored *A. cryaerophilus* numbers in sewage effluents undergoing two secondary treatments (air, activated sludge) in a Bologna, Italy, treatment plant. Air and activated sludge treatments reduced numbers by 97.1% and 97.8%, respectively. Tertiary treatment with 2 ppm chlorine dioxide removed 99.9%, bacteria. On average, for all sewage types, *A. cryaerophilus* levels were three times higher than thermophilic campylobacter levels.

Additional characteristics and studies on individual members of the genus

***Arcobacter*.**

Arcobacter butzleri

From 16S rRNA analysis, it was determined that *A. butzleri* was most closely related to *A. skirrowii*. It can reduce nitrates, has negative to weak catalase activity, can grow on MacConkey agar and in the presence of 8% glucose (Wesley, 1994). Lior serogroups 1 and 5 were the most common human isolates, followed by 2, 6 and 12 (Mansfield and Forsythe, 2000). In 1983, several children in an Italian school, between 3-7 years of age, suffered from an outbreak of gastrointestinal illness showing symptoms of recurrent abdominal cramps but no diarrhea, lasting between 5 and 10 days. Three of the children were hospitalized for up to 8 days. Symptoms occurred 2-3 times daily and did not respond to anti-spasmodic drugs. In the subsequent investigation *A. butzleri* Lior serogroup 1 was the only enteric pathogen isolated from the feces of all the children involved. Since there was no common food or water source of the outbreak that could be identified (although farm animals were present on the school grounds), and since the children acquired the infection in succession, this particular case suggested person-to-person transmissibility of the disease. PCR-ERIC fingerprinting established relatedness of all 14 outbreak strains isolated and their distinctiveness from other *A. butzleri* stains (Vandamme et al., 1992a; Vandamme et al., 1993). *A. butzleri*-associated disease was studied in 22 patients by Kiehlbauch et al., most exhibited symptoms of abdominal pain and nausea, and less commonly fever, chills, vomiting and malaise (Vandamme et al., 1992a). *A. butzleri* bacteremia was also reported in a patient with liver cirrhosis (Yan et al., 2000). On et al. (1995) reported the infection of a U. K. newborn with multiple-

antibiotic resistant *A. butzleri* together with *C. freundii* and group B streptococci. Infection seemed to have occurred *in utero* and treatment with intravenous penicillin and cefotaxime resolved the problem. A study involving Thai children suffering from diarrhea revealed that 16% of the *Campylobacter*-like isolates involved were *A. butzleri*, also called Group 2 aerotolerant *Campylobacter* spp. Children infected with this organism were 1-2 years old and had mild diarrhea with loose or mucoid stools. Infection with *C. jejuni* on the other hand, results in a more acute, watery diarrhea (Taylor et al., 1991). Lerner et al. (1994) reported some of the few documented primary clinical associations of *A. butzleri* in two immunosuppressed patients with underlying chronic disease. They were hospitalized with persistent diarrhea and severe abdominal cramps. The sole pathogen isolated from fecal samples was *A. butzleri*. In both instances, the symptoms reduced after antibiotic administration. The preceding case reports point strongly to the pathogenic and invasive capacity of this species.

In a study of 131 strains of *A. butzleri*, water and sewage were noted as the most frequent nonhuman sources of the organisms, underlining the fact that water supplies (possibly unchlorinated) are one of the key agents of disease transmission. Over a period of several months, Jacob et al. (1993) detected ‘campylobacter-like organisms’ at various stages of a German drinking water reservoir supplied by surface waters. The organisms were later identified as *A. butzleri*. Jacob et al. (1998) isolated 147 *Campylobacter*-like strains from six German drinking water treatment plants over a two-year period. One hundred of these were identified as *A. butzleri*, 24 as *Arcobacter* spp., 17 as *A. butzleri*-like and 6 as *Campylobacter* spp. *A. butzleri* serotype 1 was predominant, followed by serotypes 17, 19 and 2, with some of these serotypes being plant-specific. Biotypes 8A,

8B and 4A were the most common, with *A. butzleri* one, biotype 8A being detected across all plants. The organism was isolated at all treatment phases, including drinking water, though more frequently from raw than from pure water. Rice et al. (1999) reported the recovery of *A. butzleri* from a contaminated well (that experienced a breakdown in its automated chlorination system) that was the source of water implicated in an outbreak of gastrointestinal illness at a Girl Scout camp, in Idaho. The organism survived in the well water for 16 d at 5°C and declined by less than 0.5 log₁₀ units therein. The isolate was sensitive to chlorine, the mean log₁₀ reduction for three strains was 5.06 min /1min/5°C.

A. butzleri has been recovered from up to 100% of chicken carcasses (Lammerding et al., 1994), up to 91% mechanically separated turkey samples (Manke et al., 1998) and 14 macaques with diarrhea (Anderson, 1993). *A. butzleri* (predominantly Lior serogroup 1) was detected on 96.8% poultry carcasses and in 50% turkey drumsticks, 57% ground chicken, 86% ground turkey and 100% whole frying chickens studied by Lammerding et al. (1996). The authors suggest that the organism may have a significant reservoir in poultry products. Interestingly, they recognized slight variations in colony morphology from a single plate/sample (up to four types) that were identified as different serogroups of *A. butzleri*. It is possible that genomic rearrangement could have occurred, a feature also noted in *A. butzleri* isolated in a study involving swine (Hume et al., 2001).

Arcobacter cryaerophilus

This organism possesses a distinctive fatty acid profile (with 16:1 ω 7c and 16:1 ω 7t) that distinguishes it from other bacteria (Moss and Daneshwar, 1992; Vandamme et al., 1992b). It has been isolated from canal waters in Bangkok. More

recently, blood-culture of a traffic accident fatality in Hong Kong revealed the cause of a bacteremia to be a slow-growing organism, unidentifiable by conventional means. 16S rRNA sequencing identified the organism as a strain of *A. cryaerophilus* (Woo et al., 2001). A study of aborted bovine fetuses in a cattle herd in North-Rhine-Westfalia established a definite etiological association between infectious abortions and *A. cryaerophilus* (Parvanta, 1999). This study also emphasizes the difficulties encountered in identifying the organism and differentiating it from other *Campylobacter* spp., in this case from *C. fetus* subsp. *venerealis*. Fernández et al., (1995b) reported isolation of *A. cryaerophilus* from the abomasum of an aborted bovine fetus in Chile. Hsueh et al. (1997) reported the isolation of *A. cryaerophilus* 1B from the blood of a uremic patient with hematogenous pneumonia, the infection was controlled with ceftizoxime and tobramycin. Boudreau et al. (1991) attempted to devise a serotyping scheme for *A. cryaerophilus* strains using homologous and heterologous antisera developed in rabbits. Only 35% of the strains were typed with heat-labile formalinized whole cell antigen and 61% were typed with the heat-stable boiled whole cell antigen.

Arcobacter skirrowii

This species was included in the genus in 1991. It has been recovered from the feces of diseased lambs and from cases of abortions in swine, sheep and cattle (Vandamme et al., 1992b). 16S rRNA analysis has determined that it is more closely related to *A. cryaerophilus* than to *A. butzleri* (Wesley, 1994).

Arcobacter nitrofigilis

Covert and Moran (2001) determined that *A. nitrofigilis* made up 26% of the bacterial population enriched from low-molecular weight fractions of dissolved organic carbon obtained from a Southeastern U.S. estuary.

Unidentified *Arcobacter*-like Proteobacteria

Gevertz et al. (2000) isolated an obligately chemolithotrophic nitrate-reducing *Arcobacter*-like organism (92% similarity) from oil field brine in Saskatchewan, Canada. Although this isolate was catalase and oxidase negative unlike other arcobacters, 16S rRNA analyses suggest that this organism is a novel member of the genus and plays a significant role as a sulfur reducer in its petroleum-rich habitat. Snaidr et al. (1997) found potentially pathogenic *Arcobacter* spp. (4% of bacterial population studied, 98-99% similarity to *A. cryaerophilus*) in activated sewage sludge in Germany. They conclude that nonpathogenic environmental strains of *Arcobacter* spp. may exist and may be common in sewage sludge. Voordouw et al. (1996) while studying oil field bacteria in western Canada identified 47% of clones as a sulfide-oxidizing *A. nitrofigilis*-like organism. Zubkov et al. (1999) isolated an *Arcobacter* spp. most closely resembling *A. nitrofigilis* from the North Sea. A 1997 study conducted on Romanian Black Sea shelf sediments (Thamdrup et al., 2000) to characterize dissimilatory manganese reducing bacteria, determined by molecular screening methods that two of the clone types studied were affiliated with the *Arcobacter* branch, part of the ϵ subclass of Proteobacteria, and played a significant role in these sediments. However, continued culturing of the organisms from the initial enrichments was not successful. In another study (Watanabe et al., 2000), 11 clones obtained during the study of petroleum-contaminated groundwater

from underground crude oil storage sites in Japan were most closely related to *Arcobacter* spp., in particular to *A. nitrofigilis*. Recently, a novel coastal marine isolate ‘*Candidatus Arcobacter sulfidicus*,’ that produces hydrophilic, rigid, irregular sulfur filaments as a metabolic end product, was described by Wirsén et al. (2002). ‘Candidatus’ is the provisional status ascribed to incompletely described prokaryotes. This isolate rapidly excreted sulfur filaments (0.5-2 µm by 20-500 µm) in laboratory culture systems, and the filaments became thickened through sulfur deposition by radially attached members of the population. The considerable ecological impact of this metabolic activity, which may contribute significantly to primary production in marine environments, has been hypothesized by the authors. Thus, it is probably accurate to say that the majority of the studies to date that have involved *Arcobacter* spp. have focused more on its pathogenic role, however, environmentally significant species do exist, and need further investigation.

Survival characteristics

Limited information is available on the survival of *Arcobacter* spp. when exposed to variable laboratory-induced or environmental conditions, as compared to the wealth of information available on the characteristics of some other pathogenic species. Thus comparisons continue to be drawn between this genus and the best-known related species, *Campylobacter*. *A. butzleri* was found to possess a slightly higher resistance ($D_{10} = 0.27$ kGy) to irradiation in ground pork as compared to *C. jejuni* ($D_{10} = 0.19$ kGy). Thus, even low irradiation doses of 1.5 to 4.5 kGy would therefore, be sufficient to eliminate its presence in ground pork (Collins et al., 1996a). Exponential phase *A. butzleri* cells (NCTC 12481) were reported to be less heat sensitive than stationary phase

cells, with observed z values of 8.1 and 7.4°C for exponential and stationary phase cultures, respectively. (Hilton et al., 2001). With respect to disinfectant action, in water, *A. cryaerophilus* was sensitive to pure oxygen, but showed some resistance to 2 ppm ClO₂ (Stampi et al., 1993). *A. butzleri* was sensitive to chlorine in well water (0.46 ppm free Cl₂), undergoing a mean 5.06 log₁₀CFU/ml reduction in 60 s (Rice et al., 1999). Zanetti et al., (1996a) reported that *A. butzleri* was more sensitive to disinfectant action in secondary sewage effluent, as compared to other bacteria (heterotrophs, coliforms, fecal streptococci). A higher reduction of *A. butzleri* occurred with 4 ppm ClO₂ than BrCl (4/8/12 ppm) after an approximate contact time of 20 min. Phillips (1999) examined the antimicrobial properties of preservatives at the concentrations usually found in foods, and reported that 0.5, 1 and 2% lactic and citric acids inhibited growth in culture of *A. butzleri*, with citric acid being the more effective and yielding no viable cells after 8 h. Sodium lactate (1/1.5/2 %) inhibited growth up to only 7-8 h, while sodium citrate (0.5/1/1.5 %) was more effective than sodium lactate. Nisin inhibited growth at 500 IU/ml over 5h. Nisin also enhanced the effects of lactic and citric acids and sodium lactate, but did not enhance the effect of sodium citrate. In a subsequent study, Phillips and Duggan (2001) reported that EDTA (1-20mM) alone, or in combination with nisin (500 IU/ml) or trisodium phosphate (0.5 mM) inhibited growth of *A. butzleri*, short-term simultaneous exposure with the agents being more effective than sequential treatment.

Campylobacters are very sensitive to drying, especially at room temperature, and to chlorine treatment. Reduction in cell numbers due to drying effects is influenced by the suspending medium, temperature, and the extent of drying. This is probably the reason why the organisms are less commonly isolated from red meats (with a lower surface

humidity) than poultry (Nachamkin and Blaser, 2000). The organisms were recovered from the surface of tiles (aluminum, stainless steel, formica, ceramic) in experiments, only as long as the surfaces were visually moist (Oosterom et al., 1983). For chlorine, more rapid destruction of *C. jejuni* in buffer, by both free chlorine (0.1 ppm) and monochloramine (1 ppm) was observed at pH 6 than at pH 8, at both 4° and 25°C. However, on poultry carcass surfaces, campylobacters were detected in spite of the presence of up to 340 ppm chlorine in chilling water. Thus, the protective effects of food surfaces or tissues on the survival of these organisms during intervention techniques, is significant (ICMSF, 1996). Initial freezing (-20 °C/ 7 d) reduces numbers on poultry carcasses by 1-2 log cycles, though the residual cells are reported to survive freezing for up to 6 months (Yogasundram and Shane, 1986). Also, campylobacters are more heat sensitive and γ irradiation-sensitive than most other vegetative Gram-negative bacteria and are killed quite easily by routine cooking processes (Corry and Atabay, 2001; ICMSF, 1996). Thus, by association, the resistance/sensitivity profiles of arcobacters may resemble the above-mentioned patterns observed for campylobacters.

Cultivation methods and associated problems

Initial isolation and studies on arcobacters were conducted using a medium originally designed for the growth of *Leptospira* species (Bey and Johnson, 1978; Ellis et al., 1977; Johnson and Harris, 1967) i.e., EMJH P80 (Ellinghausen-McCullough-Johnson-Harris Polysorbate 80) supplemented with 2% rabbit serum or (2% rabbit serum + 0.15 % agar) or (2% rabbit serum + 0.15% agar + 100 μ g/ml 5-fluorouracil). Dickson et al. (1996) advocated the use of a biphasic culture system to increase cell numbers for growth of pathogenic *Arcobacter* spp. Their system was made up of a basal layer of 10%

bovine blood agar overlaid with brain heart infusion broth. Increasing the surface area of the culture system reportedly increased population levels achieved. It was determined that an enrichment step was necessary for the successful detection of arcobacters from food or environmental sources and various researchers worked, with varying degrees of success, to create enrichment and selective media. Among these were de Boer et al., (1996), Collins et al., (1996b), Johnson and Murano (1999a), and Lammerding et al., (1996). Also, filtration of enriched broth through a 0.45 µm syringe-type membrane filter unit onto solid media was found to aid the isolation process. Modified cefoperazone charcoal deoxycholate agar (mCCDA), cefoperazone amphotericin teicoplanin (CAT) agar and CIN (*Yersinia*-selective agar) successfully supported the growth of a number of campylobacters and arcobacters (Atabay and Corry, 1997, Wesley, 1994). A subsequent study by the same authors (Corry and Atabay, 1997) reported that CAT promoted better growth of both *Arcobacter* and *Helicobacter* strains. It has been established that high cell numbers in liquid culture is a hard-to-achieve feat with this genus. Primary isolation necessitates a microaerobic environment of 3-10% O₂ (Mansfield and Forsythe, 2000). Use of Oxyrase[®] to create this environment has been highly recommended as a cost and time-effective procedure. Oxyrase[®] is a commercially available biocatalytic agent composed of *E. coli* membrane fragments and associated enzymes in suspension (Tran, 1995).

Johnson and Murano (1999a) developed a medium for the isolation of *Arcobacter* spp. by serially testing the efficacy of inclusion of various basal medium ingredients, detoxifying agents, reducing and growth-promoting agents, antibiotics and color-enhancing compounds. Evaluation of the above yielded five formulae showing promising

results, which were further tested in detail and compared with Brucella agar. The final recommended medium contained 0.05% thioglycolic acid, cefoperazone (32 µg/ml), 0.05% sodium pyruvate and 5% sheep's blood added to a basal nutrient mixture. The added advantage of this medium was that a deep red color was observed around desired colonies. Engberg et al. (2000) tested 1376 human fecal clinical and nonsymptomatic specimens on four media: mCCDA, CAT, Skirrow's blood-based medium and yeast blood agar (with filtration). *A. butzleri* and *A. cryaerophilus* were isolated on mCCDA, followed by CAT and the filter technique in that order of frequency.

In 1996 de Boer and others described the Arcobacter selective broth (ASB, with a Brucella broth base) and semisolid Arcobacter selective medium (ASM, Mueller-Hinton broth base) for the aerobic enrichment and isolation of *Arcobacter* spp. from store-bought meats, including poultry. This technique was based on the detection of swarming of the organisms (swarming distance of 30-40 mm) on a semisolid medium and the added antibiotics were cefoperazone, cycloheximide, trimethoprim and piperacillin. The latter helped prevent the outgrowth by *Pseudomonas* spp. at the 24°C incubation temperature used. Some success has also been obtained by isolating the organisms using a cellulose acetate filter (0.45-0.65 µ APS) placed directly on an antibiotic-free non-selective medium (Taylor et al. 1991). The 48 h growth of twenty strains of *Arcobacter* (*butzleri*, *cryaerophilus*, *skirrowii*) and *Campylobacter* spp. in an Arcobacter enrichment medium (AM) developed by Oxoid Inc. was compared and evaluated against two media designed for enrichment of *Campylobacter* spp. namely, Preston broth (Oxoid Inc.) and LabM broth. This study used Arcobacter basal medium (ABM) as control with subculture from liquid media done on blood agar plates. No *Campylobacter* spp. showed good growth

characteristics in either AM or ABM. All three *Arcobacter* spp. showed good growth in AM, none grew in Preston broth and nine grew in LabM broth (Atabay and Corry, 1998). Thus, choice of media used should take into consideration the sample source including possible competing flora, and the target *Arcobacter* species desired.

Additional characteristics of *Campylobacter* spp. pertinent to *Arcobacter* comparisons

Though campylobacters are generally spiral shaped with a darting, corkscrew-like motility, antibiotic addition induces long forms. Also, coccal forms occur in old cultures and may be induced by certain inhibitory agents. In comparison, Ellis et al., (1977) reported the occurrence of ‘tightly wound and loose spiral filaments up to 20 μm long, in addition to coccoid forms’ in *Arcobacter* cultures.

The infective dose for *Campylobacter* infection was determined to be 500 cells in milk; the incubation period is commonly 2-5 d, but feces may remain positive for 2-7 weeks after the illness in the absence of chemotherapy (Butzler et al., 1992). The organisms mainly colonize the small intestine (the colon may also be colonized), and are invasive, producing enterotoxins and cytotoxins. A cholera-like toxin has been described by some investigators and a Shiga-like toxin is produced *in vitro* by a small number of strains. Most strains are susceptible to the nonspecific bactericidal activity of normal human serum; interestingly, some healthy persons have elevated serum titers to campylobacters. *Campylobacter* enteritis has often mimicked appendicitis because of the severe abdominal pain involved. Other diseases or syndromes caused by the genus include meningitis, reactive arthritis, Reiter’s syndrome and Guillain-Barré syndrome.

Erythromycin remains the treatment of choice for most *Campylobacter* infections (Butzler et al., 1992).

Skirrow's selective *Campylobacter* isolation medium for fecal samples is still widely used today. Enrichment processes are generally required for food samples, because of low numbers of cells, but not for diarrheic fecal samples. Blood or charcoal are usually recommended for inclusion as media ingredients, as they have the capacity to absorb toxic products of oxygen metabolism, thus sufficiently altering the microenvironment of the medium for the growth of microaerophilic cells. Corry et al., (1995) provide an excellent source for the comparison of various media used in the isolation of campylobacters.

The much-used Lior *Campylobacter* typing scheme is a slide agglutination serotyping test based on the thermolabile flagella antigen, that detects 100 different serotypes of *Campylobacter*. (Griffiths and Park, 1990). Lior and Woodward (Lerner et al. 1994) serotyped 131 *A. butzleri* strains, 44 belonged to serogroup 1, the predominant human serogroup. Early attempts to classify arcobacters often included the use of the Lior typing scheme in routine tests. In contrast, the Penner and Hennessy O serotyping scheme detects lipopolysaccharide antigens of about 60 different types (Nachamkin and Blaser, 2000).

Role of predictive microbiological modeling in foods

Microbial modeling is the 'use of mathematical expressions to describe microbial behavior' that 'depict how bacterial populations change with time and how the rate of change is influenced by environmental conditions' (Whiting and Buchanan, 1994). Increasing choices in varieties of raw, minimally processed or ready-to-eat foods,

necessitates the use of a wide range of extrinsic or intrinsic intervention or preservative techniques, including chemicals, low pH or inhibitory temperatures or gas mixtures. The hurdle concept in food processing uses combinations of several factors to synergistically enhance the unsuitability of the food microenvironment to microbial survival and/or growth. Although mandated quality control and shelf-life testing activities confirm the susceptibility of a food product to microbial activity, it would be invaluable in the product development stages, to be able to predict the integrity against microbial proliferation in a particular formulation, or the effect of a change in processing parameters. The ultimate goal is the maximization of product stability and shelf life. Also, the very real problem of shelf life testing multiple food formulations, which is cost prohibitive, can be overcome. Microbial growth or survival predictive modeling techniques provide this prognostic ability. They can also determine potential risks from pathogens after an expected or abusive storage time, and can aid in deciding on a 'use by' or 'best before' date for perishable foods. Effects of combinations of existing or new preservatives, both direct and indirect, can also be predicted using mathematical equations (Skinner and Larkin, 1994). Identification of critical control points in a food industry HACCP plan can be made possible using the predictive ability of such models. The subsequent decision on the fate of a food batch that has experienced processing abnormalities can be influenced through use of a model. Predictive models also are invaluable in microbial risk assessment procedures, where they provide realistic food borne exposure estimates, to susceptible human and animal populations; microbial risk assessment is a currently much-studied research area (Whiting and Buchanan, 1994).

Predictive processes have been studied for several years dating back to the 1920s and 1930s. Some of the oldest ‘models’ are the ‘Thermal Death Time’ calculations and the ‘botulinum cook’ predicting survival of *Clostridium botulinum* in heated foods (Baird-Parker and Kilsby, 1987, Stumbo et al., 1983, Whiting and Buchanan, 1994). In the 1980’s, this field received renewed attention for several reasons: occurrence of several major foodborne disease outbreaks which underlined the need for safety in the food supply, increasing time constraints in food production and distribution processes, and the availability of computer-based analytical tools (Ross and McMeekin, 1994). Studies targeting microbial spoilage have though, been somewhat limited (growth of yeasts in soft drinks, lactic acid bacteria in vacuum packaged meats, shelf-life prediction of fresh fish and cod are a few available examples) as compared to studies modeling the presence of pathogens in foods (Betts, 1997). Also, some models are concerned with the effect of parameters on microbial inhibition, others predict microbial growth or survival (Baird-Parker and Kilsby, 1987). Numerous models are available for thermal inactivation processes, but few describe microbial activity at chilled or ambient temperatures (Whiting and Buchanan, 1994). Predictive models are currently available for several foodborne bacterial pathogens including *Salmonella* (Gibson et al., 1988; Oscar, 1999), *Clostridium botulinum* (Gibson et al., 1987; Roberts et al., 1981), *Clostridium perfringens* (Juneja et al., 1996), *Listeria monocytogenes* (Buchanan et al., 1989; Buchanan and Phillips, 1990; Duh and Schaffner, 1993), *Shigella flexneri* (Zaika et al., 1991), *Escherichia coli* O157:H7 (Buchanan and Klawitter, 1992; Juneja et al., 1999; Sutherland et al., 1995), *Pseudomonas fluorescens* (Wilcox et al., 1993), *Yersinia enterocolitica* O:3 (Lindberg and Borch, 1994), *Staphylococcus xylosum* (McMeekin et

al., 1987), *Staphylococcus aureus* (Eifert et al., 1996), *Aeromonas hydrophila* (Palumbo et al., 1991; 1996). Some have been incorporated into the Pathogen Modeling Program, an online resource made available by the USDA (USDA, 2001). A similar 'expert system' program is available in the U.K.: the "Food Micromodel" program (McClure et al., 1994; Whiting and Buchanan, 1994) and in Europe the FLAIR (Food Linked Agricultural and Industrial Research) program is a collaboration between laboratories in 15 countries to study growth responses of organisms in many natural foods (Ross and McMeekin, 1994).

Data is collected from laboratory studies in growth media, of single strains or a cocktail, assuming that the fastest growing or the most resistant cells in the population would influence the observations, as the case may be. Experimental conditions include chosen variables within a selected range, and the data obtained is used to create a mathematical equation/s. The resulting predictive equations can then be extrapolated to predicting microbial growth or survival under a much wider selection of the variables under consideration, albeit within the predetermined range. Growth curve data in terms of viable cell counts can be analyzed by different nonlinear regression methods. One that is commonly used is the modified Gompertz equation (Gibson et al., 1988) and response-surface methodology. The Gompertz equation is a double-exponential function that traces an asymmetrical sigmoidal curve (Buchanan, 1993). Fitting this equation to the data points produces a four-phase sigmoidal curve with individual areas representing the stages of microbial growth, and yields values of the Gompertz parameters A, C, B, M. These (mainly B, M) are then modeled iteratively against controlling environmental variables and the resulting predictive equation describes both single and interactive

effects of variables studied. Predictions are made from an evaluation of the fitted function and the model explains how the derived parameters of each curve were affected by the controlling factors (Gibson et al., 1988; Buchanan, 1993; Eifert et al., 1996; Roberts, 1997). The modified Gompertz equation used, is as detailed below:

Gompertz Equation: $L(t) = A + C [\exp \{-\exp (-B(t-M))\}]$, where,

$L(t)$ = \log_{10} count of bacteria at time (h) t , (\log_{10} CFU/ml)

A = asymptotic log count of cells as time decreases indefinitely (i.e., initial level of cells), (\log_{10} CFU/ml)

C = asymptotic amount of growth that occurs as t increases indefinitely (i.e., number of log cycles of growth, or difference in the initial and final number of cells at the stationary phase) (\log_{10} CFU/ml)

B = relative growth rate at M (\log_{10} [CFU/ml]/h) where

M = time at which absolute growth rate is maximal (h)

From these, the following *derived* growth kinetic characteristics can be calculated:

EGR (exponential growth rate) = $(B \cdot C)/e$ [\log_{10} (CFU/ml)/h]

GT (generation time) = $\log_{10} 2 \cdot e / B \cdot C$ h

LPD (lag phase duration) = $M - 1/B$ h

MPD (maximum population density) = $A + C$ \log_{10} CFU/ml

Additional parameters can be modeled if necessary, among them are ‘time taken for a specific increase in the number of cells’, ‘time to toxigenesis’, or ‘specific growth rate’.

Selection of model types

Historically used models to measure growth include the Ratkowsky model used for temperature effects and the Schoolfield equation for a_w , pH and temperature effects singly, or in combination. The latter was found to give predictions closer to the actual data (Baird-Parker et al., 1987), though Buchanan (1993) describes its use as ‘complex and cumbersome’. Whiting and Buchanan (1993) have classified model types as *primary* (those which describe changes in microbial numbers with time under a specified set of conditions, e.g., D-values), *secondary* (those which describe how parameters of primary models change in accordance with culture conditions or environmental factors, e.g., z-values) and *tertiary* level applications that are mainly used to assist in the display of the previous two levels (e.g., software like the PMP, mathematical equations). Growth and inactivation/survival models are classified as primary level models. The Gompertz equation, a primary level, often-used model has received much approval as it provides a better ‘fit’ for microbial data and is easier to use, as compared with other sigmoidal equations (Zwietering et al., 1990). Recently, mechanistic-based growth models have also received some attention. While they are considered to be ‘inherently superior’, most currently used models are empirical (Buchanan, 1993).

Changes in parameters of primary models (e.g., Gompertz parameters A, C, M, B) may be described by different types of secondary models. These may be one of the following types (Ross and McMeekin, 1994; Skinner and Larkin, 1994; Whiting and Buchanan, 1994):

- a) a response surface equation (polynomial regression equation) that has quadratic or cubic terms and their interactions. Here, a linear model is developed, which

has the form of a polynomial equation in its modeled parameters. Response-surface models were primarily designed to optimize industrial processes. Often, increased homogeneity in the variance is achieved through modeling the natural logarithm of the parameter under consideration. These types of equations are used to describe and fit data composed of several factors (Whiting and Buchanan, 1994).

- b) the Arrhenius equation, which is the logarithm of the rate constant versus the reciprocal of temperature in ° K, may have additional terms when pH and a_w are also being studied. This type would also include the ‘modified Arrhenius’ or ‘Davey’ model. Extensive analysis led to the conclusion that the original Arrhenius equation did not adequately describe the rate response to temperature in most biological systems. The Schoolfield equation, based on the original Arrhenius equation, is the reparameterized modification that has received much support.
- c) the Bélehrádek-type (or square root) model is based on the linear relationship between the square root of the rate and temperature, and may also include additional terms for pH and a_w studies. This equation is best suited for temperatures below the optimum growth temperature of a microorganism. Modification of the original Bélehrádek model of the 1930’s was described by Ratkowsky et al. (Whiting and Buchanan, 1994, Ross and McMeekin, 1994), and includes a term that compensates for the reduced growth rate of a microorganism above its optimum growth temperature.

Studies have determined that when growth rate is governed by two variables, independent of each other, modifications of either the Davey or Ratkowsky equations can be used (Buchanan, 1993). An exhaustive explanation and comparison of several model types is available in the publication by McMeekin et al., (1993). Another classification scheme for models is to separate them as probability-based (prediction of the likelihood of an event, like spore germination or toxin production) or kinetics-based (modeling the rate and extent of microbial growth). The choice depends on the bacterium being studied and its growth impact on the product safety. In 'kinetic' models, the 'response variable' is recorded in time units (i.e., a rate, or the time taken for a particular response). Probability-based models are usually used when any growth whatsoever is considered a danger (e.g., spore formers like *C. botulinum*) while kinetics-based models are used for non sporeformers (Buchanan, 1992; Ross and McMeekin, 1994). Traditionally, probabilistic equations have described individual contributions of variables, more recently, terms have been included, that allow prediction of when an event is likely to take place. For example, Genigeorgis et al., modeled the effects of temperature, inoculum size and percentage brine on lag phase to toxin production of *C. botulinum* B and E in cooked turkey (Buchanan, 1993). In a scheme that draws from various sources, Skinner and Larkin (1994) classified predictive equations as probabilistic, regression, Arrhenius, and square root models.

Early models often described first-order decreases in logarithmic numbers. However, realistic observations have often recognized non-linear trends in microbial reductions, and subsequent combination models were developed. (e.g., increases/decreases in spore populations). These took into account first-order decreases in

susceptible spores in a population, followed by activation of the more resistant spores, ending finally with inactivation of the residual spores in the population. Sublethal environmental parameters often act singly or in combination to cause shoulders or tailing in inactivation or survivor curves. Models have been devised to take such situations into consideration. One such equation for thermal destruction of *L. monocytogenes* and *S. aureus* in the presence of lactoperoxidase exhibits a shoulder and dual slopes with the second slope describing a tailing effect (Whiting and Buchanan, 1994). Another area of concern for scientists developing models, has been the prediction of microbial behavior in non-ideal conditions as found in food environments (e.g., cycling temperature levels, pH changes, varying a_w or other food parameter changes during storage). Laboratory experiments for model construction obtain data under constant conditions. Although attempts have been made to accurately model sinusoidal-type fluctuations, it was noticed that abrupt experimental transitions induce non-classical bacterial responses (e.g., an interim adjustment period as opposed to an abrupt response). Additional research is thus necessary to model such fluctuations (Whiting and Buchanan, 1994).

Data collection and factors influencing growth models

Several factors may influence growth models, including inoculum level and method, interference through the presence of normal flora, data collection methodology including experimental replications (Baird-Parker et al., 1987) and range of variables selected. Liquid medium is the preferred substrate, as it can be easily manipulated, and supports similar or faster growth than that observed in foods. Thus the models obtained would be independent of food type, and predictions would err perhaps, on the side of safety (McClure et al., 1994). While the majority of microbial growth data was obtained

by a count of cell numbers, some researchers have used conductance measurements as an estimate of microbial activity (Lindberg and Borch, 1994). There are indications that pre-experimental growth conditions of the inoculum used also has a significant effect on test growth or survival patterns. Thus 'priming' of inoculum to best represent the conditions likely to be encountered in the food environment of that bacterial species is recommended (e.g., growth to stationary or late log phase, acid or alkali adaptation, etc.) (Whiting and Buchanan, 1994; Baranyi and Roberts, 1994). Though the recommended number of data points for each growth curve obtainable is around 10, Eifert et al., (1996) obtained equivalent results with a reduced dataset of 5 observations per curve. Since the magnitude and sign of parameters were similar to those obtained for the full model, this study provides justification for a reduction of the labor-intensive data collection process. Models are known to provide the most accurate predictions in the central area of their design, where all variable values are close to the midpoint of their respective ranges, thus using a model for prediction outside the set ranges is not recommended (Whiting and Buchanan, 1994). Ratkowsky et al. (1991) expressed concern over the inherent variability observed in replicated bacterial growth responses, and the fact that the variability increased at slower growth rates. This effect was characterized in the following equation: $\text{Variance} = 4 \times (\text{response time})^3 \times \text{variance (square root of } k)$, where k is the reciprocal of the response time (McMeekin et al., 1993).

Types of predictions or observations obtainable from predictive models

When used to determine microbial activity in a selected product, a predictive model estimates values of Gompertz parameters B and M , which together with the relevant A and C values, are then used to calculate the derived growth kinetic

characteristics (e.g., LPD, EGR, MPD, GT). In addition, probabilistic derived characteristics may also be estimated. Several individual characteristic values predictively obtained by changing one or more variable values can be used to plot response-surface diagrams or contour plots that provide an at-a-glance interpretation of the interactive effect of experimental variable conditions on the response variable.

Reliability/confidence in predictive micromodels

It is essential to recognize that predictive models are developed using an experimental system, with pure cultures under largely controlled conditions, thus practical usability of predictive micromodels should be decided through validation studies. In these studies, the microorganism is inoculated into laboratory media or food products at variable levels covered under the model, but not included in initial data collection, and microbial growth or survival is noted in terms of cell counts. Observed growth kinetic characteristics and Gompertz parameter values from validation studies are compared with predicted values obtained from the model. Close agreement between the two suggests a realistic, workable model and model limitations if any, are determined at this stage. (Betts, 1997; Eifert et al., 1996; Pin et al., 1999; Walls et al., 1996; Walls and Scott, 1996). The results of all such studies, when utilized in food industry situations, should therefore be subject to the analytical abilities and judgment of trained individuals, who by experience are able to interpret scientific predictions in a 'real life' scenario. When less-than desirable predictions are obtained, additional factors may be necessary to widen the applicability of the model (Whiting and Buchanan, 1994). Often, to obtain a 'best-fit' for a model, it is necessary to perform a suitable transformation on the dataset, in order to 'normalize' the variance. Using the logarithm, inverse, square or square-root

are some of the commonly used methods to normalize the data. Natural logarithmic transformations ensure that predicted values for A, C, M, B, are positive and the model has decreased variability (Baker and Genigeorgis, 1993). Root mean square error (RMSE) and percentage goodness of fit are used to assess the adequacy of any model to fit the dataset. RMSE may arise from various sources including natural variability (inherent microbial variability), systematic errors (laboratory methodology) and bias (model choice and fitting processes) (McClure et al., 1994; Baranyi and Roberts, 1995). However, Ratkowsky et al., 1991, state that mean square error between observed and predicted values is, depending on the type of model used, not an appropriate tool to compare the reliability of predictive models. They note that responses like generation time and lag phase duration become increasingly variable as their 'mean magnitude' increases, or near the 'limiting values' (stressful conditions like low pH, high salt) for survival. While several researchers have noted good agreement between observed and predicted values of parameters (Gibson et al., 1988), some have noted that microorganisms (*S. aureus* and *B. cereus*) grew better in actual foods or food homogenates than in laboratory media (Ross and McMeekin, 1994).

Choice of variables used in modeling studies

Baranyi and Roberts (1994) note that variables in a mathematical model fall into one of three classes: a) extracellular conditions unaffected or negligibly affected by cell growth or the 'growth independent' environment; b) extracellular conditions that are altered by cell growth or 'growth dependent' environmental factors; and c) intracellular concentrations of substances, (e.g., DNA, RNA) which change during growth, and characterize the physiological state of the cells. Thus, the selection of variables that are to

be factors in the building of a predictive model should be governed by: a) the characteristics of the microorganism; b) the environment with which it is usually associated or has a reservoir; c) the food product characteristics in which it is likely to be of significance as spoilage flora or as a pathogen, including harvesting, processing, packaging and storage conditions; and d) intervention strategies known to be traditionally associated with the food product/s of concern. Factors that have been used in various modeling studies have included atmospheric conditions (aerobic/anaerobic/microaerophilic/ CO_2 levels), pH, a_w , chemical preservatives (NaCl, nitrites, nitrates, ascorbates, lactates phosphates), and most importantly, growth or storage temperature. These criteria were used in the selection of variables used in modeling experiments for *Arcobacter* spp. The strong association of the genus with meat products, in particular with swine/pork and thus the potential for its existence in pork products led to the selection of sodium chloride, sodium nitrite and sodium tripolyphosphate (STPP) as model factors, as these three are commonly used preservatives in processed pork products (e.g., ham, bacon, sausage). It has been reported that Gram-negative bacteria are relatively resistant to bacteriostatic effects of nitrite, and that other factors interacting with nitrite (e.g., salt, temperature and pH) play an important preservative role (Zaika et al., 1991). Phosphates, while originally thought to play mainly a functional role in food (e.g., increased tenderness, moisture retention, emulsification, color development and leavening; reduced shrinkage during cooking, prevention of oxidative rancidity), have also been investigated for their antimicrobial (and antibotulinal) properties (Post et al., 1963; Tompkin, 1983; Wagner, 1986; Zaika et al., 1997). Sodium pyrophosphate and sodium tripolyphosphate are the most popular

phosphates used in the meat industry, as moisture binding agents (Zaika et al., 1993). Some clinically important *Campylobacter* spp. have a restricted higher growth temperature range, (notably the thermophilic species like *C. jejuni* and *C. coli*) of 32-45°C (ICMSF, 1996). *Arcobacter* spp. on the other hand reportedly grow over a wider temperature range of 15-37°C (Wesley, 1994). Thus, at the temperatures that may be used in food processing or storage that may allow growth or survival of microorganisms, arcobacters might be expected to have an edge over competing campylobacters. Temperature range for the modeling study was thus selected on this basis. Finally, pH (native or developed) is always a significant governing factor for microbial growth, and pH was the fifth variable chosen for the modeling study. Thus in the model being developed, it will be possible to study the main and interactive effects of the five environmental variables.

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CHAPTER 3

EFFECT OF pH, NaCl CONTENT AND TEMPERATURE ON GROWTH AND
SURVIVAL OF *ARCOBACTER* SPP.¹

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Abstract

Arcobacter spp. have been linked to cases of gastroenteritis in humans and abortions in livestock. Growth and survival characteristics of six human isolates in the presence of selected environmental factors were studied. Four strains of *A. butzleri* and two strains of *A. cryaerophilus*, one from each of the two hybridization groups 1A and 1B were exposed to pH levels of 3.5-8.0. Most strains grew between pH 5.5-8.0, with optimal growth of most *A. butzleri* and *A. cryaerophilus* strains at pH 6.0-7.0 and 7.0-7.5, respectively. The 24 h optimal growth range in the presence of NaCl was 0.5-1% for *A. cryaerophilus*. However, after 96 h the optimum was between 0.5-2% NaCl. The optimum range for growth of *A. butzleri* strains was 0.09-0.5% NaCl after 96 h. The upper growth limits were 3.5% and 3.0% NaCl, for *A. butzleri* and *A. cryaerophilus* respectively. Survival at 25°C up to 5% NaCl was noted for *A. butzleri* 3556, 3539 and *A. cryaerophilus* 3256. Decimal reduction times (D-values) at pH 7.3 in phosphate buffered saline for three *A. butzleri* strains studied were 0.07-0.12 min at 60°C, 0.38-0.76 min at 55°C, and 5.12-5.81 min at 50°C. In combination with a lowered pH (5.5), a decrease in thermotolerance was observed, with D-values of 0.03-0.11 min at 60°C, 0.30-0.42 min at 55°C, and 1.97-4.42 min at 50°C. Calculated z-values ranged from 5.20 to 6.28 °C. D-values of a three-strain mixture of *A. butzleri* in raw ground pork were estimated to be 18.51 min at 50°C and 2.18 min at 55°C. Mild heat (50°C) followed by cold-shock (4° or 8°C exposure) were found to have a synergistic lethal effect, bringing about a greater reduction in cell numbers than with an individual 50°C treatment or with cold-shock temperatures of 12° or 16°C. Elucidation of the inhibitory effects of pH, NaCl or

temperature on *Arcobacter* spp. will facilitate the creation of appropriate intervention strategies aimed at controlling growth or survival of these strains in food environments.

Introduction

Members of the genus *Arcobacter* often described as ‘emerging pathogens’ are comprised of four species, *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. nitrofigilis* (Vandamme and DeLey, 1991; Vandamme et al., 1991). These Gram-negative, aerotolerant, vibrio-like bacteria were first observed by Ellis et al. (1977) in veterinary specimens. They are closely related to, and were formerly designated as members of *Campylobacter*. Strains of *Arcobacter* have been detected in drinking water (Jacob et al., 1993; Musmanno et al., 1997) and muscle foods, including pork, beef and poultry (Atabay et al., 1998; Collins et al., 1996b; de Boer et al., 1996; Lammerding et al., 1996). They have been clinically associated with abortions and mastitis in livestock (Butzler et al., 1992; Wesley, 1994) and diarrhea in non-human primates (Anderson et al., 1993). *Arcobacter*-associated illness in humans takes the form of persistent diarrhea, gastroenteritis or occasionally septicemia (Hsueh et al., 1997; Wesley, 1994). *A. butzleri* is the species most often linked to outbreaks of disease in humans, the more significant reports include an outbreak among Italian schoolchildren (Vandamme et al., 1992a) and acute diarrheal disease in Thai children (Taylor et al., 1991). Severe or long-term diarrhea, sometimes with accompanying bacteremia was reported among 29 U.S. patients suffering from *A. butzleri* infection (Wesley, 1994). *A. butzleri* has also been isolated from a newborn with bacteremia (On et al., 1995) and enteritic patients with chronic underlying disease (Lerner et al., 1994). Studies have revealed the varied antibiotic response of *Arcobacter* strains (Atabay and Aydin, 2001; On et al., 1995), while

virulence studies on *A. butzleri* detected the production of cytotoxins by almost all strains (Musmanno et al., 1997).

Foodborne illness affects 76 million people in the U.S. each year. *Campylobacter* is the leading cause of bacterial foodborne disease, according to statistics for the year 2000 released by FoodNet, the Emerging Infections Program Foodborne Diseases Active Surveillance Network at the Centers for Disease Control (CDC). The organism was the most frequently detected pathogen in all 5 years of the survey, and was isolated in 36.7% of laboratory-tested cases of diarrhea. *Campylobacter* infection-rates surpassed the incidence of *Salmonella* and *Shigella*-associated disease (CDC-MMWR, 2001). Though the mortality rate is low in the estimated 2.4 million affected population, 124-500 *Campylobacter*-related deaths occur in the U.S. each year, while 40% of the cases of Guillain-Barré syndrome are thought to be initiated by *Campylobacter* infections (CDC, 2001a; CDC, 2001b). The closely related *Arcobacter* spp. are similarly found in foods and are also associated with gastritis. A role for *Arcobacter* spp. as potentially important human pathogens has thus been discussed (Wesley, 1997). Their similarity to *Campylobacter* may extend to infective capacity, and possibly the range and extent of foodborne illness caused. The objective of this study was to determine the susceptibility of *Arcobacter* spp. to various environmental and food-related stress factors. Since this genus comprises species that are considered emerging human pathogens, it was important to determine the growth and survival capabilities of these organisms, as a means to relating to their potential presence and survival in contaminated foods.

Materials and methods

Preparation of inoculum: Human strains of *Arcobacter* spp. (*A. butzleri* NADC 3556, 3257, 3494, 3539; *A. cryaerophilus* NADC 1A 3252, *A. cryaerophilus* NADC 1B 3256) were obtained from Dr. Irene Wesley at the USDA ARS-NADC Laboratory, Ames, IA, and stored at -70°C in Mueller-Hinton broth supplemented with 20% glycerol. Before use, they were subcultured onto 5% bovine blood agar plates (prepared with Blood agar base with low pH, Acumedia Manufacturers Inc., Baltimore, MD; defibrinated bovine blood, Metro Medical Supply, Inc., Burlington, NC) and incubated for 36-48 h in a controlled gas atmosphere (10% CO_2 , 5% O_2 , 85% N_2) in a CO_2 water-jacketed incubator (Model 3130, Forma Scientific, Marietta, OH). Using 10 μL polystyrene loops (VWR Scientific, Westchester, PA), isolated colonies of each culture were individually inoculated into liquid growth media (1:5 dilution with sterile distilled water). The medium used was Ellinghausen McCullough Johnson Harris (EMJH) PLM-5 medium, pH 7.35, (Intergen Inc., Purchase, N.Y.) supplemented with 1% Oxyrase[®] (Oxyrase Inc., Mansfield, OH), which was dispensed into sterile 250 ml polystyrene tissue culture flasks (Becton Dickinson and Company, Sparks, MD) and incubated on an orbital shaker, with gentle shaking (50 rpm) at 30°C (*A. cryaerophilus*) or 37°C (*A. butzleri*) for 30 h. This incubation period was selected based on preliminary growth curve experiments that determined that the target stationary-phase cells were obtained in this period. Cultures were centrifuged (8000 x g, 25 min, 4°C , Sorvall RC-5B refrigerated centrifuge, DuPont Instruments, Newtown, CT), washed twice with 0.1M phosphate buffered saline (PBS, pH 7.4) and were resuspended in PBS to yield a final concentration of 10^9 CFU/ml. Prepared cultures were stored at 4°C until used, but for no more than 72 h.

pH tolerance: EMJH medium with 1% Oxyrase[®] was distributed into a series of 500 ml flasks and the pH of each aseptically adjusted to 3.5-8.0 at 0.5 unit intervals using 1N HCl or 0.1N NaOH. Four ml portions from each flask were distributed into (80 mm x 12 mm) test tubes and inoculated with appropriately diluted cultures of *A. butzleri* or *A. cryaerophilus* to yield a final concentration of 10⁵ CFU/ml in each tube. Uninoculated controls were maintained for each pH level studied and were used as ‘blanks’ for OD₅₃₀ measurements. Tubes were incubated at 25° or 37°C, and the OD₅₃₀ was measured at time 0, 24 h, 48 h, 5 days and 7 days. A loopful of medium from each tube was ‘spot-tested’ onto blood agar plates and inoculated into EMJH medium with Oxyrase[®], to particularly test for survival of cells near the growth limits of the strains. The experiment was repeated twice.

NaCl tolerance: The methods used were similar to the pH study, with the exception that appropriate amounts of a 35% NaCl solution (in distilled water adjusted to a pH of 7.35) were added to the flasks, to provide a final NaCl content of 0.09-5%, and inoculated tubes were incubated at 25° or 37°C for up to 96 h. It was determined from the manufacturer of EMJH medium that the base-level NaCl content in the medium was 0.09%. Thus experimental media at higher NaCl contents received suitably adjusted supplementation with the 35% NaCl stock solution. A loopful of medium from each tube was ‘spot-tested’ onto blood agar plates and inoculated into EMJH medium with Oxyrase[®], as described in the pH study. The experiment was repeated twice.

D-value determination (in liquid medium): Fifty-μL amounts of phosphate buffered saline suspensions of *A. butzleri* 3257, 3556 or 3494 were filled by capillary action into 50 μL capillary tubes (Micropipets[®], VWR Scientific, Westchester, PA) that were sealed

at both ends with a high-temperature flame from a National[®] Air Gas torch (Model 3A-B, Wale Apparatus Co., Hellertown, PA). For experiments at pH 5.5, the suspension of cells grown up as described in the 'preparation of inoculum' section was centrifuged prior to use (8000 x g, 25 min, 4°C), and resuspended in 0.1M PBS adjusted to pH 5.5, to yield a final concentration of 10⁹ CFU/ml. The suspension was stored at 4°C until used, but for no more than 1h. Filled capillary tubes were inserted into a plastic mesh covering on a test tube rack, so as not to be exposed to possible direct conductive heating effects of the base of the water bath. Test tube racks were placed in a circulating water bath (Model M20B, Lauda Circulating water bath) at either 50, 55 or 60°C and capillary tubes removed at time intervals, ranging from 2 s to 20 min, depending on the temperature used. These were immediately immersed in cooling water at 20°C for a period of 2 min. Capillary tubes were surface sterilized by dipping in 95% ethanol for 10 s, with any adhering ethanol rinsed off by swirling the capillary tubes in sterile distilled water. Unheated tubes were maintained as initial inoculum controls. The ends were broken off and the capillary tube was crushed with a sterilized glass rod into 4.95 ml PBS, appropriate dilutions were made and aliquots plated onto 5% bovine blood agar plates which were incubated in the controlled gas mixture atmosphere for up to 48 h. Log₁₀ counts obtained (CFU/ml) were plotted against time (min) and a linear regression was obtained, from which a calculation of the D-value (negative reciprocal of the slope of the linear regression equation) was done. Three replications of each experiment were carried out.

D-value determination (in ground pork): Suspensions of *A. butzleri* 3257, 3556 and 3494 were prepared as above. Based on predetermined counts on blood agar plates,

appropriate aliquots of the three cultures were mixed together to obtain a three-strain cocktail approximating 10^9 CFU/ml, with equivalent counts (CFU/ml) of all three strains. Ground pork (75% lean) was obtained from a supermarket (HyVee Inc., West Des Moines, IA) and irradiated (30 kGy) at the Linear Accelerator Facility, Iowa State University, Ames, IA in order to eliminate background microflora that were likely to confound the results. Frozen packages of irradiated pork were shipped to Athens, GA and stored at -18°C until used. As needed, portions of ground pork were thawed overnight at 4°C , mixed for homogeneity, injected into 13 mm collagen sausage casings (Devro Teepak™, Swansea, SC) using a sausage stuffer attached to a mixer (Model K5-A, Kitchenaid® Inc.), and individual 10 g portions of ground pork sausage were tied off. One ml portions of *Arcobacter* suspensions were injected into the center of each sausage portion using a 5 ml syringe. Inoculated sausages were inserted through a plastic wire mesh covering over a test-tube rack, which was placed in a circulating water bath at either 50 or 55°C . Come-up, hold, and cooling times were measured using a data recorder (Model RD106, Omega Engineering, Stamford, CT) and sausage portions were removed at selected intervals for up to 45 min, depending on the temperature used. Each treated portion was quickly placed in a stomacher bag, 100 ml PBS added, and the sample was stomached (Tekmar model 400, Tekmar, Cincinnati, OH) for 2 min at normal speed. Aliquots of appropriate dilutions were plated on 5% bovine blood agar plates, which were incubated in a modified gas atmosphere (10% CO_2 , 5% O_2 , 85% N_2) and D-values were obtained as described above. Three replications of each experiment were carried out.

Cold-shock experiments: Fifty- μL capillary tubes were filled with a suspension of *A. butzleri* 3556, prepared as outlined in the D-value section, and placed in a circulating

water-bath at 50°C for 30 s or 1 min, and then immediately immersed in an ice-water or cold water bath maintained at 4, 8, 12 or 16°C for 15s, 30s, 45s or 1min. Capillary tubes were then treated for recovery of cells as outlined above. The experiment was repeated twice.

Results

The growth and survival responses of *A. butzleri* and *A. cryaerophilus* strains to varying pH, NaCl and temperature influences were elucidated in these studies. Strains of *A. butzleri* and *A. cryaerophilus* grew in Oxyrase[®]-supplemented EMJH medium between pH 5.5-8.0; however the optimum pH for most *A. butzleri* strains is in the range of 6.0-7.0, while the optimum for *A. cryaerophilus* is pH 7.0-7.5, since the experimental inoculations reached a maximum cell density at these pH levels (Fig. 3.1, 3.2).

Differences in cell densities at different pH levels were observed between the *A. butzleri* strains studied, with *A. butzleri* 3556 exhibiting the lowest cell density across all growth pH levels, at both 37 and 25°C for the 48 h incubation period. On the other hand, *A.*

butzleri 3539 achieved higher cell densities as compared to the other *Arcobacter* strains studied, at 37°C for the 48 h incubation period, and at both temperature levels for the 5d incubation period. At 48 h, *A. butzleri* strains were able to tolerate a lower pH of 5.0 at 25EC, but not at 37EC. This additional tolerance was not exhibited at the 5 d incubation.

The temperature-dependence of growth at levels near the lower pH limits is also exhibited at 48 h, wherein a temperature of 25°C yielded higher cell numbers at pH 5.5 for all *A. butzleri* strains, as compared to 37°C. Also, for *A. butzleri* strains, while the lower temperature of 25EC did not initially (48 h) appear to be as optimum as 37EC, on prolonged incubation (5 days), these strains achieved higher numbers at 25EC. At both

25EC and 37EC, for an incubation period of either 48 h or 5 d, growth of both strains of *A. cryaerophilus* was minimal, even when it did occur. This trend was observed through the entire period of experimentation with these strains. A temperature of 25EC and prolonged incubation however, appeared to favor the growth of both *A. cryaerophilus* 3252 (1A) and 3256 (1B). A related interesting observation was that *A. butzleri* strains on agar media averaged a colony size of 2-5 mm for incubation periods of up to 72 h. *A. cryaerophilus* strains, on the other hand did not achieve a colony size larger than pinpoint within the same incubation period. It has been noted that *Arcobacter* spp. in general were reportedly difficult to grow to high cell numbers with some variation in maximum cell numbers achieved, depending on the medium used (Mansfield and Forsythe, 2000).

The range of growth was between 0.09-3% and 0.09-3.5% NaCl, for each of *A. cryaerophilus* and *A. butzleri* strains studied (Table 3.1, 3.2). Individual strains exhibited varying growth and tolerance ranges over the 96 h study. Tolerance was noted as the occurrence of growth on subculture, for primary experimental inoculations not exhibiting growth as turbidity in OD₅₃₀ measurements. Strains *A. butzleri* 3556, 3539 & *A. cryaerophilus* 1B survived an NaCl concentration of 5.0% after 48 h at 25EC. These tolerance levels dropped to 4.0% NaCl after a 96 h incubation, with the exception of *A. butzleri* 3539, which continued to show high NaCl tolerance. An interesting feature was the additional tolerance exhibited by *A. butzleri* strains over their growth levels at 25EC, in light of the fact that 37EC, and not 25EC is the preferred growth temperature for *A. butzleri* strains. The optimum NaCl level for *A. cryaerophilus* 1B was 0.5-1% after a 24 h incubation period, and was 0.5-2% after 96 h. For the four *A. butzleri* strains, optimum

Table 3.1: Response of *Arcobacter butzleri* (A.b.) and *Arcobacter cryaerophilus* (A.c.) strains to varying concentrations of NaCl at 25°C, when incubated in Ellinghausen McCullough Johnson Harris (EMJH) medium (pH 7.3) supplemented with Oxyrase[®]. Values are representative of two replications.

Strains	48 h response (% NaCl)			96 h response (% NaCl)		
	Optimum growth ^a	Maximum growth	Tolerance up to ^b	Optimum growth ^a	Maximum growth	Tolerance up to ^b
A.b. 3556	0.09	2.5	5.0	0.09	3.5	4.0
A.b. 3257	1.00	2.0	3.0	0.09	2.0	3.0
A.b. 3494	1.00	2.0	3.0	1.00	3.0	3.5
A.b. 3539	0.09	2.0	5.0	0.09	2.5	5.0
A.c. 3256	0.09	2.0	5.0	0.50	2.0	- ^c

^a maximum cell density observed at this NaCl(%) content

^b tested by subculture onto blood agar plates and in EMJH growth medium

^c no additional tolerance observed above growth level

Table 3.2: Response of *Arcobacter butzleri* (A.b.) and *Arcobacter cryaerophilus* (A.c.) strains to varying concentrations of NaCl at 37°C, when incubated in Ellinghausen McCullough Johnson Harris (EMJH) medium (pH 7.3) supplemented with Oxyrase[®]. Values are representative of two replications.

Strains	48 h response (% NaCl)			96 h response (% NaCl)		
	Optimum growth ^a	Maximum growth	Tolerance up to ^b	Optimum growth ^a	Maximum growth	Tolerance up to ^b
A.b. 3556	0.09	3.5	- ^c	0.09	3.5	- ^c
A.b. 3257	0.09	2.0	3.0	1.00	3.0	- ^c
A.b. 3494	0.50	3.0	3.5	0.09	3.5	- ^c
A.b. 3539	0.09	3.0	- ^c	0.50	3.0	3.5
A.c. 3256	2.00	3.0	- ^c	2.00	3.0	- ^c

^a maximum cell density observed at this NaCl(%) content

^b tested by subculture onto blood agar plates and in EMJH growth medium

^c no additional tolerance observed above growth level

Table 3.3: D-values (decimal reduction times, min, \pm standard deviation) and z-values ($^{\circ}$ C) of three human isolates of *Arcobacter butzleri* in phosphate buffered saline (PBS) at pH 7.3, and in PBS acidified with 1N HCl (pH 5.5). These numbers are representative of three replications.

Strain number	D values (min)						Z value ($^{\circ}$ C)	
	60 $^{\circ}$ C		55 $^{\circ}$ C		50 $^{\circ}$ C		pH 5.5	pH 7.3
	pH 5.5	pH 7.3	pH 5.5	pH 7.3	pH 5.5	pH 7.3		
3556	0.06 \pm 0.001	0.12 \pm 0.005	0.42 \pm 0.01	0.76 \pm 0.01	3.77 \pm 0.23	5.12 \pm 0.19	5.55	6.11
3257	0.03 \pm 0.0005	0.07 \pm 0.003	0.30 \pm 0.01	0.38 \pm 0.02	1.97 \pm 0.05	5.81 \pm 0.33	5.61	5.20
3494	0.11 \pm 0.005	0.10 \pm 0.002	0.40 \pm 0.032	0.56 \pm 0.011	4.42 \pm 0.52	5.31 \pm 0.15	6.28	5.81

NaCl levels ranged between 0.09-0.5%, based on the maximum population density that was observed at these levels.

Decimal reduction times (D-values) and z-values for the three *A. butzleri* strains studied are outlined in Table 3.3. It was observed that strains of *A. butzleri* varied slightly in their sensitivity to the lethal effects of the heating temperatures. The combination of heat and reduced pH brought about a decrease in the D-values. For strains 3556 and 3257, this reduction at pH 5.5 was 26-50% and 21-66%, respectively. Strain 3494, however, showed a lower D-value reduction at pH 5.5 of 0-28% (Fig. 3.3, 3.4, 3.5). The z-values calculated from D-value observations, were similar for all three strains of *A. butzleri* studied. At pH 5.5, the range was between 5.55-6.28° C, and at pH 7.3, the range was between 5.20-6.11°C. The decimal reduction time for the three-strain mixture of *A. butzleri* in heated ground pork at 50°C and 55°C was found to be 18.51 min and 2.18 min, respectively (Fig. 3.6). The mean come-up times at 50° and 55°C were 4.25 min and 4.11 min, respectively, while the mean cooling times from 50° and 55°C were 2 min and 2.38 min, respectively.

Heating *A. butzleri* 3556 to 50EC for 30 s or 1 min, followed by immediate low-temperature treatment decreased the numbers of surviving cells, compared with a control receiving no low-temperature treatment (Fig. 3.7). This effect was more pronounced at lower temperatures of 4EC and 8EC as compared to 12EC or 16EC, and was observed in both sets of results. The cold shock effect was more a function of degree of low temperature used, than the magnitude of heating time involved, for the treatment times studied. A 3.2- 4.0 log₁₀ decrease in cell numbers at a cold-shock temperature of 4°C was the maximum reduction observed, followed by a 2.27-3.34 log₁₀ decrease at 8°C, a 1.31-

2.73 log₁₀ decrease at 12°C and a 0.33-1.12 log₁₀ decrease observed at 16°C. The reduction in cell numbers observed when heat was the only stress factor applied, averaged 0.14-0.17 log₁₀ for 30 s or 1 min.

Discussion

Survival of pathogens in the environment and in food products is governed by a complex array of factors. Several of these factors are inherent in the genotypic composition of the genus and are reflected in the ability to adapt to adverse conditions commonly encountered in their reservoir area (soil, water, animals) or in the environment into which they have been artificially introduced (foods, susceptible unnatural host animals, etc.). These adaptive mechanisms are often transferable between genera, or more commonly species, conferring 'potential pathogen' status on 'newly emerging' microorganisms. *Arcobacter* spp. have aroused interest only within the last two decades (Vandamme et al., 1992b). This is largely because of their close phenotypic and genotypic relationship to *Campylobacter* spp., a somewhat elusive species in the laboratory, yet known to be responsible for a large number of cases of foodborne disease especially in the developed world (Phillips, 2001). While substantial research data has been accumulated on the characteristics of *Campylobacter* spp., notably on the thermophilic, pathogenic species *C. jejuni* and *C. coli*, research on *Arcobacter* spp. has centered around establishing its reservoirs in nature and its distribution within the food industry. This study has, therefore elucidated important characteristics of the genus, in particular of strains of the primary human pathogen, *A. butzleri*. Its similarity to *Campylobacter* warrants a comparison of characteristics between the two genera.

Much has been discussed on the role of campylobacters as VNCs (viable-but-nonculturable) cells. This has been based on the rapid decline in cell numbers observed in starvation or reduced a_w environments (Colwell and Grimes, 2000), as in unchlorinated water, poultry or beef surfaces (ICMSF, 1996). Similar observations were made in our experience of handling of *Arcobacter* species, which experienced a rapid decline in cell numbers on the surface of 5% bovine blood agar plates, becoming unculturable on agar media transfers, in about 3-4 weeks at 4°C. Subsequent inoculations into liquid growth media (EMJH) and prolonged incubation (for up to 1 week) resulted in resuscitation of the strains. Also, experimental inoculations of three *A. butzleri* strains in raw ground pork at levels of 4-6 log₁₀ CFU/g, followed by incubation at 37°C for up to 72 h exhibited strain-dependent patterns. While one strain showed neither growth nor survival in pork, the other two strains were able to survive, with no increase in cell numbers, at the highest inoculation level for periods of up to 72 h. Thus, the significance of *Arcobacter* spp. in foods and as potential pathogens may be related to their ability to survive under various environmental conditions, while not necessarily experiencing an increase in cell numbers.

Thermal tolerance is a characteristic not usually associated with campylobacters, in spite of the high growth-range preference of the 'thermophilic species' (Corry et al., 1995). Reported D-values at pH 7.0 in phosphate buffer of two human enteritic *Campylobacter jejuni* strains (ICMSF, 1996) were in the range of 0.88-1.63 min at 50EC, while D-values of *Campylobacter* in foods ranged from 0.79-2.25 min at 57-55°C in cooked chicken, to 0.21-13.3 min at 60-50°C in lamb cubes, to 0.62-5.9 min at 56-50°C in ground beef (ICMSF, 1996). From our experiments, *Arcobacter* strains studied were somewhat more thermotolerant, with D-values ranging from 0.03-5.81 min at 60-50°C in

phosphate buffered saline, to 2.18-18.51 min at 55-50°C in ground pork. This apparent enhanced resistance factor, coupled with its published ability to survive and grow under aerobic conditions makes *Arcobacter* spp. significant as potential foodborne pathogens, when compared with available literature on *Campylobacter* studies. A combination of heat and acid pH (5.5) decreased heat resistance of *A. butzleri*, though one strain appeared to be less susceptible (*A. butzleri* 3494) to these combined effects as compared to the others (*A. butzleri* 3556 and 3257). The pH of 5.5 was chosen since one of the primary reservoirs of arcobacters is swine/raw pork products, which have pH values of 5.5-5.6 (Brown, 1982; Collins et al., 1996b). The closest comparisons available are the D-values of *Campylobacter jejuni* in poultry scald water which were 0.4 min and 8.72 min at 52°C, at a pH of 4.0 and 6.0, respectively (ICMSF, 1996).

An interesting feature noted, was the apparent effect of pre-experimental growth medium conditions on defining the thermotolerance of *A. butzleri*, in our studies. Early determination of D-values was conducted using Leptospira medium (WCG Serum Products, Centerville, UT). Subsequent to non-availability of this medium, experiments were conducted using EMJH medium (Intergen Inc., Purchase, NY). Though cultures grown in both media were washed twice in PBS before experiments, there was a consistent 'tail' region observed in survivor curves at 50°C for the strain 3556 under study, when the WCG Serum products medium was used for pre-experimental growth of cells (Fig. 3.8), as compared to the EMJH medium. A comparison of the ingredients of both media is available in Table 3.4. It is possible that presence or levels of one or more ingredients may be a contributing factor in this observation. The main differences between the two media are the use of Tween 80 and copper sulfate in EMJH, as opposed

Table 3.4: Comparison of ingredients of media recommended for growth of *Arcobacter* spp.

Media Ingredient	EMJH formulation ^a (1X)	WCG Serum products formulation ^b (1X)
Ammonium chloride (NH ₄ Cl)	0.225 g/L	0.225 g/L
Thiamine	4.5 mg/L	4.5 mg/L
Glycerol	0.09 g/L	0.09 g/L
Calcium chloride (CaCl ₂ ·2H ₂ O)	0.015 g/L	0.015 g/L
Magnesium chloride (MgCl ₂ ·7H ₂ O)	0.015 g/L	0.015 g/L
Zinc sulfate (ZnSO ₄ ·7H ₂ O)	0.004 g/L	0.004 g/L
Vitamin B12	0.002 g/L	0.0002 g/L
Sodium phosphate, dibasic	0.9 g/L	0.9 g/L
Potassium phosphate, monobasic	0.27 g/L	0.27 g/L
NaCl	0.9 g/L	0.9 g/L
Pyruvate	0.09 g/L	0.1 g/L
30% albumin solution	-	1 % (v/v)
Tween 20	-	0.125 % (v/v)
Ferrous sulfate (FeSO ₄ ·7H ₂ O)	0.05 g/L	0.05 g/L
Bovine albumin fraction V	1 % v/v	-
Copper sulfate (CuSO ₄ ·5H ₂ O)	0.0003 g/L.	-
Tween 80	0.125 % (v/v)	-

^a add 996 ml distilled water, pH of medium = 7.4

^b volume raised to 1 L with deionized, glass distilled water; pH of medium = 7.35

to Tween 20 and no copper sulfate in the WCG Serum products formulation. Withell (1942) conducted an in-depth study on the variation in shape of time-survivor curves, and has noted the existence of 'lag times' or 'tailing' in survivor curves. While it is well known that a difference in the age of cells can bring about a curve shape variation, it is possible that certain media ingredients can also emphasize cell thermal variations, as seen in our study. Tailing has been observed with a number of different microorganisms and experimental techniques. Humpheson et al. (1998) reported reproducible biphasic thermal survivor curves with higher resistance in 'tail' subpopulations, in *Salmonella* Enteritidis PT4 cultures studied, at 60°C. The authors report that de novo protein synthesis of heat shock proteins is responsible for the observed tailing in *S. Enteritidis*. Moats et al. (1971) have stated that the variations observed in tail populations were of physiological rather than of genetic origin, since subcultures of heat-resistant cells exhibited no more heat resistant than the parent cultures.

A successive combination of two potential stress-inducing effects, i.e., heat followed by cold-shock may work synergistically to bring about a large reduction in microbial cell numbers. This effect was distinctly observed on heating *A. butzleri* to 50EC, followed by low temperature treatments of 4EC and 8EC. Yogasundram and Shane (1986) mention the susceptibility of *Campylobacter* spp. wherein freezing reduces cell numbers by 1-2 log₁₀ cycles on the surface of poultry carcasses. Though these synergistic effects have not been tested 'in situ' with *Arcobacter* spp., spraying carcasses with chilled water or disinfectant sprays after scalding would be a cheap, effective intervention method. Humphrey and Cruickshank (1985) described a related study on *C. jejuni* strains wherein altered resistance to subsequent treatment with chemical

antibacterial agents was observed, following either freezing at -20°C or heating at 50°C . Populations surviving these sublethal treatments were found to be more sensitive to rifampicin and sodium deoxycholate. The authors suggest that a loss of barrier properties of the bacterial outer membrane was responsible for the noted responses.

Studies conducted in our laboratory also noted growth of some *A. butzleri* strains in EMJH medium at 10°C , at pH levels of 6.5-8.0, for a minimum incubation period of 7 d. Earlier studies have reported the lower growth limit for *Arcobacter* spp. to be 15°C (Wesley, 1994). Additionally, we observed that all *A. butzleri* isolates studied were able to survive prolonged incubation at -20°C (6 mo, EMJH medium) or -70°C (up to 24 mo, Mueller Hinton medium with 20% glycerol) with, depending on the strain, a 0-1.5 \log_{10} decrease in cell numbers.

Arcobacter spp. were found to have a pH growth range of 5.5-8.0, with an optimum for growth of 6.0 -7.5, depending on the species and strain. Some strains can tolerate a pH of 5.0, especially at non-optimal growth temperatures (25EC), for up to 48 h. In comparison, the reported pH growth range for *Campylobacter* is 4.9-ca. 9.0, with an optimum of 6.5-7.5. (ICMSF, 1996). The consistently low maximum cell numbers attained by *A. cryaerophilus* strains in comparison to *A. butzleri* strains, as observed in the pH study, is probably a reflection of the inability of this species to attain sufficiently high numbers in natural and food environments. This is perhaps the reason why reported isolation rates, in literature, of *A. butzleri* strains are higher.

Arcobacter spp. can grow at NaCl levels up to 3.0% or 3.5%, depending on the species and strain of concern. Some strains survive NaCl levels of up to 5.0%, again, especially at non-optimal growth temperatures (25EC) for periods of up to 48 h. The

reported optimum NaCl concentration for growth of *Campylobacter* spp. was 0.5%, with a maximum of 1.5% (ICMSF, 1996). Thus, in comparison, *Arcobacter* spp. can grow in and tolerate higher NaCl concentrations. In conclusion, our studies indicate that strains of *Arcobacter* are aerobic to microaerophilic species, with higher NaCl and temperature resistance when compared with *Campylobacter* spp., and with a pH growth and survival level comparable to that of *Campylobacter*. It is possible that under the same environmental conditions, arcobacters would be capable of enhanced survival against competing campylobacters and could be responsible for foodborne diseases that are mistakenly identified as *Campylobacter*-related. Further research is necessary to determine the interacting effects of environmental stresses that have been noted in this paper, with a view to designing a cost-effective strategy for control of campylobacter-like organisms (CLOs) in food products.

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Fig. 3.1: Growth response of *Arcobacter butzleri* (A.b.) and *Arcobacter cryaerophilus* (A.c.) strains incubated in Ellinghausen McCullough Johnson Harris (EMJH) medium supplemented with Oxyrase[®] at varying pH levels at 37°C (A) and 25°C (B) for 48 h. All values are representative of two replications.

Figure 3.1(A)

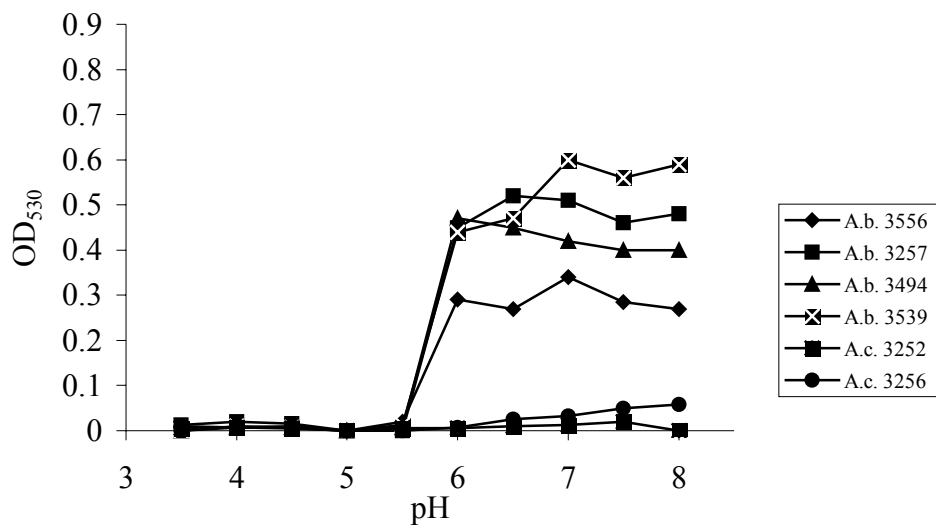


Figure 3.1(B)

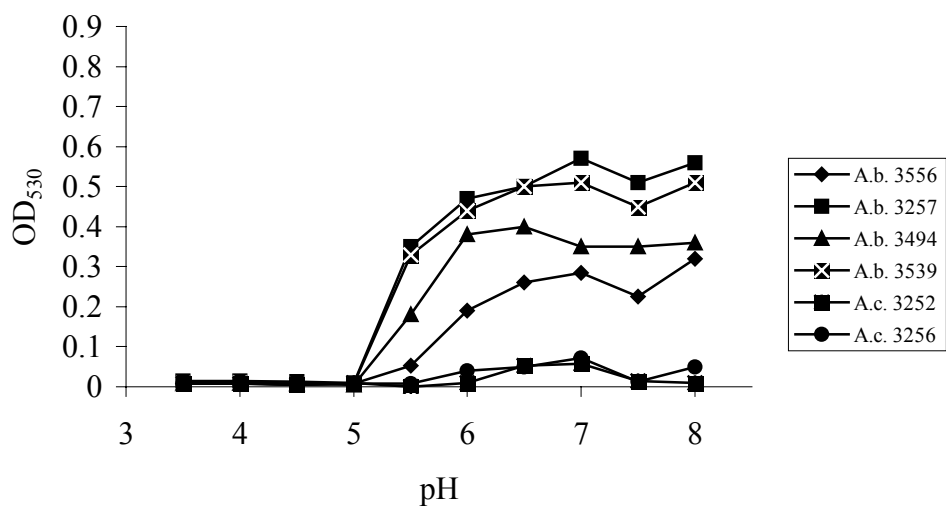


Figure 3.2: Growth response of *Arcobacter butzleri* (A.b.) and *Arcobacter cryaerophilus* (A.c.) strains incubated in Ellinghausen McCullough Johnson Harris (EMJH) medium supplemented with Oxyrase[®] at varying pH levels at 37°C (A) and 25°C (B) for 5 d. All values are representative of two replications.

Figure 3.2(A)

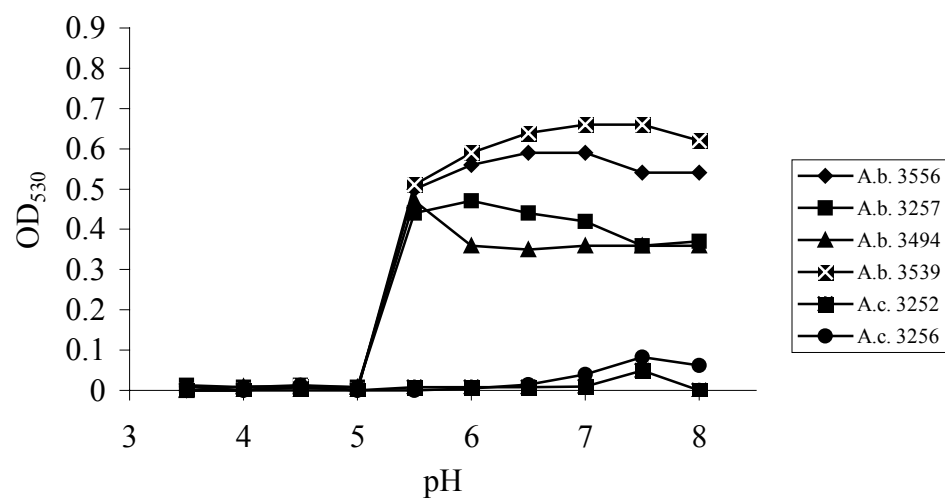


Figure 3.2(B)

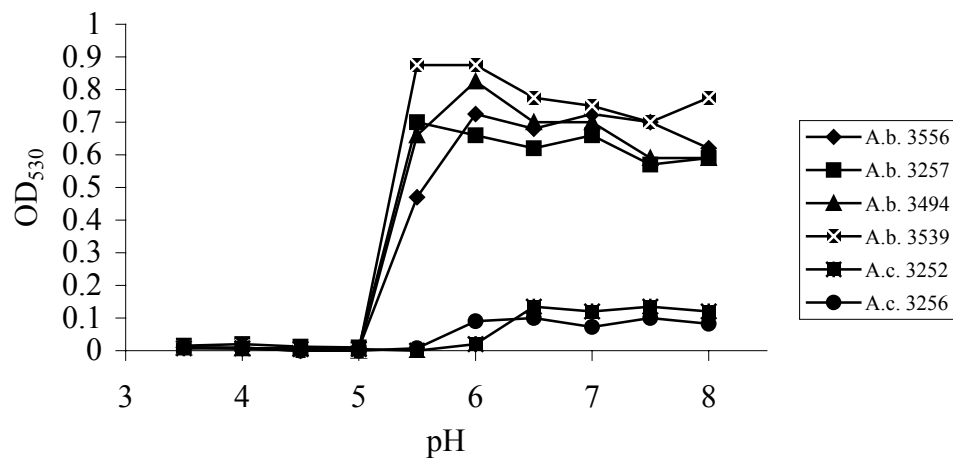


Figure 3.3. Thermal survivor curves for *Arcobacter butzleri* 3556, 3494 and 3257 at 60°C, at pH A) 7.3 and B) 5.5. Values plotted at each time point are representative of three replications.

Figure 3.3(A)

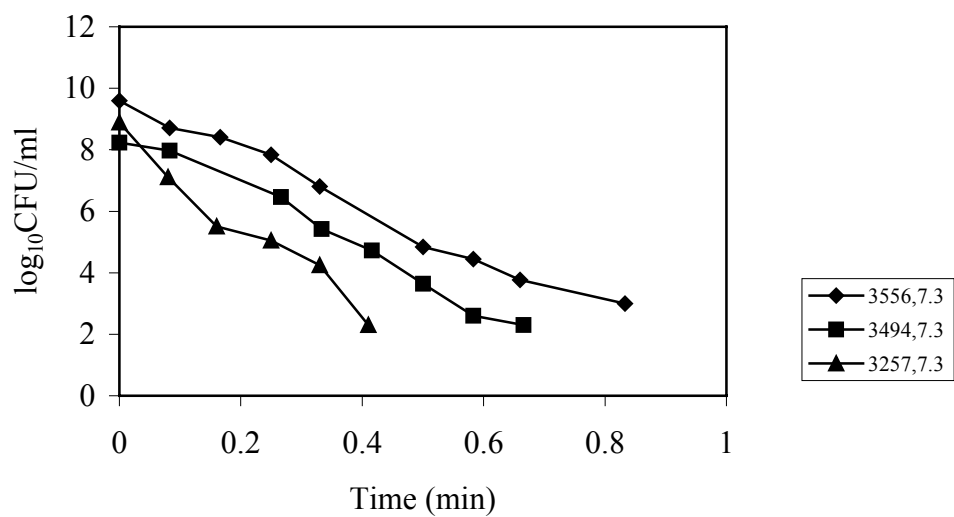


Figure 3.3(B)

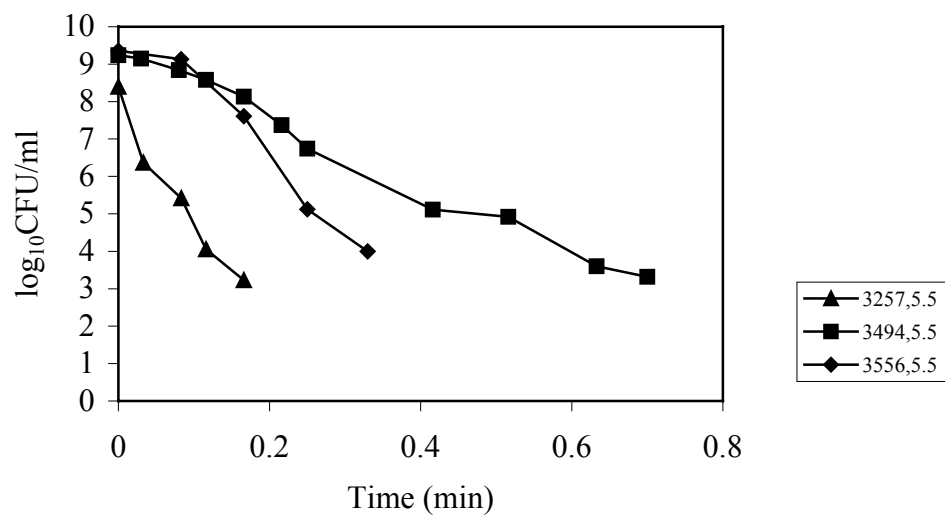


Figure 3.4. Thermal survivor curves for *Arcobacter butzleri* 3556, 3494 and 3257 at 55°C at pH A) 7.3 and B) 5.5. Values plotted at each time point are representative of three replications.

Figure 3.4(A)

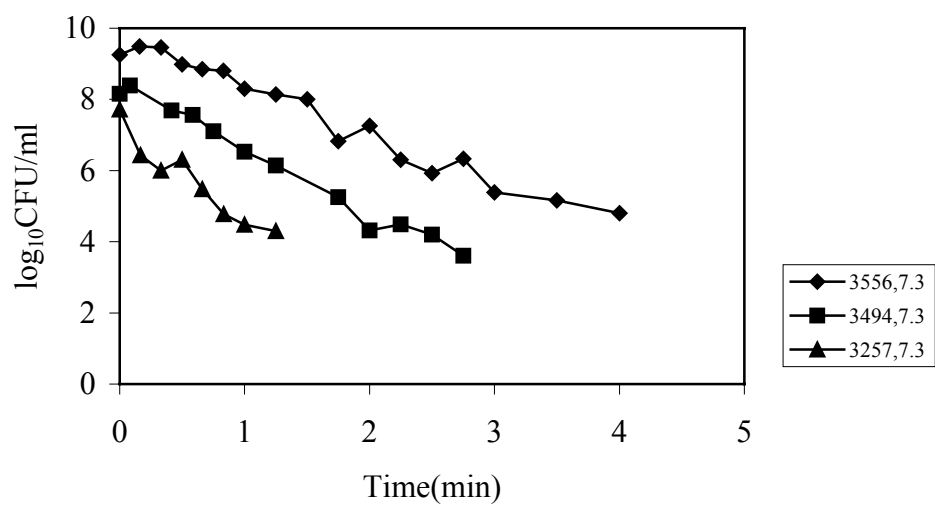


Figure 3.4 (B)

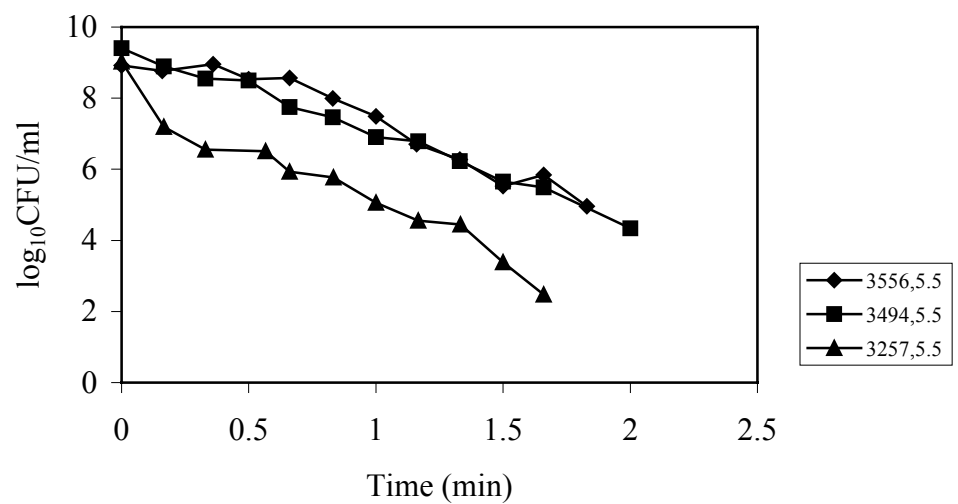


Figure 3.5. Thermal survivor curves for *Arcobacter butzleri* 3556, 3494 and 3257 at 50°C at pH A) 7.3 and B) 5.5. Values plotted at each time point are representative of three replications.

Figure 3.5 (A)

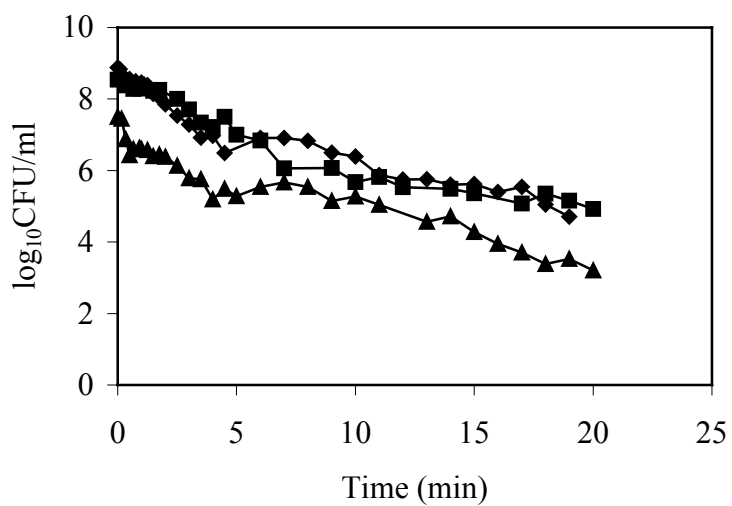


Figure 3.5(B)

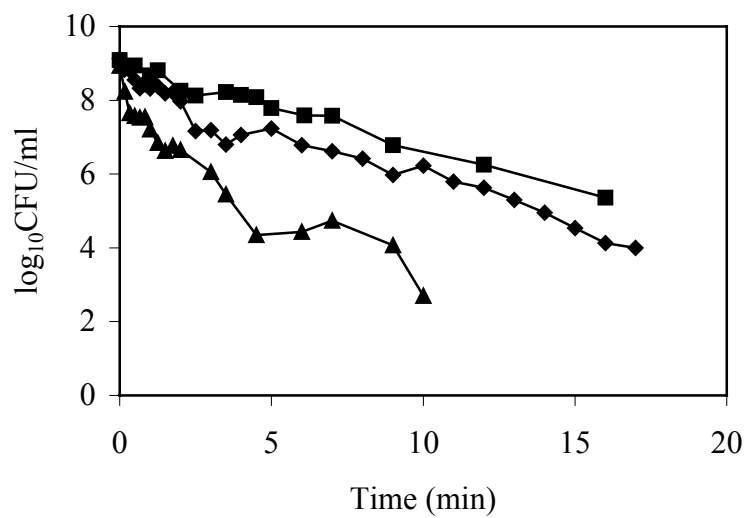


Figure 3.6: Thermal survivor curves for a three-strain mixture of *Arcobacter butzleri* in raw ground pork at 50° and 55°C. Values plotted at each time point are representative of three replications.

Figure 3.6

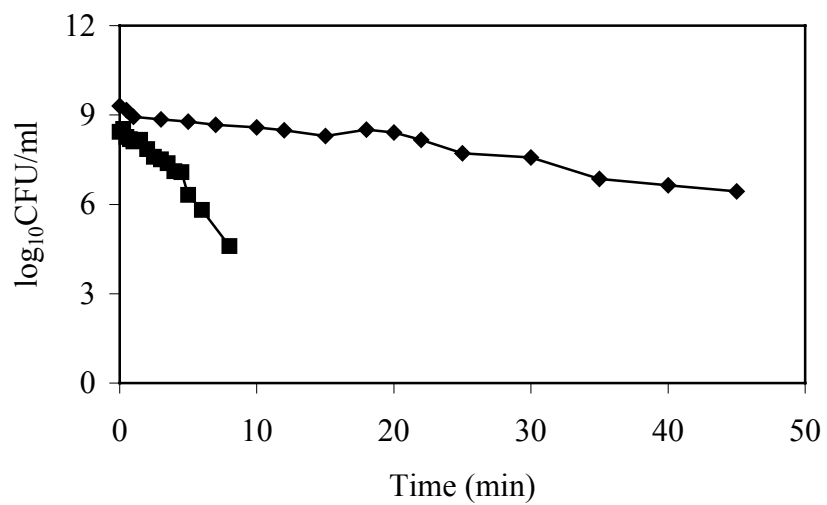


Figure 3.7: Synergistic effect of application of mild heat-shock at 50°C for either 30s or 1 min, followed by cold treatment at 4, 8, 12, and 16°C on reduction in numbers of *A. butzleri* 3556 in phosphate buffered saline at pH 7.3. Values on the X-axis represent temperature-time combinations for each treatment. Values are representative of two replications.

Figure 3.7

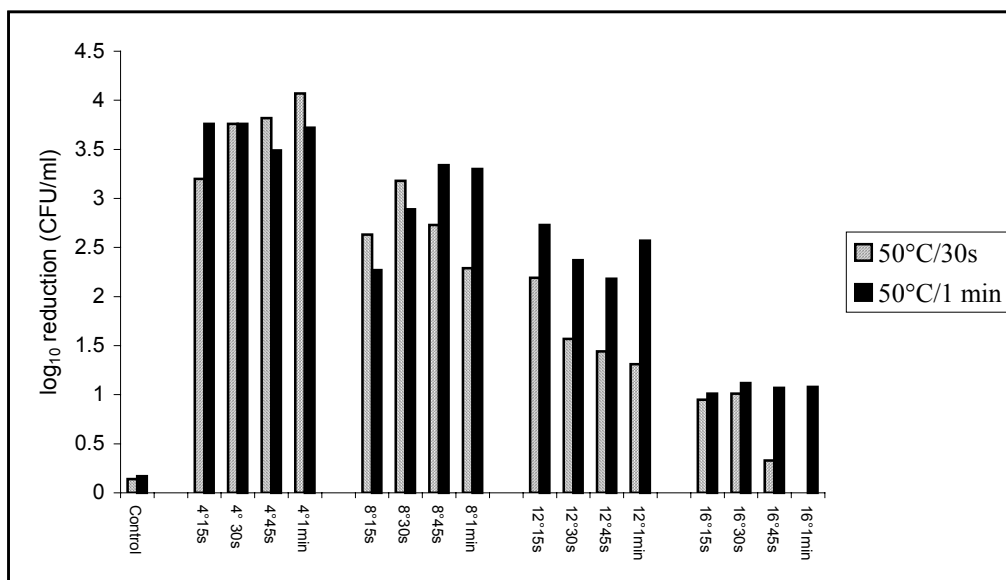
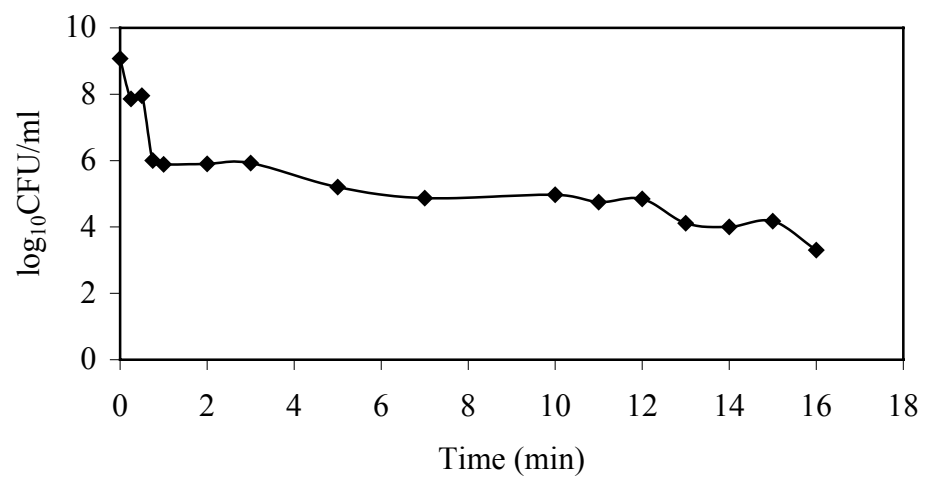


Fig. 3.8. Thermal survivor curve for *Arcobacter butzleri* 3556 at 50°C at pH 7.3 in phosphate buffered saline. *Leptospira* medium was used for pre-experimental growth of cells. Values plotted at each time point are representative of three replications.

Figure 3.8



CHAPTER 4

PREDICTIVE MODELING OF EFFECTS OF pH, TEMPERATURE, SODIUM
CHLORIDE, SODIUM NITRITE AND SODIUM TRIPOLYPHOSPHATE CONTENT
ON GROWTH OF *ARCOBACTER BUTZLERI*¹

¹ D'Sa, E. M., M. A. Harrison, and V. K. Juneja. To be submitted to the *Journal of Food Protection*

Abstract

Arcobacter spp. are considered to be emerging foodborne pathogens implicated in disease transmission through raw and improperly handled foods. Growth characteristics of a three-strain mixture of *A. butzleri* were studied under multiple stress conditions, to develop a mathematical model predicting their growth in environments. The effect of combinations of ambient temperature (12-37°C), initial pH (6.0-7.5), sodium chloride (0.09-3.5%), sodium nitrite (0-180 ppm) and sodium tripolyphosphate (0-0.012%) content on *A. butzleri* in EMJH medium were studied using a fractional factorial plus central composite experimental design. Growth curves from experimental data generated using cell counts obtained on blood agar plates, were fitted to the modified Gompertz function and Gompertz parameters A, C, M & B were calculated for each treatment combination. From these, derived growth characteristics: LPD (lag phase duration), GT (generation time), EGR (exponential growth rate) and MPD (maximum population density) values were calculated. Quadratic and cubic response surface models were constructed from the curve-fitting data using regression analysis on Gompertz parameters and derived growth characteristics. Data were analyzed using both untransformed values and natural logarithm transformations. Significant main and interaction effects for each model were identified. Evaluation and analysis of the predicted derived growth characteristics indicate that the quadratic logarithmically-transformed models gave a better fit. Most MPDs achieved were largely independent of treatment combination conditions and averaged 8.0 log₁₀ CFU/ml. *A. butzleri* isolates studied were found to be highly sensitive to low levels of sodium tripolyphosphate (STPP) and do not grow or survive at STPP levels above 0.016% and 0.02%, respectively. Thus, pH, sodium

chloride, sodium nitrite and sodium tripolyphosphate can be used in combination with low temperature to influence the growth characteristics of *A. butzleri*.

Introduction

Organisms belonging to the *Campylobacter*-related genus *Arcobacter* have been clinically associated with cases of gastroenteritis (Lerner et al., 1994; Phillips, 2001; Vandamme et al., 1992) and occasionally septicemia (Huseh et al., 1997; On et al., 1995) in humans, and with enteritis, abortions and mastitis in animals (Anderson et al., 1993; Ellis et al., 1977; Wesley, 1994). *A. butzleri* is the species most commonly isolated from clinical specimens and foods. *Arcobacter* spp. have been recovered from drinking water (both surface & reservoir) and raw food including red meats and poultry (Collins et al., 1996a; 1996b; de Boer et al., 1996; Mansfield and Forsythe, 2000; Wesley, 1997). These organisms share several characteristics in common with *Campylobacter*. Thus, it has been postulated that the role of *Arcobacter* in foodborne illness is not currently well understood, and that misdiagnosis is possible, given the close relatedness of the genus to *Campylobacter* and the similarity of disease syndromes caused (Wesley, 1997).

Foodborne illness associated with *Campylobacter* is considered to have the highest incidence for any bacterial foodborne disease in the U.S., with an estimated 2 million persons affected each year. The economic implications of these infections, and the associated Guillain-Barré syndrome, are considerable (CDC, 2001; CDC-MMWR, 2001).

Increasing awareness and concern about the safety of the food supply, together with a growing reliance on processed, nationally and internationally-distributed convenience foods has led to a need for rapid, broad-based process-control and monitoring techniques, by food industries. Current HACCP programs provide much of

this process management, but additional time saving risk-assessment programs are necessary, especially in product-development and formulation stages.

Predictive microbiology is a useful tool that involves obtaining detailed knowledge about the growth and survival responses of microorganisms in their niches, which subsequently allows evaluation and projection of the effects of processing and post-processing conditions on microbial food safety. This is achieved through use of a series of mathematical relationships (McMeekin et al., 1993). Predictive models have been developed from studies on several microorganisms including, but not limited to *Salmonella* (Gibson et al., 1988), *Clostridium botulinum* (Gibson et al., 1987), *Clostridium perfringens* (Juneja et al., 1996), *Escherichia coli* O157:H7 (Buchanan and Klawitter, 1992), and *Aeromonas hydrophila* (Palumbo et al., 1991). Concerted efforts have made available the Pathogen Modeling Program (USDA, 2001), an online predictive database tool that allows analysis of food product-type environmental parameter combinations and an assessment of the microbiological hazards. Models for microbial growth, inactivation (thermal, nonthermal), gamma irradiation, cooling, time-to-toxigenesis and time-to-turbidity are made available through this program.

The concept of ‘hurdle technology’ or using combinations of preservative effects (e.g., low temperature, acidity, chemicals, reduced moisture) that act synergistically at lowered individual levels to eliminate or reduce microbial growth, has been long used in the food industry. A predictive model would help define recommended levels of these factors in foods, the choice of factors being governed by regulations, anticipated microbial hazards and product category. The objective of this study was to construct a predictive mathematical model describing the interactive effects of five factors on the

growth responses of *A. butzleri* in EMJH culture medium. Since *A. butzleri* has been detected in poultry and red meats (Wesley, 1994; 1997), sodium chloride, sodium nitrite and sodium tripolyphosphate (STPP) were selected as factors in the model, together with pH and ambient temperature. Sodium nitrite is commonly added to pork products for control of *C. botulinum* spores, and to maintain cured meat color and texture. Allowable levels are up to 156 ppm (120 ppm for bacon) and up to 0.5%, for sodium nitrite and polyphosphates respectively, in meat and poultry products (Brown, 1982; Zaika and Kim, 1993).

Materials and methods

Preparation of inoculum: Human strains of *Arcobacter butzleri* (3556, 3257, 3494) were obtained from Dr. Irene Wesley at the USDA ARS-NADC Laboratory in Ames, Iowa and stored at -70°C in Mueller-Hinton broth supplemented with 20% glycerol. Before use they were subcultured onto 5% bovine blood agar plates (prepared with blood agar base with low pH, Acumedia Manufacturers Inc., Baltimore, MD; defibrinated bovine blood, Metro Medical Supply, Inc., Burlington, NC) and incubated for 36-48 h in a controlled atmosphere (10% CO_2 , 5% O_2 , 85% N_2). Isolated colonies of each culture were then individually inoculated, using 10 μL polystyrene loops (VWR Scientific, Westchester, PA) into a 1:5 dilution of Ellinghausen McCullough Johnson Harris medium [EMJH, (Intergen Inc., Purchase, NY),] and sterile deionized water, supplemented with 2% Oxyrase[®] (Oxyrase Inc., Mansfield, OH) in 250 ml polystyrene tissue culture flasks (Becton Dickinson and Company, Sparks, MD). These were incubated on an orbital shaker, with gentle shaking (50 rpm) at 37°C for 30 h. Cultures were centrifuged (8000 x g, 25 min, 4°C , Sorvall RC-5B refrigerated centrifuge, DuPont

Instruments, Newtown, CT), washed twice with 0.1M phosphate buffered saline (PBS), and were resuspended in PBS to yield a final concentration of 10^9 CFU/ml. Prepared cultures were stored at 4°C until used, but for no more than 72 h.

Effect of sodium nitrite and STPP: Cultures were appropriately diluted and mixed in suitable amounts to provide an equal-concentration mixture of the three strains. Ten-ml portions of EMJH (1:5) medium were mixed with Oxyrase[®] at a 2% level, and selected amounts of an appropriately diluted filter-sterilized stock solution of 1% sodium nitrite (Sigma–Aldrich Co., St. Louis, MO) or 1% STPP (FMC Corp., Philadelphia, PA) were added, to provide test solutions within the range of 0-200 ppm sodium nitrite or 0-0.1% STPP. Solutions were inoculated with the culture cocktail at a level of $3 \log_{10}$ CFU/ml, and incubated at 20 and 37°C with gentle shaking (50 rpm) for 72 h. Presence of visible turbidity was noted as growth of the populations. To determine whether or not the bacteria survived in the treatments regardless of the presence or absence of growth, a loopful of medium was transferred to a blood agar plate which was incubated for 48 h.

Growth curve experiments: Solutions of NaCl (35%), sodium nitrite (1%), STPP (1%), HCl (2.25N) and NaOH (1N) were prepared in sterile deionized water, filtered through 0.2 µm cellulose nitrate filters and stored at 4°C until used. EMJH medium was combined with appropriate amounts of NaCl, sodium nitrite and STPP (Table 1) and the pH was adjusted as necessary. Oxyrase[®] (2%) was added to each 20 ml volume in individual tissue culture flasks. Appropriately diluted volumes of the three strains were added to yield equivalent levels of each, to a cell density of approximately $3 \log_{10}$ CFU/ml. Samples were withdrawn at time zero, microdiluted with PBS as necessary, in 1.5 ml polypropylene microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA), plated on 5%

bovine blood agar plates and incubated at 37°C in a CO₂ incubator with a gas atmosphere of 5% O₂, 10% CO₂ and 85% N₂ for up to 48 h. Colonies were counted and converted to log₁₀ CFU/ml counts. The experimental flasks were incubated at the selected temperatures, samples from each flask were subsequently withdrawn at selected intervals (based on pre-trial runs), similarly plated, incubated at 37°C and counts determined for up to 10 days.

Mathematical Model Experimental Design: Based on previous research, levels of pH, temperature, sodium chloride, sodium nitrite and STPP were selected and the outer limits of the experimental model were defined as follows: temperature: 12-37°C, initial pH: 6.0-7.5, NaCl (%):0-3.5, sodium nitrite (ppm):0-180, STPP (%): 0-0.012. In comparison, published growth limits for *Campylobacter* include a minimum and maximum temperature of 32 and 45°C, respectively; a pH range of 4.9-9, and an NaCl (%) optimum and maximum of 0.5 and 1.5, respectively (ICMSF, 1996). A randomized fractional factorial plus central composite type model design was selected, which included: a) one full-factorial design that tested all possible combinations of the following variables (32 combinations): temperature: 19 or 30°C, initial pH: 6.45 or 7.0, NaCl: 1 or 2.5%, sodium nitrite: 52 or 128 ppm, STPP: 0.0035 or 0.0085%; b) One center point which represented midpoint range values for all five variables; c) Ten ‘star’ points that tested each extreme variable values successively against midpoint values of the other four variables; d) controls (EMJH and inoculum): at 19, 24.5 and 30°C at each initial pH of 6.0, 6.45, 6.75, 7.0 and 7.5. Two replications were done for each combination, with five replications tested for the center point combination. The variable combinations used can be seen in Table 4.1.

Table 4.1: Experimental design (fractional factorial plus central composite type) used for growth curve experiments for *Arcobacter butzleri* in EMJH medium. L = lattice point, S = star point, C = center point

Treatment number	Temperature (°C)	pH	NaCl (%)	NaNO ₂ (ppm)	STPP (%)	Design point
1	19	6.44	1.0	52	0.0035	L
2	19	6.44	1.0	52	0.0085	L
3	19	6.44	1.0	128	0.0035	L
4	19	6.44	1.0	128	0.0085	L
5	19	6.44	2.5	52	0.0035	L
6	19	6.44	2.5	52	0.0085	L
7	19	6.44	2.5	128	0.0035	L
8	19	6.44	2.5	128	0.0085	L
9	19	7.06	1.0	52	0.0035	L
10	19	7.06	1.0	52	0.0085	L
11	19	7.06	1.0	128	0.0035	L
12	19	7.06	1.0	128	0.0085	L
13	19	7.06	2.5	52	0.0035	L
14	19	7.06	2.5	52	0.0085	L
15	19	7.06	2.5	128	0.0035	L
16	19	7.06	2.5	128	0.0085	L
17	30	6.44	1.0	52	0.0035	L
18	30	6.44	1.0	52	0.0085	L
19	30	6.44	1.0	128	0.0035	L
20	30	6.44	1.0	128	0.0085	L
21	30	6.44	2.5	52	0.0035	L
22	30	6.44	2.5	52	0.0085	L
23	30	6.44	2.5	128	0.0035	L
24	30	6.44	2.5	128	0.0085	L
25	30	7.06	1.0	52	0.0035	L
26	30	7.06	1.0	52	0.0085	L
27	30	7.06	1.0	128	0.0035	L
28	30	7.06	1.0	128	0.0085	L
29	30	7.06	2.5	52	0.0035	L
30	30	7.06	2.5	52	0.0085	L
31	30	7.06	2.5	128	0.0035	L
32	30	7.06	2.5	128	0.0085	L
33	12	6.75	1.75	90	0.006	S
34	37	6.75	1.75	90	0.006	S
35	24.5	6.00	1.75	90	0.006	S
36	24.5	7.50	1.75	90	0.006	S
37	24.5	6.75	0.00	90	0.006	S
38	24.5	6.75	3.5	90	0.006	S
39	24.5	6.75	1.75	0	0.006	S
40	24.5	6.75	1.75	180	0.006	S
41	24.5	6.75	1.75	90	0	S
42	24.5	6.75	1.75	90	0.012	S
43	24.5	6.75	1.75	90	0.006	C

Data analysis: Data obtained from the growth curve experiments was fitted to the modified Gompertz equation using nonlinear regression analysis with a Gauss Newton iteration in SAS (SAS, 1989). Fitted curves generated the Gompertz parameters A, C, M and B, which were then used to calculate the derived growth characteristics: maximum population density (MPD), generation time (GT), lag phase duration (LPD) and exponential growth rate (EGR) of *A. butzleri* for each combination studied. Experimental inoculations that showed less than one- \log_{10} cycle increase over inoculated levels were considered to be non-growing.

Modified Gompertz Equation: $L(t) = A + C [\exp \{-\exp (-B(t-M))\}]$, where,

$L(t)$ = \log_{10} count of bacteria at time (h) t , (\log_{10} CFU/ml)

A = asymptotic log count of cells as time decreases indefinitely (initial level of cells), (\log_{10} CFU/ml)

C = asymptotic amount of growth that occurs as t increases indefinitely [number of log cycles of growth, or difference in the initial and final number of cells at the stationary phase (\log_{10} CFU/ml)]

B = relative growth rate at M (\log_{10} [CFU/ml]/h) where

M = time at which absolute growth rate is maximal (h)

From these, the following *derived* growth kinetic characteristics were calculated:

EGR (exponential growth rate) = $B \cdot C / e$ \log_{10} (CFU/ml)/h

GT (generation time) = $\log_{10} 2 \cdot e / B \cdot C$ h

LPD (lag phase duration) = $M - 1/B$ h

MPD(maximum population density)= $A+C$ \log_{10} CFU/ml

The data was subjected to regression analysis using second (quadratic) and third (cubic) order response surface models with temperature, pH, NaCl, sodium nitrite and STPP levels as independent variables. Regressions were performed on the following parameters: A, C, M, B, LPD, EGR, MPD and GT. The parameters were first modeled 'as is' and then using the natural logarithm transformation. In addition, data was analyzed by an analysis of variance (ANOVA) using SAS (SAS, 1989), F-values and significance for main effects and interaction effects were calculated for the individual equations. For the development of the cubic models, stepwise regression using backwards elimination (BE) was used to eliminate superfluous and nonsignificant terms from the model for each response. The resulting equations thus use a subset of the 'general form of the regression' and these subsets are specific to each response being analyzed.

Results

Effect of sodium nitrite and STPP: *A. butzleri* was more sensitive to lower levels of STPP than previously described in literature for other organisms in presence of various polyphosphates. For the three *A. butzleri* strains studied, growth occurred in the range of 0-0.016%, while survival was seen up to 0.02% STPP. Thus, the minimum inhibitory concentration was 0.018% STPP, under the experimental conditions studied. Under the pre-experimental conditions chosen, sodium nitrite allowed growth of *A. butzleri* up to 200 ppm. Higher levels were not selected for further study, as 200 ppm is the maximum permissible level for this preservative in meat and poultry products (Anonymous, 2001).

Mathematical modeling experiments: A total of 104 growth curves were generated and used in the data analysis for this study. The forms of the regression equations are noted in

Table 4.2, using $\ln(\text{GT})$ as an example. All models were evaluated using the ‘Adjusted R^2 ’ value, which is an adjustment performed to remove the variation between replicate values from the Total Sum Squares (TSS) values, during the regression modeling process. Table 4.3 represents the Adjusted R^2 values for natural logarithm transformed quadratic and cubic models. The GLM procedure was able to determine equation terms in each model that were significant ($p < 0.05$) and thus highlight the effects (both main and interaction) that uniquely contribute significantly to each individual model. These are noted in Table 4.4. Traditionally, analyses of models have focused on those models expressing values of Gompertz parameters B and M, and derived growth kinetic values GT and LPD (Palumbo et al., 1991). This is because ‘A’ (initial level of bacteria), though important in determining the MPD, is an independent value, based on often incidental factors not within the scope of the model (e.g. environmental or processing-plant contamination levels, pre-processing conditions). Also, Buchanan and Phillips (1990) determined that inoculum size had no significant effect on derived growth kinetic characteristics of *L. monocytogenes* and several researchers have confirmed this observation with other pathogens used in modeling studies. For $\ln(\text{MPD})$, model analysis indicates that pH, NaCl and nitrite levels are the significant main effects governing this value, together with other interaction effects. Observed maximum population densities achieved ranged from 2.36 \log_{10} (lowest observed, 2 observations) CFU/ml to 12.27 \log_{10} CFU/ml (highest observed, 1 observation), with a mean of 8.0 \log_{10} CFU/ml. Thus, with few exceptions, MPDs achieved for treatment combinations that did exhibit growth were closely ranged.

Table 4.2: Quadratic and cubic response surface models of the natural logarithm of the generation time (h) for the growth of *A. butzleri* in EMJH medium as a function of temperature (temp, 12-37°C), initial pH (pH,6.0-7.5), sodium chloride level (NaCl, 0-3.5%), sodium nitrite level (nitrite, 0-180 ppm) and sodium tripolyphosphate level (phos, 0-0.012%).

Model type	To calculate ln(GT)
Quadratic (Second order)	$\ln(\text{GT}) = 45.868603 - 0.222923*\text{temp} - 12.548411*\text{pH} - 2.886261*\text{NaCl} - 405.726455*\text{phosp} + 0.1775522*\text{nitrite} - 0.009774*\text{temp}*\text{pH} + 0.013018*\text{temp}*\text{NaCl} + 7.532328*\text{temp}*\text{phos} - 0.000800*\text{temp}*\text{nitrite} + 0.471514*\text{pH}*\text{NaCl} + 11.300784*\text{pH}*\text{phos} - 0.022511*\text{pH}*\text{nitrite} + 45.866237*\text{NaCl}*\text{phos} - 0.003365*\text{NaCl}*\text{nitrite} + 1.424866*\text{phos}*\text{nitrite} + 0.004314*\text{temp}*\text{temp} + 0.939236*\text{pH}*\text{pH} - 0.014355*\text{NaCl}*\text{NaCl} - 521.975552*\text{phos}*\text{phos} - 0.0000310*\text{nitrite}*\text{nitrite}$
Cubic (Third Order)	$\ln(\text{GT}) = 1.0924 + 29.07248\text{NaCl} - 0.1667*\text{nitrite} - 0.0280*\text{temp}*\text{pH} - 424.7075*\text{temp}*\text{phos} + 0.0329*\text{nitrite}*\text{temp} - 3.3536*\text{NaCl}*\text{pH} + 284.0038*\text{pH}*\text{phos} + 757.3277*\text{NaCl}*\text{phos} - 0.1717*\text{NaCl}*\text{nitrite} - 46.6370*\text{nitrite}*\text{phos} - .3030*\text{NaCl}*\text{NaCl} + 762359.6917*\text{phos}*\text{phos} + 0.1269*\text{NaCl}*\text{temp}*\text{pH} - 21.9814*\text{temp}*\text{pH}*\text{phos} - 0.0022*\text{nitrite}*\text{temp}*\text{pH} + 7.7461*\text{NaCl}*\text{temp}*\text{phos} - 0.0010*\text{NaCl}*\text{nitrite}*\text{temp} + 0.2850*\text{nitrite}*\text{temp}*\text{phos} + 0.0250*\text{NaCl}*\text{nitrite}*\text{pH} + 5.4684*\text{nitrite}*\text{pH}*\text{phos} + 2.3006*\text{NaCl}*\text{nitrite}*\text{phos} + 0.0011*\text{temp}*\text{temp}*\text{pH} - 0.0658*\text{NaCl}*\text{temp}*\text{temp} + 23.0594*\text{temp}*\text{temp}*\text{phos} - 0.0004*\text{nitrite}*\text{temp}*\text{temp} + 0.6931*\text{NaCl}*\text{NaCl}*\text{temp} - 1119.3296*\text{NaCl}*\text{NaCl}*\text{phos} - 47953.4211*\text{temp}*\text{phos}*\text{phos} + 234613.3115*\text{NaCl}*\text{phos}*\text{phos} - 0.0062*\text{nitrite}*\text{nitrite}*\text{phos}$

Table 4.3. Adjusted R^2 values for natural-logarithm transformed Gompertz parameter and derived growth kinetics models, analyzed by second-order (quadratic) and third-order (cubic) regression, for *Arcobacter butzleri* in EMJH medium.

Parameter	Adjusted R^2	
	Quadratic Model	Cubic Model
ln (A)	0.576	0.972
ln (C)	0.545	0.884
ln (M)	0.791	0.943
ln (B)	0.547	0.988
ln (LPD)	0.838	0.966
ln (GT)	0.835	0.984
ln (EGR)	0.835	0.984
ln (MPD)	0.685	0.988

Table 4.4. Significant main and interaction effects ($p < 0.05$) in Gompertz parameter and derived growth kinetics characteristics models for *Arcobacter butzleri* in EMJH medium. These represent the natural logarithm transformed quadratic response surface models.

Parameter	Significant main effect	Significant interaction effect ^b
ln A	- ^a	temp*temp (4.91) ^c temp*phos (4.61) NaCl*NaCl (53.18) phos*phos (7.18)
ln C	phosphate (14.27) nitrite (16.06)	temp*phos (11.7) temp*nitrite (4.76) pH*phos (8.96) pH*nitrite (14.14)
ln B	NaCl (8.92)	pH*NaCl (12.17) NaCl*nitrite (25.59) temp*temp (6.49)
ln M	temperature (6.70)	temp*phos (6.35) phos*nitrite (6.09) temp*temp (10.22) NaCl*NaCl (11.92)
ln LPD	temperature (5.37) NaCl (5.97)	temp*pH (4.08) temp*NaCl (5.99) pH*NaCl (7.14) phos*nitrite (10.15) NaCl*NaCl (35.05)
ln EGR	PH (7.62) Nitrite (32.68)	temp*phos (4.75) temp*nitrite (12.37) pH*nitrite (25.09) phos*nitrite (6.82) temp*temp (4.79) pH*pH (8.03)
ln MPD	PH (6.4) NaCl (4.91) Nitrite (11.89)	pH*NaCl (6.47) pH*nitrite (11.73) NaCl*nitrite (22.08) pH*pH (6.25)
ln GT	PH (7.62) Nitrite (32.68)	temp*phos (4.75) temp*nitrite (12.37) pH*nitrite (25.09) phos*nitrite (6.82) temp*temp (4.79) pH*pH (8.03)

^aNo significant main effect

^b(Temperature = temp, initial pH = pH, sodium tripolyphosphate = phos, sodium nitrite = nitrite, sodium chloride = NaCl, LPD = lag phase duration, MPD = maximum population density, EGR = exponential growth rate, GT = generation time)

^c Values in parentheses represent the F-values

For $\ln(\text{GT})$ significant main effects were pH and nitrite together with several interaction effects. The effect of pH and temperature on GT at constant NaCl (2%), phosphate (0.006%) and nitrite (156 ppm) levels is shown in Fig. 4.1, and the influence of the main significant effects of pH and nitrite level on GT at constant NaCl (2%), phosphate (0.006%) and temperature (25°C) levels is seen in Fig. 4.2. These three values of constants were selected in accordance with 'most commonly used' estimates for NaCl (2%), maximum allowable levels for nitrite in sausage (156 ppm) and ambient temperature (25°C). For ease of interpretation, the plots presented represent the effects of the two parameters on generation time (GT). Additionally, plots are available for the natural logarithm transformed data, in the form used to develop the models. These are presented for comparison, if necessary, in the Appendix. The combined effect of decreasing temperature (approaching 12°C, in this study) and lowered pH (approaching 6.0) acts to increase the generation time, as observed in Fig. 4.1, with the maximum influence on GT occurring at the lowest values of these two parameters. The average generation time at a pH of 7.5, at 37°C was 1h, this was predicted to increase to up to 38 h under temperature and pH conditions that approached the lower limits for these variables. In a similar manner, the interaction effect of pH and nitrite is apparent in Fig. 4.2, where a lowered pH (6.0) at high nitrite levels (120-180 ppm) increases the predicted generation time to over 30 h (Fig. 4.2). A small increase in the predicted generation times was also seen at the portion of the response surface curve approaching pH 7.5 at a nitrite level of 0-40 ppm, which may be due to the pH conditions approaching the higher limit for growth.

For ln (LPD), significant ($p < 0.05$) main effects were temperature ($^{\circ}\text{C}$) and NaCl levels (%), and several interaction effects were also significant. NaCl levels can thus be expected to exert a significant influence in influencing lag phase durations. The effect of pH and temperature on LPD at constant NaCl (2%), nitrite (156 ppm) and phosphate levels (0.006%) is shown in Fig. 4.3, while the effects of pH and NaCl on LPD at constant temperature (25°C), nitrite (156 ppm) and phosphate (0.006%) levels is seen in Fig. 4.4. Fig. 4.5 shows the effects of temperature and NaCl on LPD at constant pH (6.0), nitrite (156 ppm) and phosphate (0.006%) levels. Combined effects of low temperature and low pH (Fig. 4.3), increasing NaCl levels and low pH (Fig. 4.4), and low temperature and increasing NaCl levels (Fig. 4.5) increased predicted LPD values. The highest values of LPDs were predicted at the combination of low temperature (12°C) and high NaCl (3.5%) levels (over 900 h, Fig. 4.5) while the effects of low temperature and low pH on extending LPDs was not as heightened (over 70 h, Fig. 4.3). Nevertheless, the overall increase in slope of the response curve appeared to be more gradual and definite in Fig. 4.3, indicating a graded and less sudden response to the influence of variable levels of temperature and pH near the lower limits. On the other hand, in Fig. 4.5, while a large number of points on the response curve have LPDs below 100 hours, a steep increase in the slope of the curve was noted as NaCl levels approached 3-3.5%, and the temperature levels decreased below 24°C . Fig. 4.3 (A, B, C) represent the effects of temperature and pH on LPD in the presence of progressively increasing levels of NaCl (A= 0.09% NaCl, B= 1% NaCl and C= 3.5% NaCl). These three figures, together with Fig. 4.3, demonstrate the effect of changing levels of a variable (in this case NaCl) on LPDs. There has been some discussion on the variability of model predictions at some variable

values near the outer limits of the model range (Ratkowsky et al., 1991). This is possibly the explanation for the response observed in Fig. 4.3A (under the influence of the four remaining variables, since NaCl levels = 0.09%), as compared to the uniform, but increasing LPDs predicted in Fig. 4.3B (1% NaCl), 4.3 (2% NaCl) and 4.3C (3.5% NaCl). Interaction effects are also apparent in the responses, especially at lower NaCl concentrations of 0.09% and 1%.

For $\ln(\text{EGR})$ the main effects of pH and nitrite were significant ($p < 0.05$) together with various interaction effects. Fig. 4.6 demonstrates the influence of the significant main effects terms of pH and nitrite levels on EGR, at constant temperature (25°C), phosphate (0.006%) and NaCl (2%) levels. Fig. 4.7 demonstrates the effects of temperature (°C) and pH on EGR at constant nitrite (156 ppm), NaCl (2%) and phosphate (0.006%) levels. Increasing nitrite levels appeared to increase EGRs in combination with a higher pH (7.5), whereas lowering the pH to 6.0 brought about a decrease of EGRs to the lowest predicted level, at higher nitrite levels (Fig. 4.6). Thus an interactive effect exists between pH and nitrite levels, in determining EGRs. Fig. 4.7 demonstrates the combined influence of high pH and increasing temperature in bringing about an increase in the predicted EGR. A low pH (6.0) influences a consistently low EGR through the entire temperature range studied. Regressions for $\ln(\text{EGR})$ and $\ln(\text{GT})$ were noted to be identical, except for the intercept term and the sign of the coefficients of the remaining terms, since these two terms are virtually reciprocals of each other, except for a multiplicative constant i.e. $\log_2 * e$.

Gompertz parameters A, C, M & B were also modeled in a similar manner and mathematical equations derived for each. Analyzing each individually, it was determined

that $\ln(C)$ was significantly ($p < 0.05$) affected by the main effects of phosphate and nitrite levels together with interaction effects. Fig. 4.8 represents the effects of temperature ($^{\circ}\text{C}$) and phosphate levels (%) on C at constant pH (6.0), NaCl (2%) and nitrite (156 ppm) levels. Increasing phosphate levels brought about a decrease in the number of log cycles of growth especially at values approaching the lower temperature of 12°C . This temperature however, favors the increase in number of log cycles of growth at low phosphate levels. The predicted values of C remained fairly constant across the range of phosphate levels, at temperatures approaching the upper model limit (37°C), indicating the interactive effect of temperature and phosphate levels in predicting C values.

$\ln(B)$ was significantly affected ($p < 0.05$) by the main effect of NaCl level and interaction effects. Fig. 4.9 demonstrates the effects of NaCl and pH levels on B at constant temperature (25°C), nitrite (156 ppm) and phosphate (0.006%), while Fig. 4.10 displays the effects of temperature and NaCl levels on B at constant pH (6), nitrite (156 ppm) and phosphate (0.006%) levels. Low pH and high NaCl levels combine to bring about a sharp increase in the predicted B parameter value (Fig. 4.9), whilst a peak in the B value (relative growth rate at the time when the absolute growth rate is maximal) is noticeable at a temperature of 24°C , particularly at high NaCl levels (Fig. 4.10).

$\ln(M)$ was significantly affected by the main effect of temperature, and other interaction effects. Fig. 4.11 represents the effects of temperature and phosphate levels on M at constant pH (6.0), nitrite (156 ppm) and NaCl (2%) levels, while Fig. 4.12 displays the effects of nitrite and phosphate levels on M at constant temperature ($^{\circ}\text{C}$), pH (6.5) and NaCl (%) levels. The influence of low temperature in bringing about a sharp predicted increase in M (time at which absolute growth rate is maximal) is apparent from Fig. 4.11,

whilst increasing nitrite levels at low phosphate levels, has the same influence on predicted values of M (Fig. 4.12). These observations are possibly the result of the interaction effects noted between terms, in model analysis.

Discussion

The response of *A. butzleri* to the combined effects of temperature, pH, NaCl, sodium nitrite and STPP levels were studied and expressed as mathematical models. In an interesting observation, high sensitivity of *A. butzleri* isolates was noted to the inhibitory effects of STPP, highlighting the potential use of this chemical as an antimicrobial agent for control of the Gram-negative CLOs (*Campylobacter*-like organisms), in addition to its present multifunctional functional use in meat and poultry products. STPP ($\text{Na}_5\text{P}_3\text{O}_{10}$) is one of the phosphates commonly added to ground meat products (Zaika and Kim, 1993). In addition to increasing the moisture binding capacity, a polyphosphate can also act as antioxidant, dispersant, chelator, buffer, emulsifier, sequestrant and may have antimicrobial properties (Zaika et al., 1997). They are widely used in precooked chicken products to achieve improved color, flavor, oxidative stability and cooking yields (Chen et al., 1973). In earlier studies carried out to determine the effects of polyphosphates, Zaika et al., (1993; 1997) reported growth of *L. monocytogenes* up to 0.5% sodium polyphosphate (Hexaphos™). Gram-positive bacteria were reportedly inhibited by polyphosphates in a study by Chen et al., (1973) whereas little or no inhibition of Gram-negative bacteria was observed. 0-3% phosphates prevented the growth of *Sarcina*, staphylococci and some strains of *Bacillus*, but Gram-negative rods tested (*E. coli* B, K37, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium) were resistant to higher concentrations of polyphosphate mixtures (up to 6%). Post et al.,

(1963) similarly determined that almost all Gram-negative bacteria studied (*Enterobacter aerogenes*, *Escherichia coli*, *Escherichia freundii*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Salmonella* Typhimurium strains) could tolerate up to 10% hexametaphosphate (HMP), with the exception of *Salmonella* Typhi which was unable to grow at a HMP concentration greater than 0.05%. Since polyphosphates are good chelating agents, their inhibitory effects may be due to removal of essential metal ions from cation binding sites on cell walls of microbes. It is thus significant that in our study, low levels of a polyphosphate (STPP) were found to inhibit this Gram negative, *Campylobacter*-like genus.

Studies have been carried out on the antimicrobial effects of sodium nitrite, including one by Zaika et al. (1991) on the effects of nitrite on the growth of *Shigella flexneri*. The organism grew at concentrations of up to 1000 ppm, though growth was dependent upon the pH and salt content of the medium. Antimicrobial activity of nitrite has been found to be pH dependent and shows a marked increase at pH levels below 6.0. This bacteriostatic effect of a weak acid (in this case nitrous acid) is apparently due to the undissociated form of the acid, and the inhibitory concentrations of the acid at all pH values have the same amount of undissociated acid. The authors also note that Gram-negative bacteria are reportedly resistant to the bacteriostatic effect of nitrite. In our study, the effect of nitrite in increasing the generation time (GT) at low pH levels was apparent from the predictions in Fig. 2. Also, nitrite was present as a significant interaction effect with other variables, in almost all the model equations developed.

Data used in modeling experiments are routinely 'transformed' in order to normalize the data. Transformations usually used are the natural logarithm, inverse,

square or square root (Myers and Montgomery, 1995). In our study, the natural logarithm transformation was deemed suitable for use in the analysis, and a comparison of the characteristics of the untransformed and transformed models indicated that the most effective models were those based on the transformed data. Cubic models, though developed through backwards elimination, were still noted to be overparameterized and were not advantageous in use over the simpler quadratic models. Though R^2 values were predictably higher with the cubic models, analysis of how well the derived values for derived growth kinetic parameters fit the observed data, led to the choice of quadratic models, for study and analysis. A comparison of observed and predicted values for the quadratic natural logarithm transformed lag phase duration analysis is displayed in Fig. 4.13.

Buchanan and Phillips (1990) described use of the Gompertz function to develop mathematical models for the quantitative description of growth kinetics as ‘particularly promising’ and researchers have used this as a model development base for several foodborne pathogens. Zwietering et al. (1990) compared several sigmoidal functions that described a bacterial growth curve and determined that the modified Gompertz equation was ‘statistically sufficient to describe the growth data and was easy to use’. Our observations indicate that the Gompertz function was adequate for curve fitting analysis, as observed in Fig. 4.14, which demonstrates the curve-fitting procedure, with ‘observed’ and ‘fitted’ growth curves that compare the effect of 1% and 2.5% NaCl at constant values of the other four variable conditions. An increase in the NaCl level was found to increase the LPD from 15.48 h to 26.42h and the GT from 1.46 h to 3.62 h.

Models that have been of greatest interest to risk-analysts are the LPD, or lag-phase duration, that essentially determines the period in which the species of concern is suppressed, under the prevailing processing, storage, or environmental conditions. Additionally, the generation time (GT) and the exponential growth rate (EGR) is also important, since it reflects the rate of increase in cell numbers, post-LPD. Buchanan and Phillips (1990) carried out a study which determined that temperature, initial pH, sodium chloride content, sodium nitrite level and atmosphere do interact to influence the growth kinetics of *L. monocytogenes*, especially, with respect to GT/EGR and LPD.

Thus, an analysis of the empirical models developed indicate that they can be used to provide “initial estimates” of the responses of the three *A. butzleri* strains studied to various environmental, processing and food formulation conditions encountered, within the limits of the model development conditions.

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Fig. 4.1. The effect of pH and temperature on generation time, GT (h) for *Arcobacter butzleri* in EMJH medium, at constant NaCl (2%), sodium tripolyphosphate (0.006%) and sodium nitrite (156 ppm) levels.

Figure 4.1

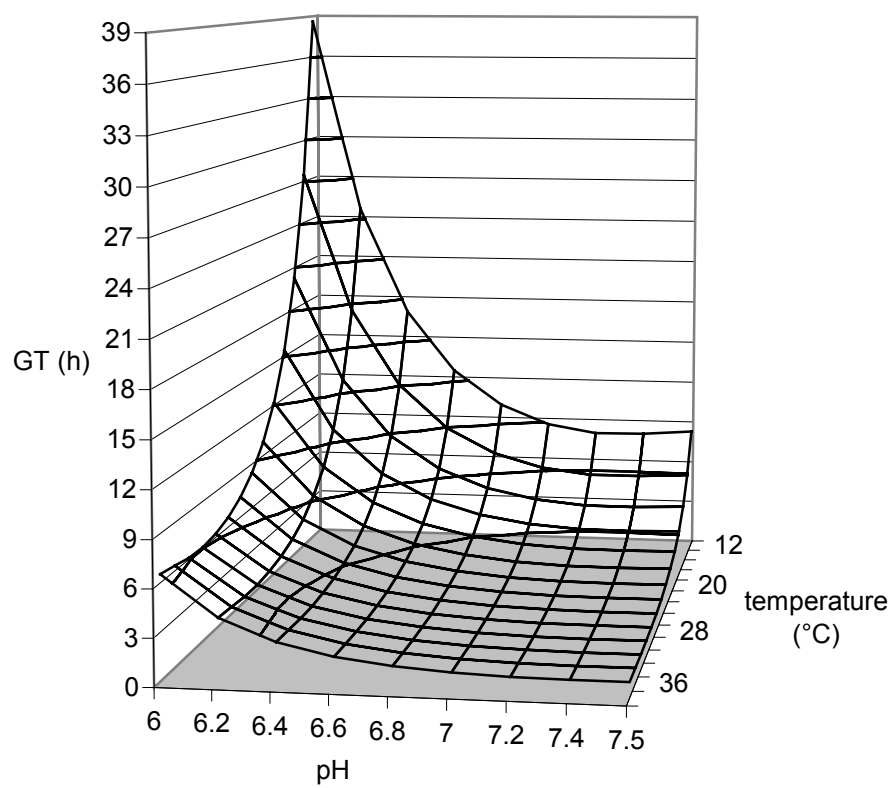


Fig.4.2. The effect of pH and sodium nitrite levels (ppm) on generation time, GT (h) for *Arcobacter butzleri* in EMJH medium, at constant NaCl (2%), sodium tripolyphosphate (0.006%) and temperature (25°C) levels.

Figure 4.2

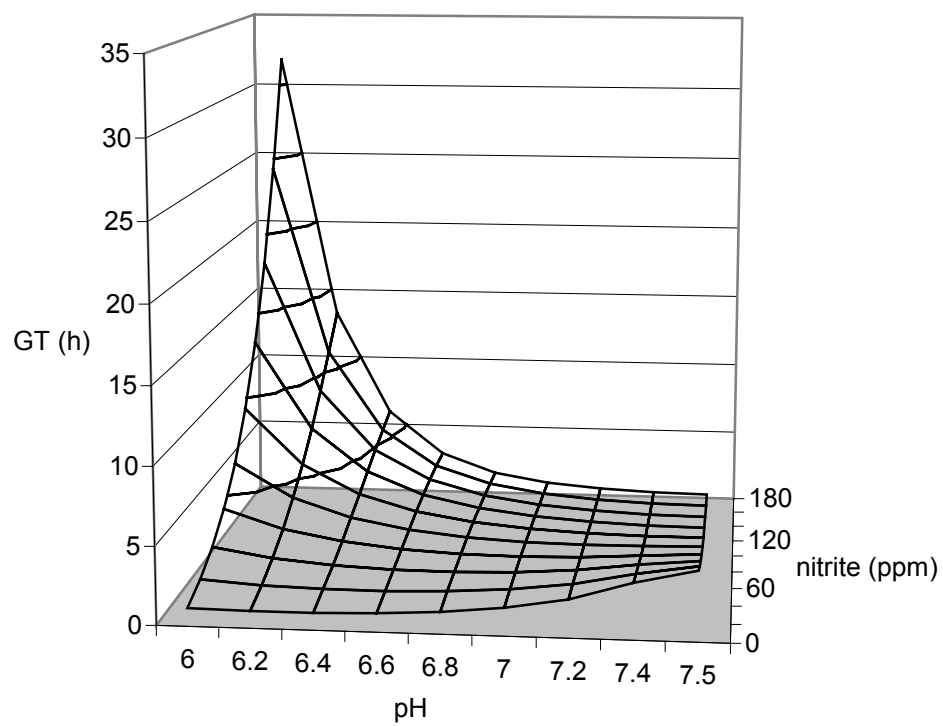


Fig.4.3. The effect of pH and temperature (°C) on lag phase duration, LPD (h), of *Arcobacter butzleri* in EMJH medium at constant NaCl (2%), sodium tripolyphosphate (0.006%) and nitrite (156 ppm) levels.

Figure 4.3

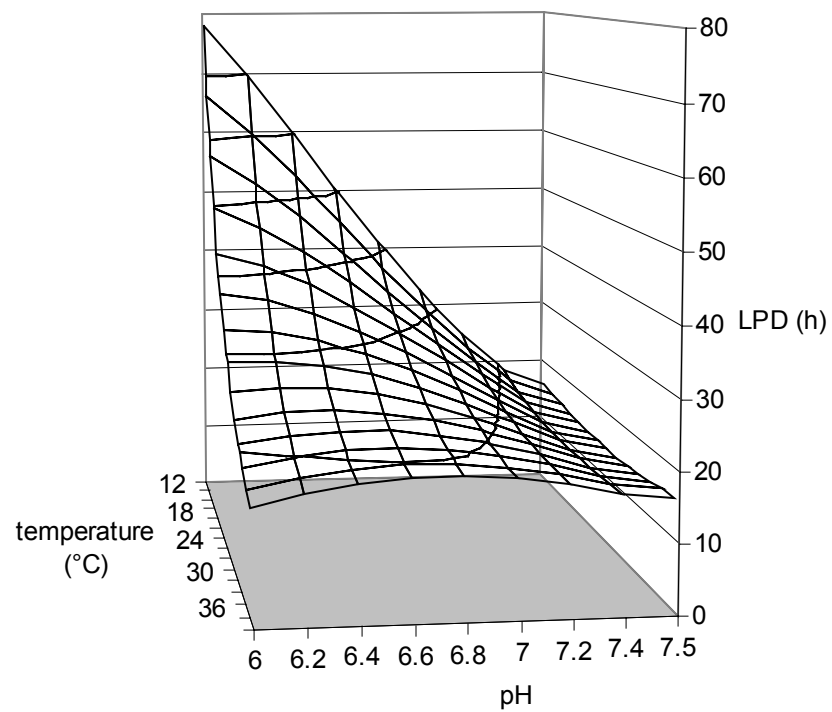


Fig. 4.4. The effect of pH and NaCl (%) levels on the lag phase duration, LPD (h) of *Arcobacter butzleri* in EMJH medium, at constant temperature (°C), sodium tripolyphosphate (0.006%) and sodium nitrite (156 ppm) levels.

Figure 4.4

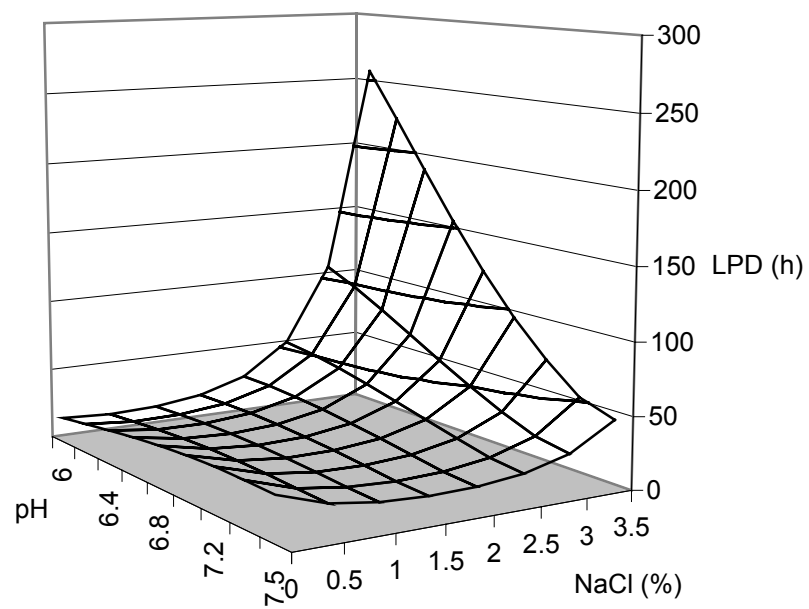


Fig. 4.5. The effects of temperature (°C) and NaCl (%) levels on the lag phase duration, LPD (h) of *Arcobacter butzleri* in EMJH medium, at constant pH (6.0), sodium tripolyphosphate (0.006%) and sodium nitrite (156 ppm) levels.

Figure 4.5

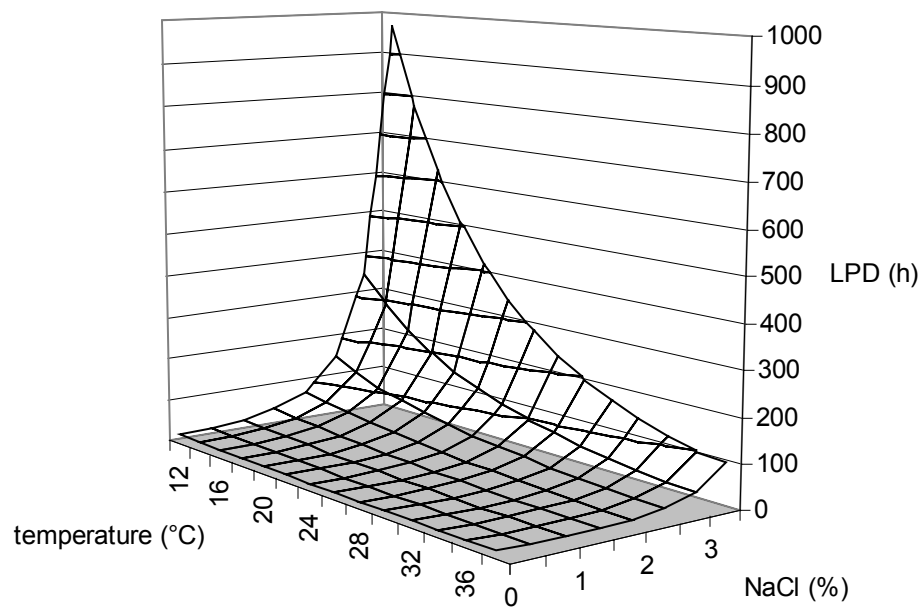


Fig.4.3 A, B, C. The effect of pH and temperature (°C) on lag phase duration, LPD (h) of *Arcobacter butzleri* in EMJH medium, at constant sodium tripolyphosphate (0.006%), sodium nitrite (156ppm) and NaCl levels. A = 0 % NaCl, B = 1% NaCl, C = 3.5% NaCl.

Figure 4.3A

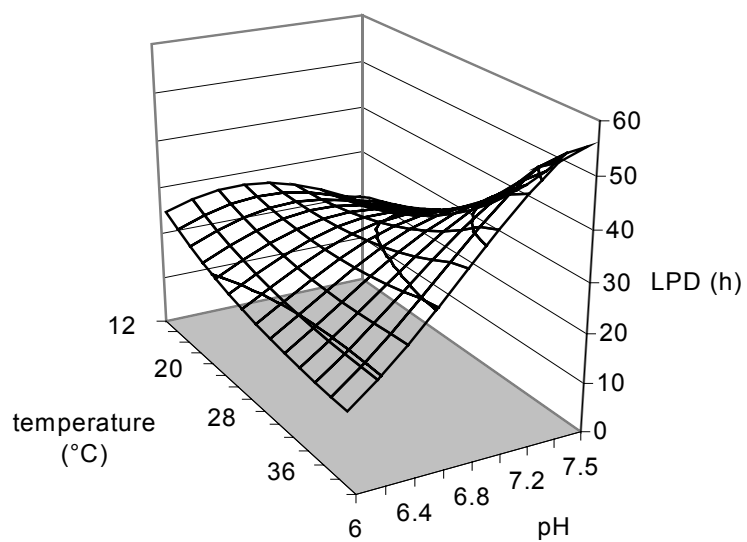


Figure 4.3B

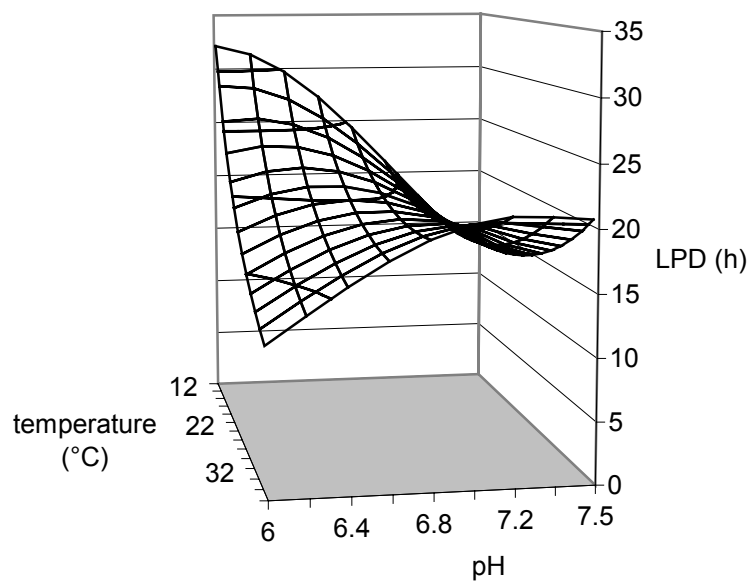


Figure 4.3C

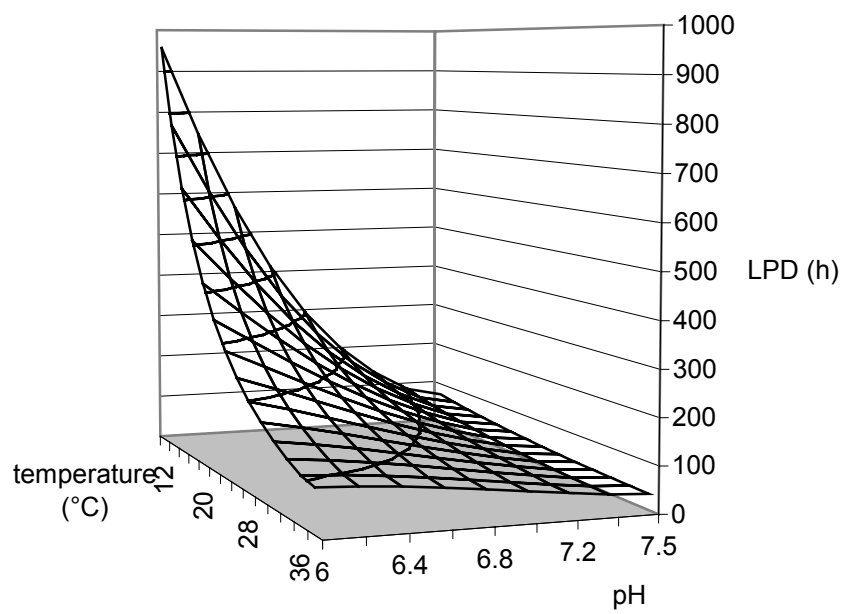


Fig. 4.6. The effect of pH and sodium nitrite level (ppm) on exponential growth rate, EGR (\log_{10} CFU/ml/h) of *Arcobacter butzleri* in EMJH medium, at constant temperature (25°C), NaCl (2%) and sodium tripolyphosphate (0.006%) levels.

Figure 4.6

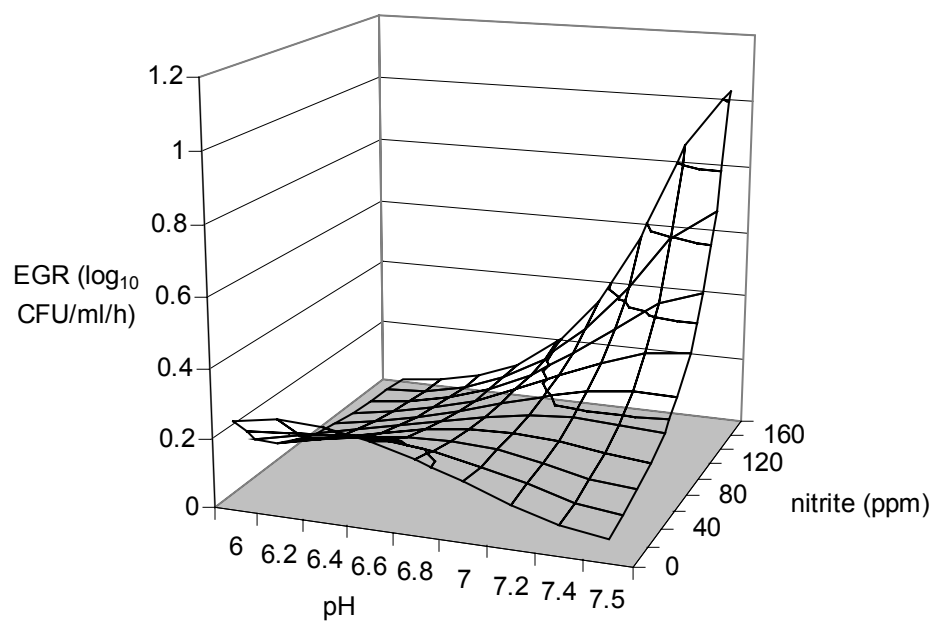


Fig. 4.7. The effect of temperature (°C) and pH on exponential growth rate, EGR (\log_{10} CFU/ml/h) of *Arcobacter butzleri* in EMJH medium, at constant NaCl (2%), sodium nitrite (156 ppm) and sodium tripolyphosphate (0.006%) levels.

Figure 4.7

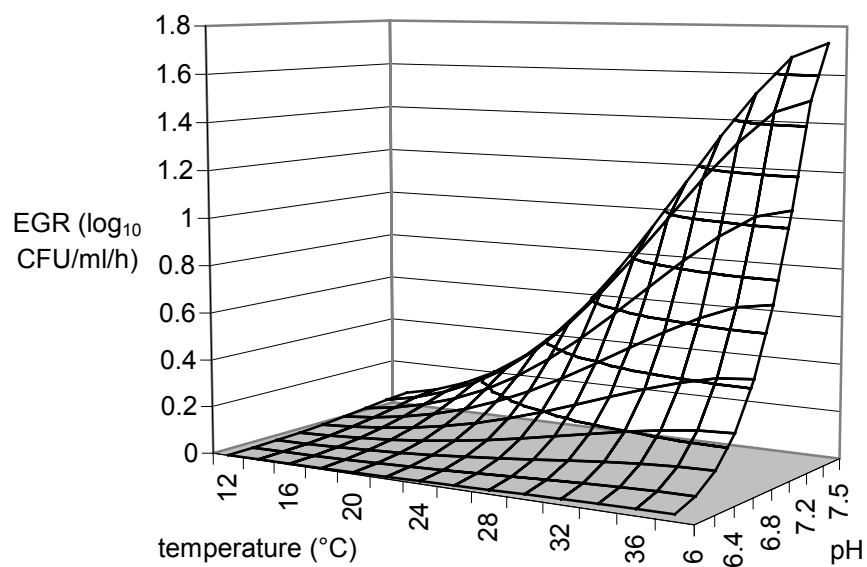


Fig. 4.8. The effect of temperature (°C) and sodium tripolyphosphate (%) on Gompertz parameter C, (\log_{10} CFU/ml), of *Arcobacter butzleri* in EMJH medium at constant pH (6.0), NaCl (2%) and sodium nitrite (156 ppm) levels.

Figure 4.8

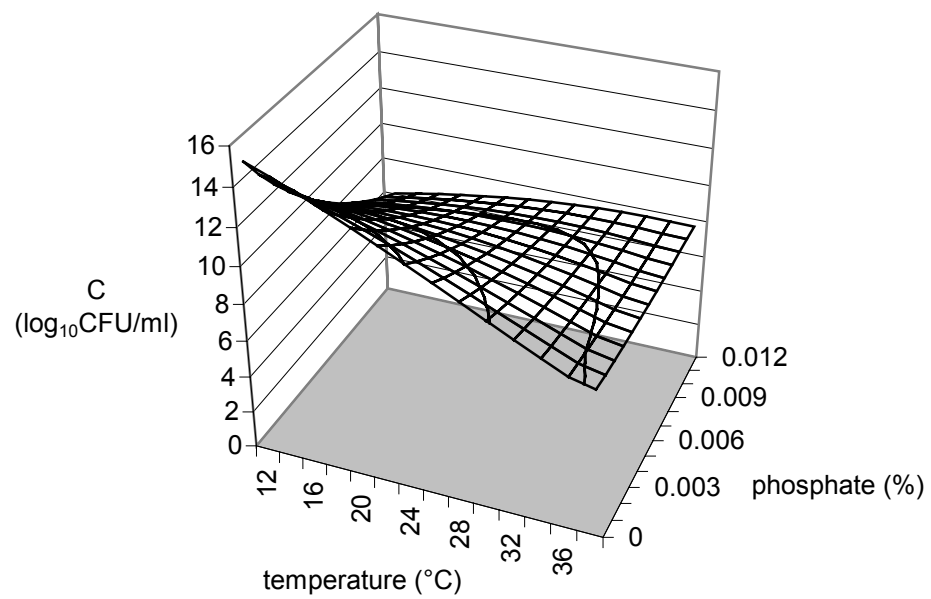


Fig. 4.9. The effect of pH and NaCl (%) levels on the Gompertz parameter B, (\log_{10} CFU/ml/h) of *Arcobacter butzleri* in EMJH medium, at constant temperature (25°), sodium nitrite(156 ppm) and sodium tripolyphosphate (0.006%) levels.

Figure 4.9

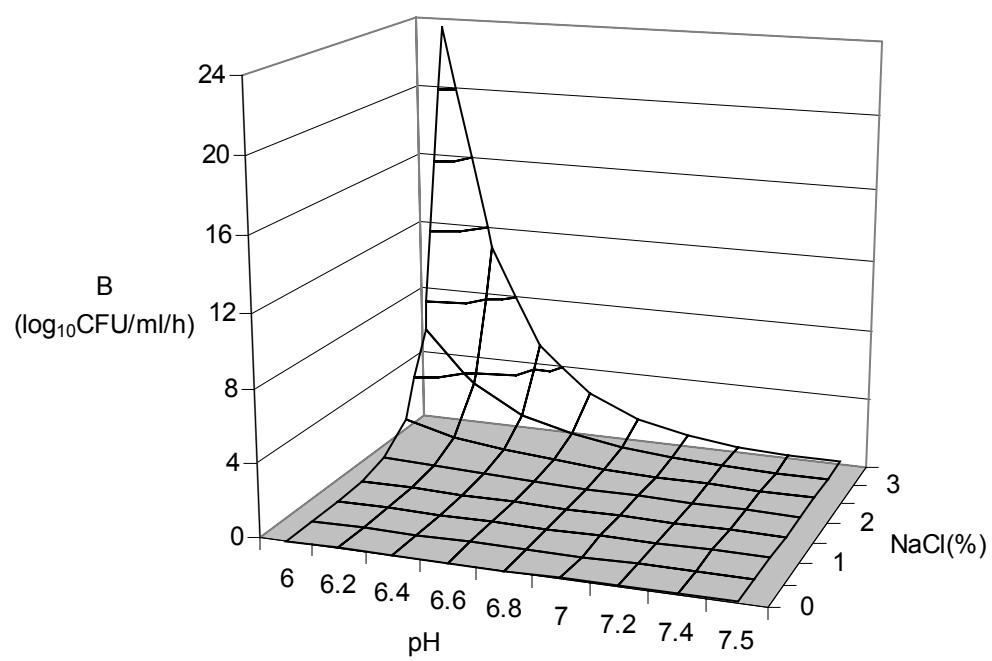


Fig. 4.10. The effect of temperature (°C) and NaCl (%) on Gompertz parameter B, (\log_{10} CFU/ml/h) of *Arcobacter butzleri* in EMJH medium at constant pH (6.0), sodium nitrite (156 ppm) and sodium tripolyphosphate (0.006%) levels.

Figure 4.10

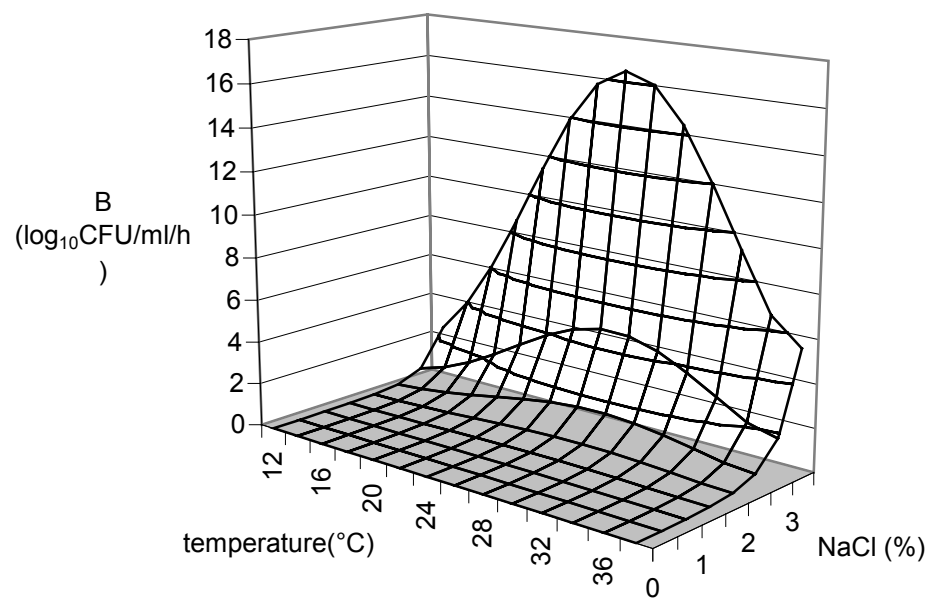


Fig. 4.11. The effects of temperature ($^{\circ}\text{C}$) and sodium tripolyphosphate levels (%) on the Gompertz parameter M (h) of *Arcobacter butzleri* in EMJH medium at constant pH (6.0), sodium nitrite (156 ppm) and NaCl (2%) levels.

Figure 4.11

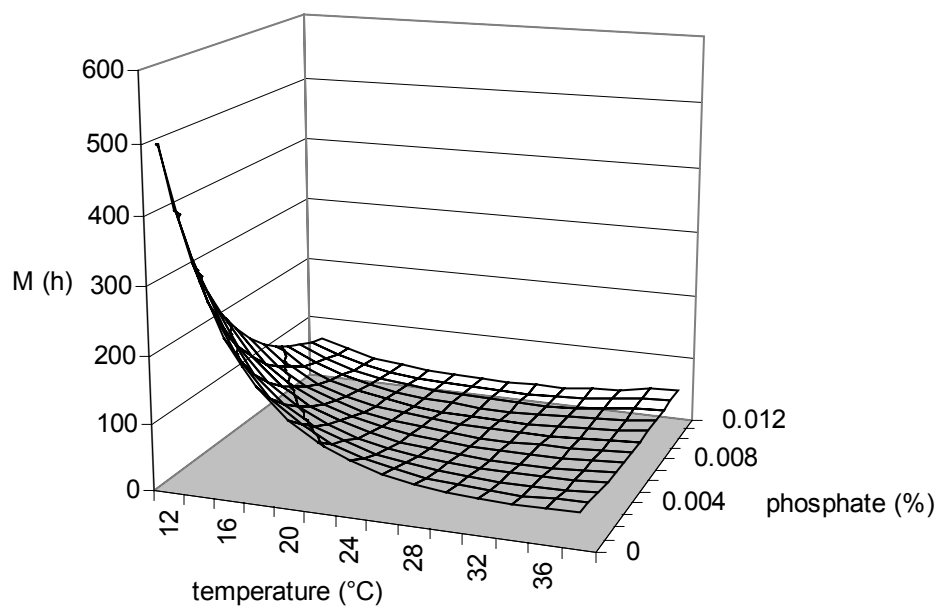


Fig. 4.12. The effect of sodium nitrite (ppm) and sodium tripolyphosphate (%) levels on Gompertz parameter M (h) of *Arcobacter butzleri* in EMJH medium at constant temperature (25°C), pH (6.5) and NaCl (2%) levels.

Figure 4.12

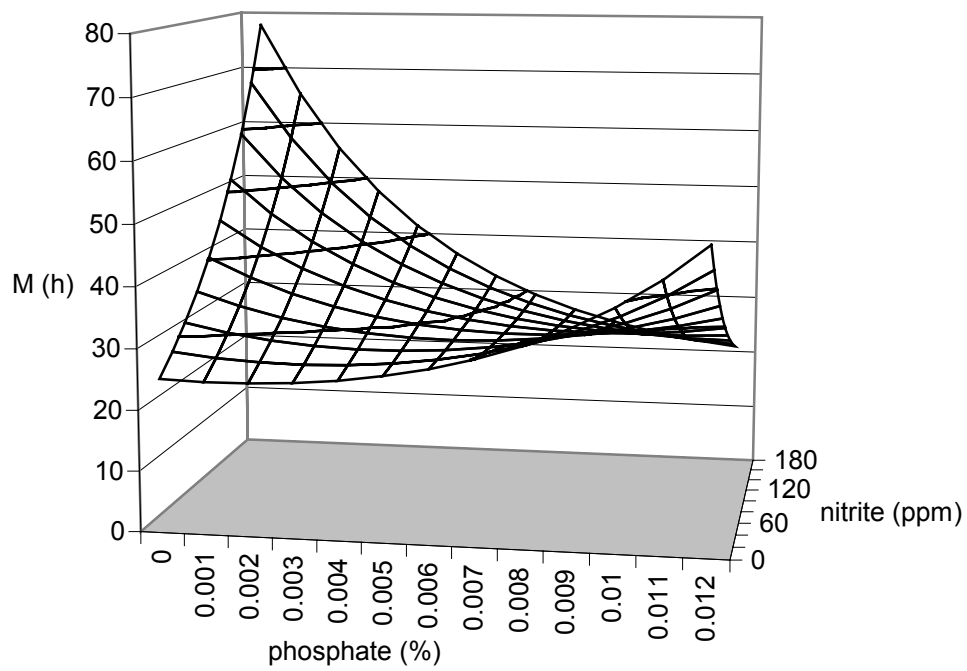


Fig 4.13. Comparison between observed and predicted lag phase durations, LPD (h), for *Arcobacter butzleri* in EMJH medium under the effects of varying temperature (°C), pH, NaCl (%), sodium tripolyphosphate (%) and sodium nitrite (ppm) levels. Predictions were made using the natural logarithm transformed quadratic response surface model for LPD. Center line is the line of identity and the two outer lines present $\pm 50\%$ of observed values.

Figure 4.13

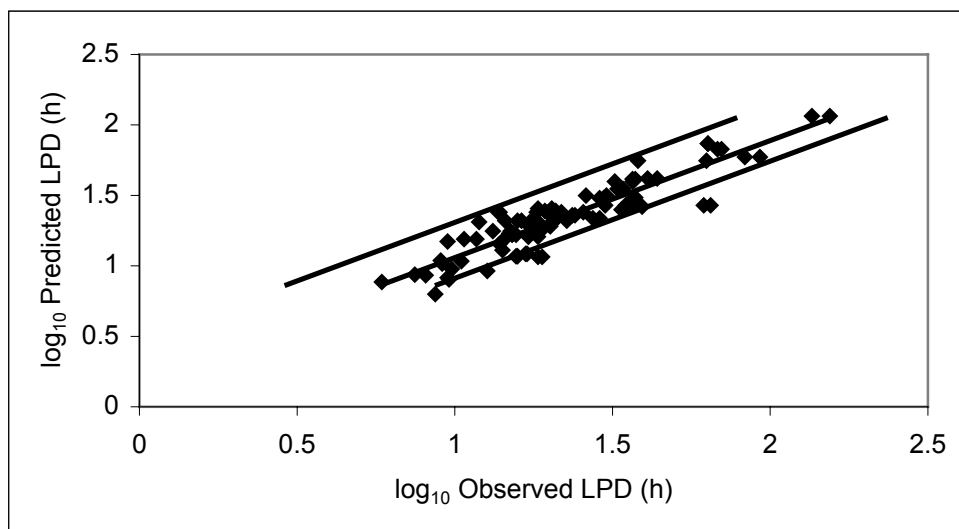


Fig 4.14. Growth curve fitting: Effect of NaCl (1%, A) and (2.5%, B) on the growth of *Arcobacter butzleri* in EMJH medium at temperature (19°C), pH (7.0) sodium nitrite (52 ppm) and sodium tripolyphosphate (0.0035%) levels.

Figure 4.14 (A)

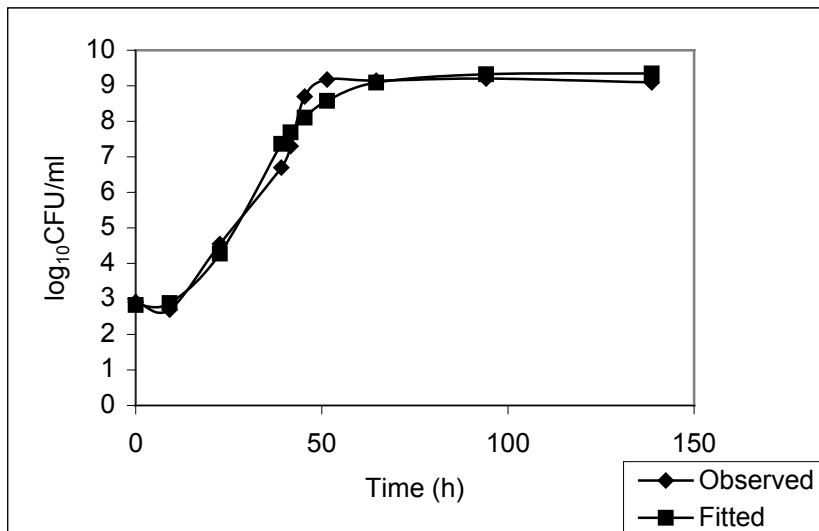
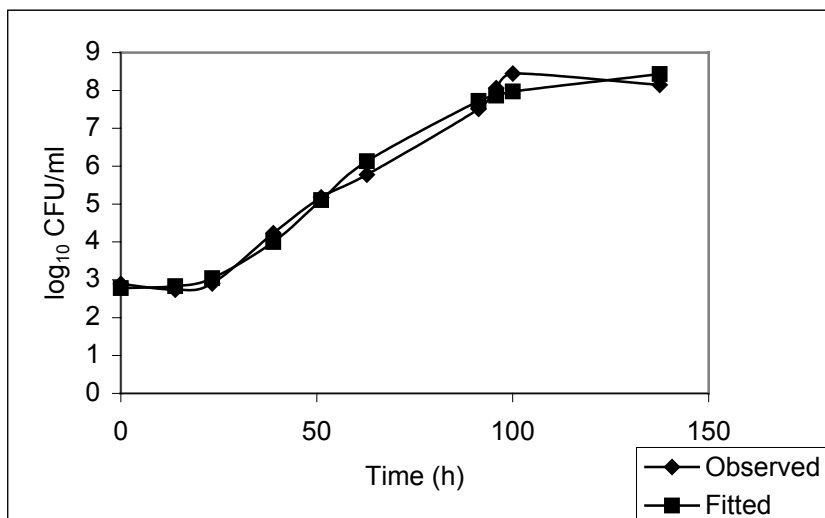


Figure 4.14 (B)



CHAPTER 5

SUMMARY AND CONCLUSIONS

Control of survival and growth of *Arcobacter* spp. in food products and in environments that may be potential contributors to foodborne disease, is dependent upon knowledge of the response of these species to extrinsic and intrinsic influencing parameters. This study investigated several controlling factors that affect the presence of this genus in laboratory and food product-type situations, and the results can be summarized as follows:

1. *Arcobacter* spp. studied have a growth range in the presence of NaCl of 0-3.0% (*A. cryaerophilus*) or 0-3.5% (*A. butzleri*). Some isolates were able to survive at levels of 5.0% NaCl for up to 48 h. Also, ambient temperature favors the survival capacity of *Arcobacter* spp. to NaCl, over their optimum growth temperature of 30-37°C. In comparison, *Campylobacter* spp. are reported to have an optimum and maximum growth response to NaCl of 0.5% and 1.5%, respectively. As a preservative, NaCl levels usually used in food products range from 1.5-3.0% in cheese, 2-4% in cured meats, to no added salt, in produce items. Thus, survival of the genus is possible, if NaCl is the only preservative used and thermal processing procedures are absent.

2. *Arcobacter* spp. studied have a pH growth range from 5.5-8.0 and additional pH tolerance was observed 0.5 units below the growth range at ambient temperatures. *Campylobacter* spp., have a reported pH growth range from 4.9-9.0, with an optimum of 6.5-7.5. Thus, the pH survival abilities of the two genera are equivalent. Nevertheless

arcobacters, owing to their aerotolerant nature probably have a survival and thus infective advantage over campylobacters, especially in low-acid, buffered food environments like raw seafood, milk and unfermented dairy products, and unchlorinated water supplies. Indeed, several outbreaks of *Campylobacter*-related human enteritis have been reported, owing to the consumption of unchlorinated water and raw or inadequately pasteurized milk (ICMSF, 1996).

3. *A. butzleri* spp. studied are sensitive to low levels of sodium tripolyphosphate, a multifunctional ingredient used in comminuted meat products up to an allowable level of 0.5%. The minimum inhibitory concentration for growth was 0.018%, with survival up to 0.02%. Thus STPP can be used effectively and in low concentrations to control *Arcobacter* spp. Sodium nitrite, on the other hand, allowed survival of *Arcobacter* up to 200 ppm. The allowable nitrite level in meat products is up to 156 ppm, or 200 ppm in bacon. Thus, sodium nitrite should be used in combination with other potential stress-inducing factors for control of CLOs in meat products.

4. Thermal resistance studies on three *A. butzleri* isolates revealed D-values in phosphate buffered saline (pH 7.3) ranging from 0.07-0.12 min (at 60°C), 0.38-0.76 min (at 55°C) and 5.12- 5.81 min (at 50°C). At pH 5.5, D-values for two of three strains were reduced, ranging from 0.03-0.11 min, 0.30-0.42 min and 1.97-4.42 min at 60, 55 and 50°C, respectively. z-values ranged between 5.20-6.11 and 5.55-6.28 °C at pH 7.3 and 5.5, respectively. Thus, the combination effect of heat and mild acid can be used to synergistically control *Arcobacter* spp. In ground pork, D-values were 2.18 min and 18.51 min at 55 and 50°C, respectively. *Campylobacter* spp., in comparison have a slightly lower thermal resistance, with z-values ranging between 4.5-8.0, for temperatures

between 60 and 48°C (ICMSF, 1996). In light of these findings, potentially hazardous foods for presence of *Arcobacter* spp. are lightly cured, uncooked or low-temperature processed meats, as the presence of the genus in raw meat and poultry products has been documented (de Boer et al., 1996; Atabay et al., 1998; de Boer et al., 1996; Wesley, 1994). Adequate cooking processes, alone or combined with a lowered pH (as in addition of acid) would sufficiently suppress growth of *Arcobacter* in these products.

5. 'Hurdle-concept' technology has been used with much success, to control the growth and survival of foodborne microorganisms in several processed and minimally-processed foods. Our study of the combined effects of temperature, pH, sodium chloride, sodium nitrite and sodium tripolyphosphate levels on the growth of *A. butzleri* in EMJH medium, investigated the use of a multi-pronged approach to food safety. Mathematical models were developed based on fitting of growth-curve data and response-surface regression analyses. Quadratic natural logarithm-transformed models were determined to give the best 'fit' and were selected for further evaluation. Significant main and interaction effects between variables were determined, for each model developed. Variables interacted, to contribute inhibitory conditions that lowered the growth of *A. butzleri*. Models predicting lag phase duration (LPD) and generation time (GT) provided valuable information about the use of interacting variables to control growth and prolong shelf life of foods. For growth-promoting variable combinations, maximum population densities (MPDs) achieved were within a fairly narrow range, with a mean of 8.0 log₁₀ CFU/ml. Thus, it is possible to utilize mathematical predictive models to obtain an estimate of growth kinetics of *A. butzleri*.

It is pertinent to note that surveys to determine the distribution of *Arcobacter* spp. in the environment and in the food chain have focused on relatively few areas – mainly raw meat and poultry products and their associated processing environments, water and sewage treatment plants; and in relatively few geographical areas (Belgium, Canada, Thailand, U.S.). Consequently, it is difficult to make a broad-based estimate about the distribution of these organisms in unprocessed/minimally processed produce items, dairy products and seafood, and their contributions therein, to foodborne disease. As *Arcobacter* spp. have reportedly been at times misidentified as *Campylobacter*, it may be useful to postulate the role of arcobacters based on the considerable research conducted on the related *Campylobacter*. Future research areas should focus on determining the distribution of *Arcobacter* in foods other than meat and poultry products and on estimating the effects of other chemicals or disinfectants that would inhibit this genus in several minimally-processed foods and high risk food-processing environments.

APPENDIX A

NATURAL LOGARITHM TRANSFORMED PLOTS OF RESPONSE SURFACES

Fig. A.1. The effect of pH and temperature on natural logarithm transformed generation time $\ln GT$ (h) for *Arcobacter butzleri* in EMJH medium, at constant NaCl (2%), sodium tripolyphosphate (0.006%) and sodium nitrite (156 ppm) levels.

Fig.A.2. The effect of pH and sodium nitrite levels (ppm) on natural logarithm transformed generation time, $\ln GT$ (h) for *Arcobacter butzleri* in EMJH medium, at constant NaCl (2%), sodium tripolyphosphate (0.006%) and temperature (25°C) levels.

Figure A.1

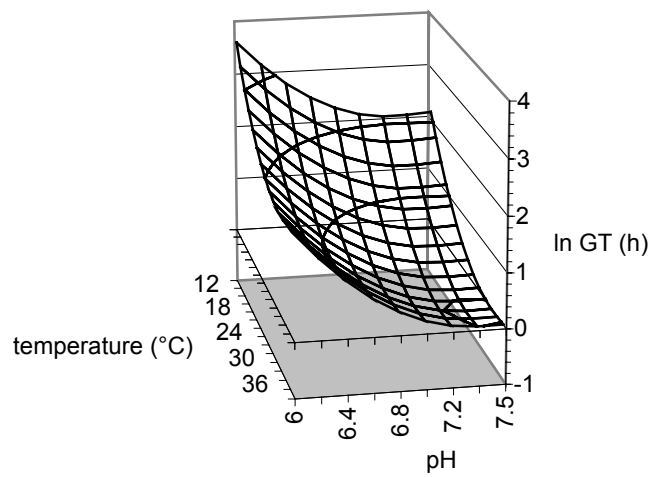


Figure A.2

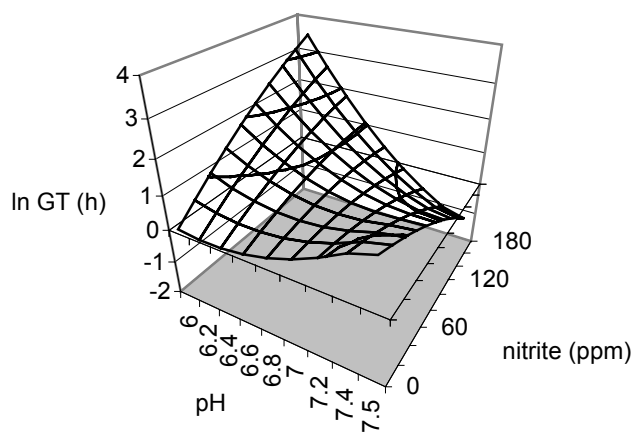


Fig.A.3. The effect of pH and temperature (°C) on natural logarithm transformed lag phase duration ln LPD (h), of *Arcobacter butzleri* in EMJH medium at constant NaCl (2%), sodium tripolyphosphate (0.006%) and nitrite (156 ppm) levels.

Fig.A.3. A, B, C. The effect of pH and temperature (°C) on natural logarithm transformed lag phase duration ln LPD (h), of *Arcobacter butzleri* in EMJH medium, at constant sodium tripolyphosphate (0.006%), nitrite (156 ppm) and NaCl levels. A = 0 % NaCl, B = 1% NaCl, C = 3.5% NaCl.

Figure A.3

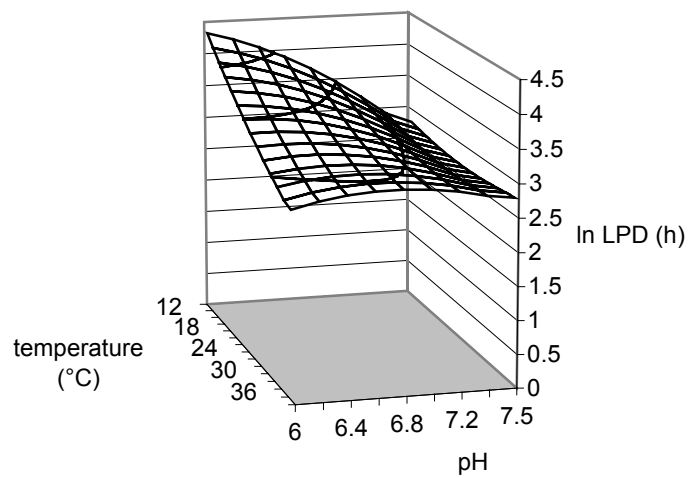


Figure A.3.A

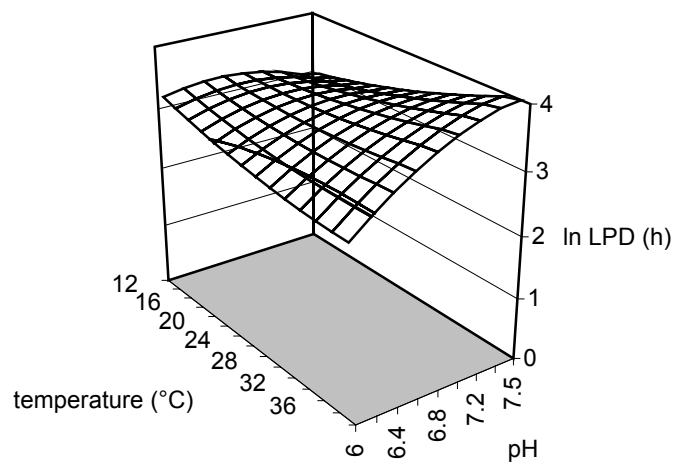


Figure A.3.B

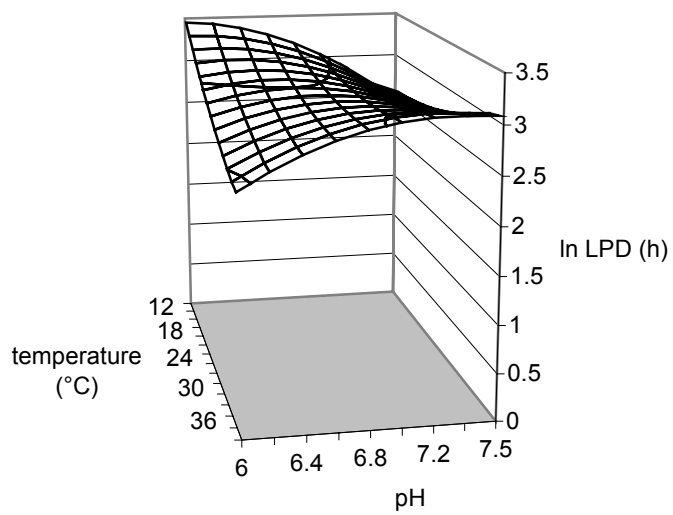


Figure A.3.C

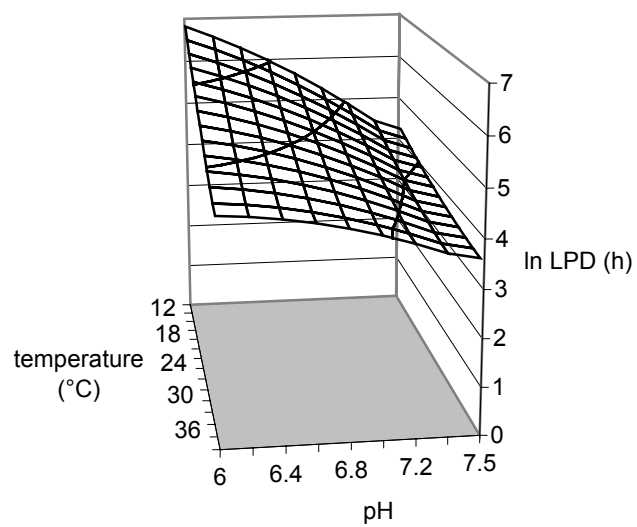


Fig. A.4. The effect of pH and NaCl (%) levels on the natural logarithm transformed lag phase duration \ln LPD (h) of *Arcobacter butzleri* in EMJH medium, at constant temperature ($^{\circ}\text{C}$), sodium tripolyphosphate (0.006%) and sodium nitrite (156 ppm) levels.

Fig. A.5. The effects of temperature ($^{\circ}\text{C}$) and NaCl (%) levels on the natural logarithm transformed lag phase duration \ln LPD (h) of *Arcobacter butzleri* in EMJH medium, at constant pH (6.0), sodium tripolyphosphate (0.006%) and sodium nitrite (156 ppm) levels.

Figure A.4

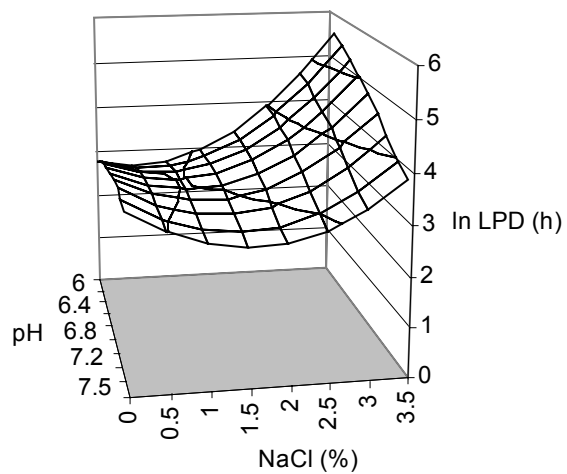


Figure A.5

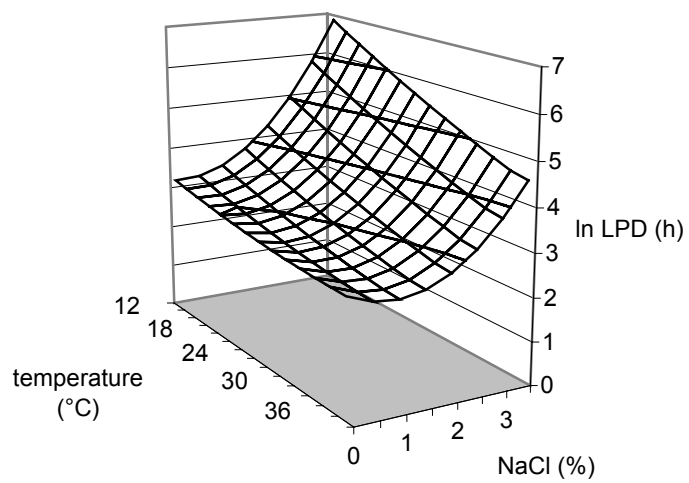


Fig. A.6. The effect of pH and sodium nitrite level (ppm) on natural logarithm transformed exponential growth rate ln EGR (\log_{10} CFU/ml/h) of *Arcobacter butzleri* in EMJH medium, at constant temperature (25°C), NaCl (2%) and sodium tripolyphosphate (0.006%) levels.

Fig. A.7. The effect of temperature (°C) and pH on natural logarithm transformed exponential growth rate ln EGR (\log_{10} CFU/ml/h) of *Arcobacter butzleri* in EMJH medium, at constant NaCl (2%), sodium nitrite (156 ppm) and sodium tripolyphosphate (0.006%) levels.

Figure A.6

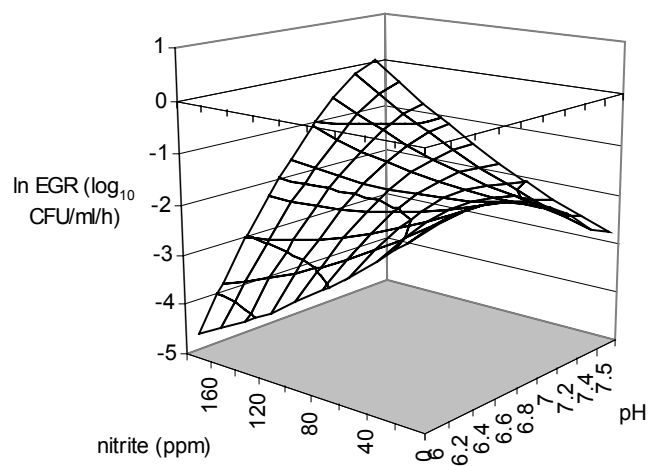


Figure A.7

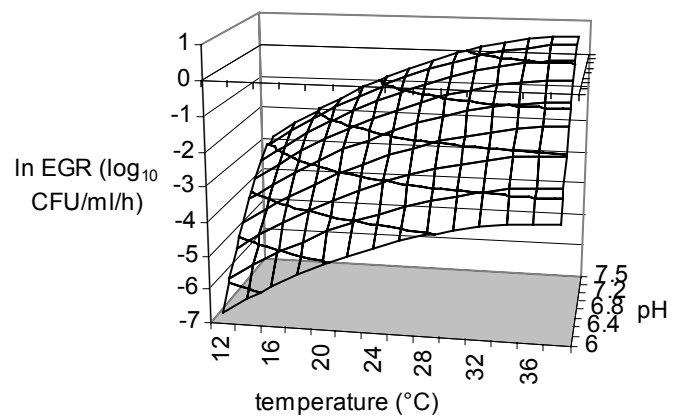


Fig. A.8. The effect of temperature ($^{\circ}\text{C}$) and sodium tripolyphosphate (%) on natural logarithm transformed Gompertz parameter C, $\ln C$ (\log_{10} CFU/ml), of *Arcobacter butzleri* in EMJH medium at constant pH (6.0), NaCl (2%) and sodium nitrite (156 ppm) levels.

Fig. A.9. The effect of pH and NaCl (%) levels on the natural logarithm transformed Gompertz parameter B, $\ln B$ (\log_{10} CFU/ml/h) of *Arcobacter butzleri* in EMJH medium, at constant temperature (25°C), sodium nitrite (156 ppm) and sodium tripolyphosphate (0.006%) levels.

Figure A.8

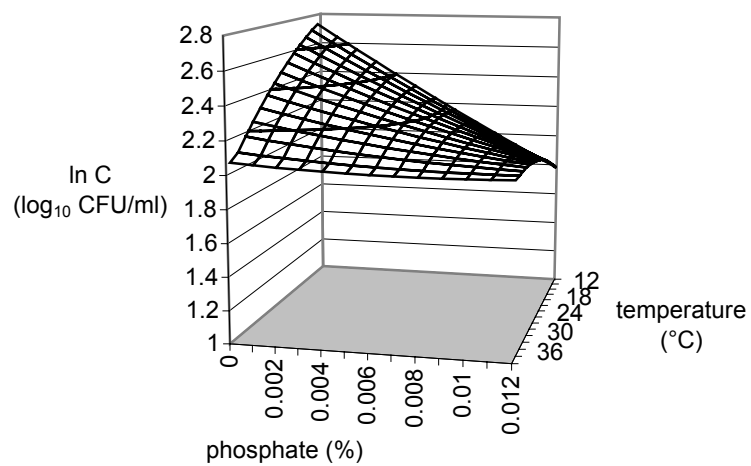


Figure A.9

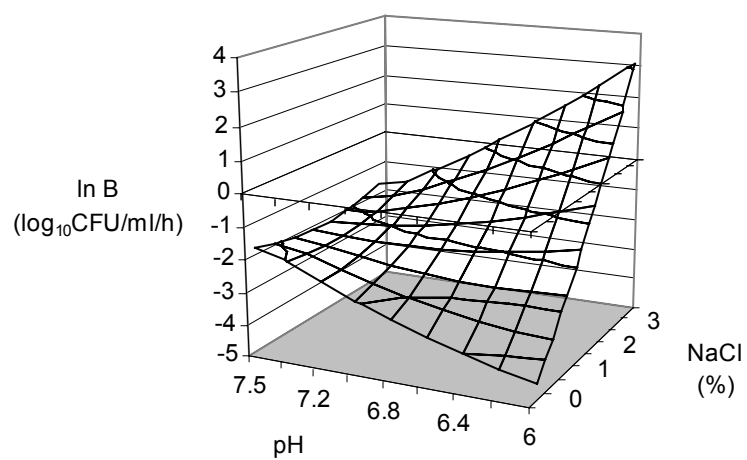


Fig. A.10. The effect of temperature (°C) and NaCl (%) on natural logarithm transformed Gompertz parameter B, $\ln B$ (\log_{10} CFU/ml/h) of *Arcobacter butzleri* in EMJH medium at constant pH (6.0), sodium nitrite (156 ppm) and sodium tripolyphosphate (0.006%) levels.

Fig. A.11. The effects of temperature (°C) and sodium tripolyphosphate levels (%) on the natural logarithm transformed Gompertz parameter M, $\ln M$ (h) of *Arcobacter butzleri* in EMJH medium at constant pH (6.0), sodium nitrite (156 ppm) and NaCl (2%) levels.

Figure A.10

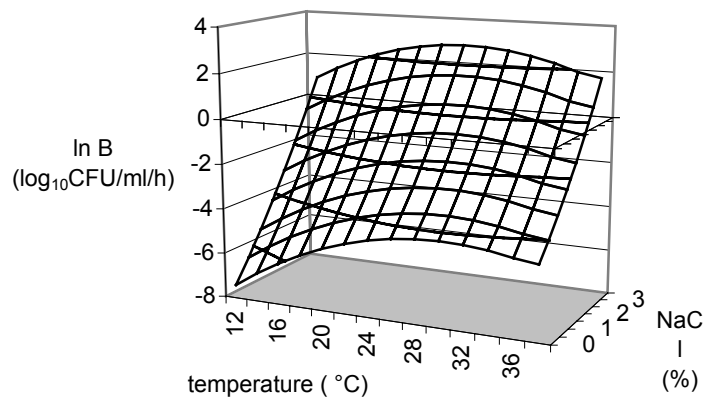


Figure A.11

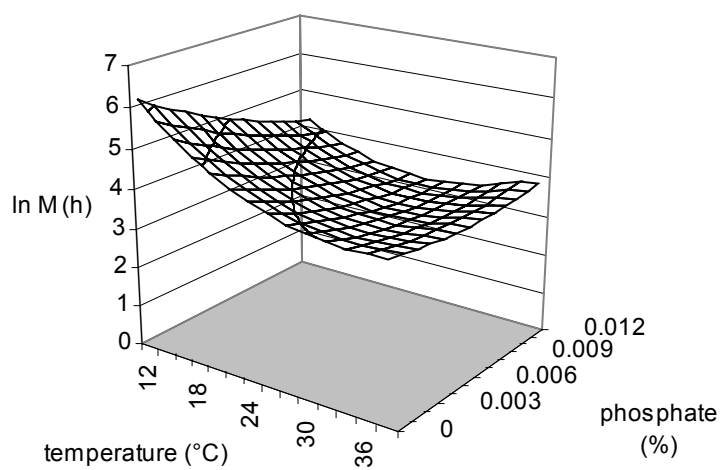


Fig. A.12. The effect of sodium nitrite (ppm) and sodium tripolyphosphate (%) levels on natural logarithm transformed Gompertz parameter M , $\ln M$ (h) of *Arcobacter butzleri* in EMJH medium at constant temperature (25°C), pH (6.5) and NaCl (2%) levels.

Figure A.12

