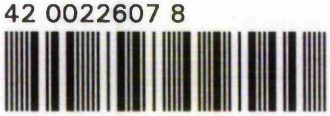

The physiology and toxicity of the newly emerging food-borne pathogen *Arcobacter butzleri* NCTC 12481.

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ABSTRACT.

The objective of this study was to investigate the physiology and toxicology of *Arcobacter* in particular the type strain *A. butzleri* NCTC 12481.

A. butzleri grew optimally at 25-35°C and pH 6-8. The presence of nitrate (0.1-5mM) in the growth medium increased the growth rate. *A. butzleri* cells in the exponential phase were more resistant to heat than stationary phase cells (D_{55} 1.1 & 0.4 min respectively) suggesting enhanced stress resistance. Heat treatment caused cell membrane damage shown by high fluorescence values due to the uptake of DNA-binding fluorescent dyes and more UV-absorbing material being released. At +4°C *A. butzleri* (stationary phase) viability decreased gradually, with increasing exposure time (1×10^9 to 8×10^5 cfu ml^{-1}, 21 day period). Freezing caused a 2 log decrease in viability after only 24 h in storage, thereafter the viability remained constant ($0.6\text{-}1 \times 10^5$ cfu ml^{-1}).

No significant cytotoxicity (as determined by the MTT assay) was detected in either cell-free extracts from stationary phase cells or in the BHI spent media. Cytotoxic activity towards N2a and to a lesser extent ECV was detected in cell-free extracts from exponential phase cells. This toxicity was greater following heat treatment of the bacterial cells (55°C, 3 min) suggesting a membrane bound cytotoxin. Morphology studies showed that the toxin caused a decrease (>50%) in the number of neurite outgrowth on N2a cells. The toxin was heat stable and resistant to proteolytic enzymes. SDS-PAGE showed the presence of low molecular weight lipopolysaccharide in material released from cells in exponential phase. The most sensitive cell line was N2a, probably due to a greater abundance of CD14 lipopolysaccharide receptor on its surface.

In conclusion, this study has quantified the growth and survival characteristics of *A. butzleri* NCTC 12481 and has demonstrated the production of an endotoxin by four human *A. butzleri* isolates.

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ABBREVIATIONS.

ACMSF	Advisory Committee on the Microbiological Safety of Food
ADP	Adenosine-5'-diphosphate
AFLP	Amplified fragment length polymorphism
ASB	*Arcobacter* selective enrichment broth
ASM	*Arcobacter* selective plating medium
AT	Adenosine and tyrosine
ATP	Adenosine-5'-triphosphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BGBA	Brilliant green bile agar
BHI	Brain heart infusion
bp	Base pair
BrCl	Bromine chloride
BSA	Bovine serum albumin
CaCo-2	Human enterocyte-like cell line
cAMP	Cyclic adenosine monophosphate
CAP	Cold acclimatisation proteins
CAT	Cefoperazone, amphoterian B, teicoplanin
CCDA	Charcoal, cefoperazone, deoxycholate agar
CDR	Communicable disease reports
CDSC	Communicable disease surveillance centre
CDT	Cytolethal distending toxin
CFE	Cell free extract
CFU	Colony forming units
ClO_2	Chlorine oxide
CHO	Chinese hamster ovary cell line
CS	Cell sonicate
CSP	Cold shock proteins
CT	Cholera toxin of *Vibrio cholerae*
CVA	Cefoperazone, vancomycin, amphoterian B
DAF	Decay accelerating factor
DMEM	Dulbecco's modified eagles medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOC	Sodium deoxycholate
EB	Ethidium bromide
ECV	Human epithelial cell line
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMJH-P80	Ellinghausen-McCullough-Johnson-Harris polysorbate 80
EPS	Extracellular polymeric substances
ERIC	Enterobacterial repetitive intergenic consensus
ECACC	European collection of cultured cells
FBP	Ferrous sulphate, sodium metabisulphate, sodium pyuvate
FBS	Foetal bovine serum
FISH	Fluorescent *in situ* hybridisation
GBS	Guillain Barre syndrome
G + C	Guanine and Cytosine

GlcN	β(1'-6)-linked D-glucosamine
GlcN3N	2,3 diamino-2,3-dideoxy D-glucose
GPI	Glycosyl-phosphatidylinositol
HCl	Hydrochloric acid
HeLa	Human cervical carcinoma cell line
Hep-2	Human laryngeal carcinoma cell line
HSP	Heat shock proteins
IL	Interleukin family
INT 407	Human intestinal epithelial cell line
J774	Mouse macrophage cell line
JM method	Johnson and Murano method
Kb	Kilobase
kDa	Kilodaltons
KDO	3-Deoxy-D-manno-octulosonic acid
K_2HPO_4	Dipotassium hydrogen orthophosphate anhydrous
LBP	Lipopolysaccharide binding protein
L-fuc	L-fucose
LMP80	Lipopolysaccharide binding membrane protein 80
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LT	Labile toxin of *E.coli*
LTB_4	Leukotriene B_4
μ'	Specific growth rate
MAbs	Monoclonal antibodies
Mbp	Mega base pairs
mCCDA	Modified Charcoal, cefoperazone, deoxycholate agar
mCD14	Membrane bound CD14 protein
mCIN	Modified cefsulodin, irgasan, novobiocin
mol%	Mol percentage
m-PCR	Multiplex polymerase chain reaction
Mr	Molecular weight
MRC-5	Human diploid lung fibroblast cell line
mRNA	Messenger ribonucleic acid
MTT	Methyl tetrazolium thiazolyl blue
NaCl	Sodium chloride
N2a	Mouse neuroblastoma cell line
NBT	Nitro blue tetrazolium
NCTC	National collection of type cultures
Neu5Ac	N-acetyl-neuraminic acid
OD	Optical density
ORFs	Open reading frames
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE_2	Prosaglandin E_2
PHLS	Public health laboratory service
PI	Propidium iodide
ppm	Parts per million
RABIT	Rapid automated bacterial impedance technique

RAPD-PCR	Randomly amplified polymorphic DNA polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNAP	Ribonucleic acid polymerase
rRNA	Ribosomal ribonucleic acid
RTX	Repeats in toxins
sCD14	Soluble CD14 protein
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
Spp	Species
Stx	Shiga toxin
3T3	Chicken embryo fibroblast cell line
t_d	Doubling time
$Tm_{(e)}$	Melting temperature of elution
TMB	3,3',5,5'-Tetramethylbenzidine
TNFα	Tumor necrosis factor α
TSB	Tryptone soya broth
TSP	Trisodium phosphate
TTC	2,3,5-Triphenyltetrazolium chloride
TTD	Time to detection
U373	Astrocytoma cell line
Vero	African green monkey kidney cell line
VNC	Viable non-culturable
VT	Vero toxin
Y-1	Mouse adrenocortical cell line

CHAPTER ONE

INTRODUCTION

1.0: INTRODUCTION

1.1. HISTORY AND TAXONOMY OF *CAMPYLOBACTER*, *HELICOBACTER* AND *ARCOBACTER*.

In 1913 McFadyean and Stockman isolated a *Vibrio*-like bacterium from aborted ovine foetuses (McFadyean & Stockman, 1913). Five years later Smith discovered a similar *Vibrio* strain from aborted bovine foetuses (Smith, 1918) and later named it *Vibrio fetus* (Smith & Taylor, 1919). In 1927 Smith and Orcutt described a group of *Vibrio*-like bacteria from the faeces of cattle with diarrhoea. These findings were confirmed by Jones *et al.* (1931) who observed a link between the presence of *Vibrio*-like organisms and bovine dysentery, these organisms were named *Vibrio jejuni.* Similar species have also been isolated from aborted sheep foetuses (Bryans *et al.*, 1960; Smibert, 1978) and the blood of patients with diarrhoea. Then in 1944 another strain called *Vibrio coli* was identified after being isolated from pigs with diarrhoea (Doyle, 1944). Florent (1959) observed two similar *Vibrio*-like organisms. One strain caused fertility problems in both cows and ewes and was named *Vibrio fetus* subsp. *venerealis.* The second type of infection caused sporadic abortions in cattle and sheep, but their infertility was unaffected and this strain was called *Vibrio fetus* subsp. *intestinalis.* These organisms caused abortion and enteritis in farm animals, and hence they were believed to be pathogens of veterinary importance.

Phenotypic and genotypic studies showed that *Vibrio fetus* and *Vibrio bubulus* had lower DNA base compositions (G+C 30–34 mol%) compared to the true *Vibrio* species (38-63 mol%). They also required a microaerophilic growth environment and had non-fermentative metabolism. Due to these fundamental differences Selbald and Veron (1963) proposed a new genus called *Campylobacter* (meaning curved rod in Greek) for these two species. In 1973 Veron and Chatelain published an in-depth taxonomy study on these microaerophilic *Vibrio*-like organisms. They proposed that *Vibrio coli, Vibrio jejuni* and *Vibrio sputorum* be also transferred to the *Campylobacter* genus with *Campylobacter fetus* being

described as the type strain. Following this study many new strains and species of *Campylobacter* were identified which included *C. hyointestinalis, C. concisus, C. mucosali, C. lari* and *C. upsaliensis*. Subsequently up to 20 species and subspecies were put into the genus *Campylobacter.*

In 1977 two phenotypically different groups of microaerophilic curved rods were isolated from aborted foetuses of cattle, pigs and sheep. This was achieved by Ellis and co-workers using a semi-solid enrichment medium originally designed for the isolation of *Leptospira* spp. (Ellis *et al.*, 1977). These strains were classified in the genus *Campylobacter,* but were members of two distinct biochemical groups. The first group was identified as *Campylobacter fetus.* Whereas the second group belonged to a previously undescribed *Campylobacter* taxon, which were aerotolerant to atmospheric oxygen after primary isolation in a microaerophilic environment (Neill *et al.*, 1978). Additional differences of the second group from true *Campylobacter* species were noted. These included optimum growth temperature of 30°C compared to the usual 37-42°C, being unable to grow on *Campylobacter* isolation media or be stained with fluorescein labelled *C. fetus* specific antisera and failure to agglutinate with antisera to *C. fetus* heat stable A, B and C antigens (Ellis *et al.*, 1978). Even though this information did not follow the true phenotypical traits of C. *fetus* the principal *Campylobacter* species, the similarities of morphology, lack of fermentation activity, darting motility and mol percent G + C content out weighed any differences. Therefore, Neill *et al.* (1985) proposed a new species of aerotolerant *Campylobacter,* which exhibited a tolerance to lower temperatures and higher oxygen concentrations than other strains. This species was named *Campylobacter cryaerophila*, meaning 'loving cold air' in Latin. Neill *et al.* (1985) observed phenotypic heterogeneity within *Campylobacter cryaerophila.* This was confirmed by Kielbauch *et al.* (1991a) who isolated two different genospecies within a group of aerotolerant *Campylobacter* strains, but no clear-cut differential phenotypic features were determined. Some strains belonged to *C. cryaerophila*, but most of the strains were members of another species, which exhibited a 40% level of DNA homology with *C. cryaerophila.* The name '*Campylobacter butzleri*' was proposed, but never validly published for this strain of *Campylobacter* which was most frequently isolated from human and non-human primates with diarrhoea (Kiehlbauch *et al.*,

1991a). A third microaerophilic *Campylobacter*-like organism was discovered in 1981, in roots and root associated sediments of salt marsh plants *Spartina alternflora* (McClung & Patriquin, 1980). It was named *Campylobacter nitrofigilis*.

Thompson *et al.* (1988) used 16S rRNA hybridisation to determine the phylogenic relationship of aerotolerant *Campylobacter.* The results showed that the organisms belonging to the genus *Campylobacter* were made up of 3 separate rRNA homology groups each deserving a separate genus status. The first rRNA group contained *C. fetus, C. hyointestinalis, C. concisus, C. mucosalis, C. sputorum, C. coli, C. jejuni, C. lari* and *C. upsaliensis*. *C. cinaedi, C. fennelliae, C. pylori* and *Wolinella succinogenes* were members of the second rRNA group. The final rRNA group contained *C. cryaerophila* and *C. nitrofigilis* only, which exhibited 87% homology with each other (Thompson *et al.*, 1988). However, only a 68% homology was observed with other *Campylobacter* strains suggesting that *C. nitrofigilis* and *C. cryaerophila* were genetically distinct organisms.

Following this a comprehensive study on the comparison of *Campylobacter* and aerotolerant *Campylobacter* isolates was performed by Vandamme and co-workers (1991). Techniques such as DNA:rRNA and DNA-DNA hybridisation, immunotyping, SDS-PAGE of cellular proteins and fatty acid analysis were used. The results obtained were similar to those of Thompson *et al.* (1988), which again suggested that the *Campylobacter* taxonomy needed to be revised. Therefore, it was proposed that the rRNA group I were the true *Campylobacter* species which contains 16 species and 6 subspecies. Whereas all of group II except *Wolinella succinogens* were renamed as the *Helicobacter* genus (Goodwin *et al.,* 1989). *Wolinella succinogenes* remains the only species in the genus *Wolinella*. Finally, the name *Arcobacter* (meaning arc-shape bacteria in Latin) was proposed for rRNA homology group III (Vandamme *et al.,* 1991). This new genus was made up of 4 species which were identified by using DNA-DNA hybridisation data.

These include:

- *A. cryaerophilus* (2 distinct electrophoretic subgroup 1A and 1B),
- *A. nitrofigilis,*
- *A. butzleri,*
- *A. skirrowii.*

A. skirrowii was proposed by Vandamme *et al.* (1992a) as the newest species of aerotolerant *Arcobacter*. Furthermore, Wirsen *et al.* (2002) has provisionally proposed the name "*Candidatus Arcobacter sulfidicus*" for a sulfide-oxidizing, filamentous-sulfur-producing vibriod organism isolated from a coastal marine area. The morphological and physiological characteristics of this organism such as cell shape, motility, location of flagella, Gram reaction, microaerophilic preference, mesophilic temperature preference, nitrogen fixation (as in *A. nitrofigilis*) and habitat are characteristics for members of the genus *Arcobacter* (Vandamme *et al.*, 1991; De Boer *et al.*, 1996; Schroeder-Tucker *et al.*, 1996). Phylogenic analysis such as 16S rRNA sequencing and *in situ* hybridisation have also placed this organism in the *Arcobacter* genus. Further investigation is required before this organism can be accepted as a true *Arcobacter* species. Nevertheless, this group could be expanding even further as an investigation of bacterial communities of oil fields and a hypersaline pond (Teske *et al.*, 1996; Voordouw *et al.*, 1996) has shown the presence of *Arcobacter*-like organisms that share less than 89% 16S rRNA gene sequence similarity with existing taxa, thus probably representing novel species (On *et al.*, 2001).

Originally the *Campylobacter* genus was grouped with *Spirillum* to constitute the family Spirillaceae (Smibert, 1978). This was mainly due to morphology similarities. However, Romaniuk *et al.* (1987) compared the partial 16S rRNA sequence from 6 *Campylobacter* strains. It was discovered that *Campylobacter* formed a previously undescribed eubacterial group, which was related to other Gram-negative bacteria only by very deep branching. Using the same 16S rRNA techniques Lau and co-workers (1987) observed similar results except they concluded that signature features of *Campylobacter-Wolinella* rRNA cluster were related to the phylum of purple photosynthetic bacteria, which is known as

Proteobacteria (Stackebrandt *et al.*, 1988; Murray *et al.*, 1990). Therefore, *Campylobacter* constitute a separate eubacterial lineage of the class Proteobacteria identified as rRNA superfamily VI. The *Arcobacter* genus is believed to be the closest relative of the genus *Campylobacter* (Figure 1.1). They are linked at an average $Tm_{(e)}$ (melting temperature of elution) of 66°C. The difference in $Tm_{(e)}$ observed between *Campylobacter* and *Arcobacter* correspond to the differences seen between members of genotypically well-defined bacterial families such as the Neisseriaceae, (Rossau *et al.*, 1989) the Aeromonadaceae, (Colwell *et al.*, 1986) the Pasteurellaceae (De Ley, 1978) and the Enterobacteriaceae (De Ley & Tytgat unpublished, as quoted by Vandamme & De Ley, 1991). This close genotypic relationship between the genera *Campylobacter* and *Arcobacter,* together with the numerous phenotypic similarities shows the importance of these organisms and suggests that a separate taxonomic and nomenclatural status is required for these taxa. Additionally, *Campylobacter* strains and reference strains from rRNA superfamily I to V have a $Tm_{(e)}$ average of 56.6 ± 3.1°C, thus confirming that *Campylobacter* and relatives do not belong to any of the 5 rRNA superfamilies. Therefore, instead of belonging to the Proteobacteria family, Vandamme and De Ley (1991) proposed that a new family should be created for these taxa called Campylobacteraceae.

1.2. MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF *ARCOBACTER* SPECIES.

Arcobacter species are fastidious Gram-negative slender helical non-spore forming rods with tapering ends (see Figure 1.2, p8). They are 0.2 to 0.9 μm wide and 1 to 3 μm long, but unusually long cells of 20 μm and greater have occasionally been observed (Wesley, 1994). When viewed by a conventional light microscope the cells appear helical, curved or S shaped, but under stressful conditions and upon ageing the cells form spherical or coccoid bodies. This state is known as a viable but non-culturable state (VNC).

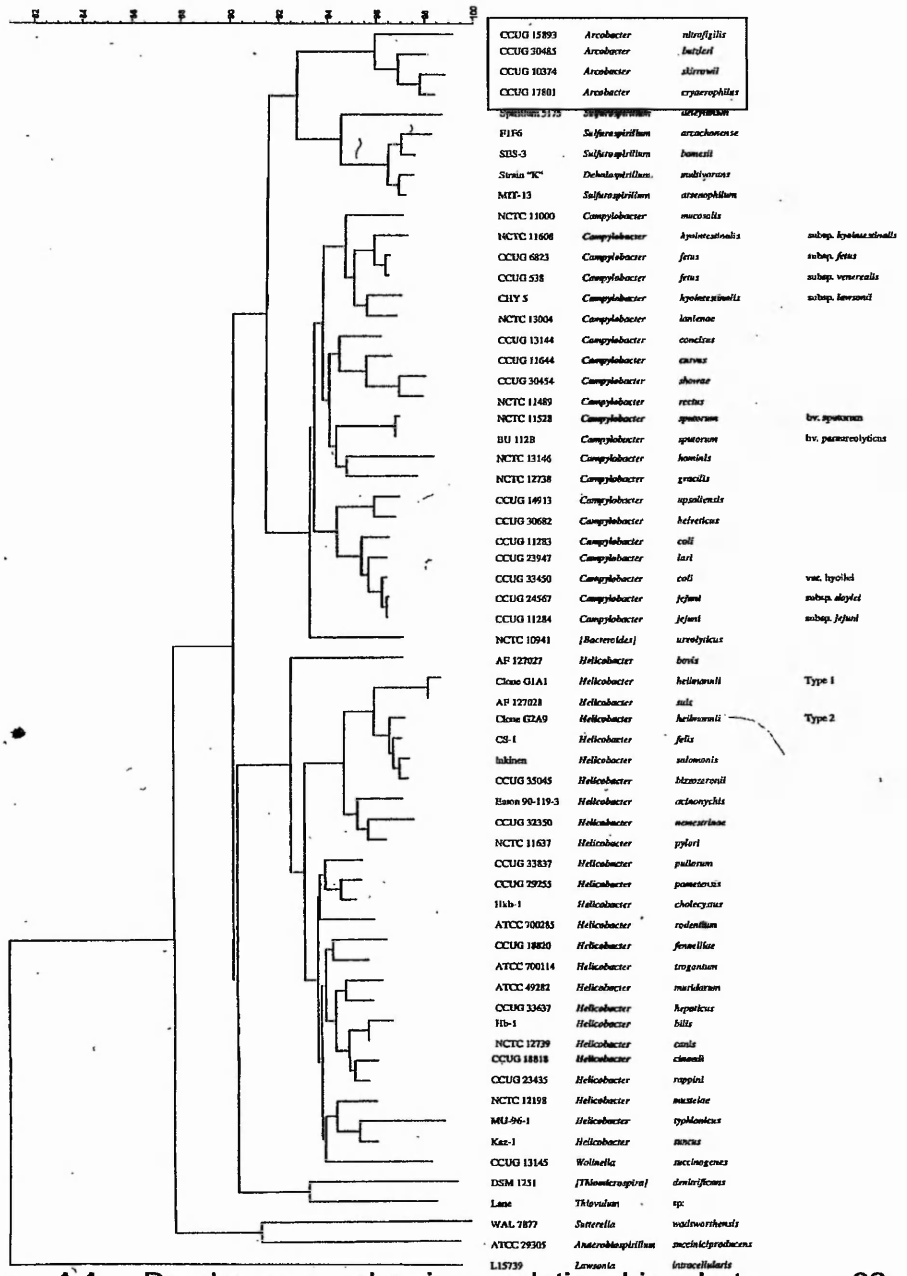


Figure 1.1: Dendrogram showing relationship between 62 strains of *Campylobacter, Arcobacter, Helicobacter, Sulfurospirillum, Wolinella, Thiovulum, Lawsonia, Sutterella, Anaerobiospirillum, Bacteroides, 'Dehalospirillum multivorans'* and *Thiomicrospira denitrificans,* as inferred by comparison of 16S rRNA gene sequences and neighbour-joining clustering. Taken from On (2001). The scale bar indicates 19% sequence similarity, whereby the higher the percentage the closer the two strains are related. Sequences were obtained via GenBank, and a multiple alignment obtained using BioNumerics software (Applied Maths, Kortrijk, Belgium). Gaps and unknown bases were not considered in the analysis.

Arcobacter are also very motile exhibiting a darting corkscrew motion due to a single unsheathed polar flagellum at one (monotrichous) or both (amphitrichous) ends of the cell. The flagella are usually 2-3 times the length of the cell (Jones *et al.*, 1985; Goossens *et al.*, 1986). The presence of the flagellum is believed to be a virulence factor that aids *Arcobacter* species in adhesion and penetration of its natural habitat.

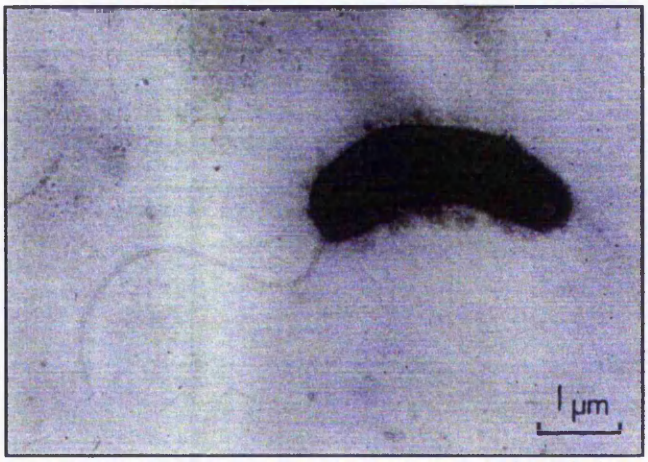


Figure 1.2: Helical rod shaped morphology of *Arcobacter* spp. with a single flagellum.

Arcobacter grow optimally under microaerophilic conditions requiring an oxygen concentration of 3-15% and a carbon dioxide concentration of 3-5% (Vandamme *et al.*, 1991). The optimum oxygen concentration for growth is 3-6%, but some strains can grow under aerobic and/or anaerobic conditions after primary isolation under microaerophilic conditions (Neill *et al.*, 1985). Furthermore, unlike *Campylobacter*, *Arcobacter* strains do not require hydrogen for growth. Their optimum growth temperature is between 30-37°C, with a lower limit of 5°C (Neill *et al.*, 1985) and an upper limit of 40°C, which is dependent upon incubation conditions. All strains are able to grow at 15°C (Vandamme *et al.*, 1991; Burnens *et al.*, 1992; On & Holmes, 1995). *Arcobacter* strains can grow between pH 5.5–9.5, but the preferred range is from pH 6.8–8.0 (Neill *et al.*, 1985).

When *Arcobacter* are grown on fresh agar a *Campylobacter*-like swarming effect can be observed. Colonies are small (1 mm in diameter), non-pigmented and

convex with entire edges. After subculturing colonies often vary in size with a more flattened appearance (Wesley, 1994). On blood agar *Arcobacter* tend to produce large (2-4 mm in diameter), rounder colonies. *A. butzleri* and *A. cryaerophilus* have a beige-whitish appearance, whereas *A. nitrofigilis* and *A. skirrowii* have a white-grey coloration (Collins *et al.*, 1996b).

Even though they grow under microaerophilic conditions they have a respiratory type of metabolism with menaquinone 6 and methyl-substituted menaquinone 6 acting as the major respiratory quinones (Vandamme & De Ley, 1991; Vandamme *et al.*, 1991; Ursing *et al.*, 1994). *Arcobacter* species are chemoorganotrophs, so they are unable to ferment or oxidise carbohydrates. They obtain their energy from amino acids and intermediates of the tricarboxylic acid cycle. *Arcobacter* species produce a negative reaction for methyl red and Voges Proskauer tests (Vandamme *et al.*, 1992a). Most species reduce nitrate and nitrite, but are unable to hydrolyse hippurate, DNA, starch or esculin. Also, all *Arcobacter* isolates hydrolyse indoxyl acetate (Harman *et al.*, 1997; Wesley, 1997). These particular organisms are both catalase and oxidase positive. Many of the *Arcobacter* strains are susceptible to nalidixic acid (40 μg/ml), colistin (0.5 μg/ml), and rifampicin (2 μg/ml), but resistant to carbenicillin (64 μg/ml), 5-fluoruracil (>256 μg/ml) and trimethoprim (>256 μg/ml) (Atabay & Aydin, 2001; Houf *et al.*, 2001a). Tetradecanoic acid, hexadecanoic acid, hexadecanonic acid and octadecanoic acid make up the major fatty acid content of *Arcobacter* species membranes (Vandamme *et al.*, 1991). Their guanine-plus-cytosine (G+C) content is low ranging from 28 to 38 mol percentage (mol%) (Veron & Chatelaine, 1973; Smibert, 1984).

Arcobacter are believed to have a small genome that is approximately 1.6-1.7 Mbp of AT-rich DNA, which is predicted to encode 1,654 proteins and 54 stable RNA species. This is half the size of the *Escherichia coli* genome (Ketley, 1997; Fields & Swerdlow, 1999; Parkhill *et al.*, 2000). Furthermore, like *Campylobacter,* the small size of the genome in *Arcobacter* is perhaps reflected in the requirements for complex media for growth and their inability to ferment carbohydrates and degrade complex substances (Griffiths & Park, 1990). However, additional genetic

information in the form of conjugative plasmids have been reported in *Arcobacter* species. Plasmids of four sizes (2, 3, 4.8 and 5 kb) were isolated from 24% (n = 170) of *A. butzleri* isolates (Harrab *et al.*, 1998).

1.3. ISOLATION OF *ARCOBACTER* SPECIES.

Arcobacter species exhibit marked similarities to *Campylobacter* with respect to phenotypic and genotypic characteristics such as morphology, nutrient requirements and the ability to induce illness (Ohlendorf & Murano, 2002b). However, differences in the fatty acid methyl ester composition, electrophoretic protein profiles and growth conditions enable *Arcobacter* species to be differentiated from *Campylobacter* species (Vandamme *et al.*, 1991). The arcobacters ability to withstand both low temperatures and atmospheric oxygen levels have been incorporated into current isolation methods (Burnens *et al.*, 1992; Corry *et al.*, 2003).

Arcobacter were originally isolated by Ellis *et al.* (1977) using Ellinghausen-McCullough-Johnson-Harris polysorbate 80 (EMJH P-80) supplemented with 5-fluorouracil (100 μg/ml) which was developed to isolate *Leptospira spp.* Since then numerous enrichment, plating media and isolation protocols have been devised for the recovery of *Arcobacter* species from food and environmental sources. These include Lammerding *et al.* (1996) who used an enrichment in peptone broth containing cefoperazone as a selective agent under microaerophilic conditions followed by filtration (0.45 μm pore size) onto modified charcoal, cefoperazone, deoxycholate agar (mCCDA) plates and incubated at 30°C under aerobic conditions. This method was then modified by Atabay and Corry (1997) where the enrichment step was in cefoperazone, amphotericin B and teicoplanin (CAT) broth and then filtered onto CAT, mCCDA and blood agar using the Steele and McDermott membrane filter technique. All *Arcobacter* species (except *A. nitrofigilis* which was not tested for) were isolated using this method, but *A. skirrowii* was only isolated on the non-selective blood agar. This may be due to a synergistic effect of cefoperazone and sodium deoxycholate. This method was

evaluated by Atabay and Corry (1997) and it was observed that an enrichment step was necessary for isolating *Arcobacter* only from poultry carcasses, whereas direct plating enabled only *Campylobacter* species to be isolated. Further investigation also showed that CAT agar outperforms mCCDA agar especially for the isolation and growth of *A. butzleri*. However, *A. skirrowii* isolates from duck were unable to grow on either CAT or mCCDA agar (Atabay *et al.*, 2001).

Atabay and Corry (1998) also used a new commercially available *Arcobacter* enrichment broth (Oxoid) with CAT selective supplement to determine its ability to isolate *Arcobacter* species compared with Preston broth and Lab M Bolton broth, which are enrichment broths for *Campylobacter* species. The new *Arcobacter* broth was able to isolate all four species of *Arcobacter* (8 strains of *A. butzleri*, 2 strains of *A. cryaerophilus,* and 1 *A. nitrofigilis* & *A. skirrowii* strain) unlike the two other methods. More importantly eight *Campylobacter* species (2 strains of *C. coli*, and 1 strain of *C. jejuni* subsp. *jejuni*, *C. lari*, *C. fetus* subsp. *venerealis*, *C. fetus* subsp. *fetus*, *C. hyoilei* & *C. hyointestinalis* subsp. *hyointestinalis*) were also used in this study and none were able to grow in *Arcobacter* broth, which is possibly due to the lack of oxygen quenching systems such as blood or charcoal. Additionally, *Arcobacter* were able to reach higher population densities in *Arcobacter* broth than those achieved in biphasic growth methods or brain heart infusion broths (Dickson *et al.*, 1996).

In 1996 De Boer and co-workers developed an *Arcobacter* selective enrichment broth (ASB) and a semi solid *Arcobacter* selective plating medium (ASM) which contained cefoperazone, piperacillin, trimethoprim and cycloheximide as selective agents. To isolate only *Arcobacter* species the plates were incubated at 24°C under aerobic conditions. This enabled *Arcobacter* to form a swarming effect on the plates. However, only *A. butzleri* and *A. butzleri*-like strains were isolated.

Collins *et al.* (1996b) were also working on a method to isolate *Arcobacter* from pork. EMJH-P80 was used as an enrichment medium followed by two selective plating methods, which were cefoperazone-vancomycin-amphotericin B (CVA) with blood medium and a modified cefsulodin-irgasan-novobiocin (mCIN) agar containing various detoxifying and growth promoting agents and 5-fluorouracil

(200 μg/ml). The plates were incubated under microaerophilic conditions, but at 30°C to prevent the growth of *Campylobacter* species. From the pork samples 149 *Arcobacter* isolates were identified by hybridisation techniques. These results compared with CVA showing a 85.2% and mCIN having a 78.5% isolation efficiency. Thus suggesting that these isolation methods were able to detect a high number of *Arcobacter* isolates from field samples.

The methods by De Boer *et al.* (1996) and Collins *et al.* (1996b) have recently been compared to an aerobic enrichment and selective plating method (JM method) developed by Johnson and Murano (1999a, b). Both media contained cefoperazone, bile salts and thioglycollate, but the enrichment broth also contained 5-fluorouracil. This method was able to isolate *A. butzleri*, *A. cryaerophilus* and *A. nitrofigilis* although *A. skirrowii* was not tested. Also, *Arcobacter* colonies had red rings surrounding them, which was due to the presence of thioglycollate in the medium. Thus providing this method with an additional phenotypical characteristic for the identification of *Arcobacter* species. This method was also superior as 42 out of 50 broiler chickens were positive for *Arcobacter* compared to the De Boer method (14/50) and the Collins method (24/50) which had considerably lower isolation rates. These values were supported by Ohlendorf and Murano (2002b) as the JM method detected *A. butzleri* to a level of 10 cfu/g in 100% (n = 4 trials, number of 'samples' was not given) and *A. cryaerophilus* in 75% of ground pork samples. These values compared to 50% and 0% for the Collins method and 25% apiece for the De Boer method for *A. butzleri* and *A. cryaerophilus* respectively. From these results the JM method looks very promising as a routine isolation method for *Arcobacter* species as it was both rapid (4 days compared to the usual 6-9 days) and specific compared to the other methods.

A more recent development in isolation methods for *Arcobacter* species was by Houf and co-workers (2001b). They observed that none of the selective supplements currently available were specific for the isolation of *A. butzleri, A. cryaerophilus* and *A. skirrowii,* which are frequently isolated from food products. Furthermore, if all three species were isolated the selective supplements were unable to fully suppress the accompanying flora present and the low numbers of

Arcobacter became masked. Therefore, Houf *et al.* (2001a) studied the susceptibility of 101 strains of *Arcobacter* species (*A. butzleri, A. cryaerophilus* and *A. skirrowii)* to a variety of selective agents. They discovered that *A. skirrowii* was the most sensitive species and that 5-fluorouracil (100 mg/L), novobiocin (32 mg/L), trimethoprim (64 mg/L) and cefoperazone (16 mg/L) were the most suitable selective agents to isolate these three *Arcobacter* species. The selective agents stated above were then used along with amphotericin B (10 mg/L) in *Arcobacter* broth (Oxoid) as a new selective liquid and solid isolation medium for the recovery of *Arcobacter* species. The media were incubated at 28°C under microaerophilic conditions. This method isolated *Arcobacter* from 100% (n = 34) of neck skin of laying hens and from 90% (n = 71) of similar samples from broiler chickens. *A. skirrowii* was not isolated from the poultry samples using this method or multiplex PCR (Houf *et al.*, 2000). Nevertheless, *A. skirrowii* was isolated from artificially contaminated poultry skin using this new method, but growth of *A. skirrowii* was hindered by the presence of the other species. The authors state that this new enrichment method was rapid and reliable for isolating *Arcobacter* species from poultry and may even be a valuable tool in studying the prevalence of *Arcobacter* species in food, animals and human biological samples (Houf *et al.*, 2001b).

Even though method development has improved over many years, a validated method does not yet exist for the isolation of *Arcobacter* species. This in turn restricts the comparison of field survey data thus preventing the true prevalence of this organism in animal and environmental sources from being determined.

1.4. IDENTIFICATION METHODS FOR *ARCOBACTER* SPECIES.

Once *Arcobacter* species have been isolated from food, human or environmental specimens it is necessary to differentiate between strains within this species. This may be important in epidemiological studies where common sources of infection need to be identified (Vandamme *et al.*, 1993). Nevertheless, identification of species within the family Campylobacteraceae using standard biochemical tests

has been problematic due to the inert metabolic activity of this family and the variability and atypical reactions of some strains. Therefore, methods to differentiate between species have been developed. These include combining a systematic range of biochemical tests with a probabilistic matrix in which the characteristics of the unknown isolate is compared to those of a defined taxa such schemes include Preston scheme (Barrett *et al.*, 1988) and API Campy (Biomerieux, S.A. France). However, these methods only include 16 and 18 named taxa respectively, and have not been revised since 1992, so do not include the most recent taxonomic changes (Atabay *et al.*, 1998). Furthermore, *A. cryaerophilus* is the only *Arcobacter* species represented in these schemes and because of strain variability it is not a reliable means of identification (Jacobs *et al.*, 1993; Ridsdale *et al.*, 1998). Therefore, these methods prevent less common *Campylobacter* and *Arcobacter* species from being identified. The lack of specificity of these schemes was also shown by Ridsdale *et al.* (1998) as API scheme misidentified 100% of the *Arcobacter* isolates from duck carcasses. Whereas the Preston scheme could only speciate *A. cryaerophilus*. However, On and Holmes (1995) developed a system of numerical analysis of phenotypic characters together with the use of extensive probabilistic identification matrix. This scheme was able to speciate the strains not identified by API or Preston methods (On *et al.*, 1996) and accurately identify to the species or subspecies level as it incorporates a large number of tests.

On and Harrington (2001) went on to use numerical analysis of amplified fragment length polymorphism (AFLP) to identify taxonomic (species and subspecies) and epidemiological (clone and strain) relationships in a wide range of *Campylobacter* species including the four species of *Arcobacter*. This method can also be used to identify genetic diversity amongst different geographical areas (On *et al.*, 2001). Comparative studies have been done by Scullion *et al.* (2001), which showed a good correlation between multiplex PCR (Houf *et al.*, 2000) and AFLP profiling. However, AFLP is a laborious technique which requires a large reference database to determine the isolates identification. Once this has been achieved the method is both reliable and highly discriminatory.

In addition to biochemical tests, 'serotyping' identification schemes were developed to differentiate strains of *Campylobacter* and *Arcobacter*. One such scheme was based on heat labile antigens (Lior & Woodward, 1991). A combination of serotyping and biotyping was successfully used to identify different *A. butzleri* isolates from poultry in France (Marinescu *et al.*, 1996).

Although these methods were able to identify *Arcobacter*, they are cumbersome to perform, time consuming and limited in specificity. Due to these drawbacks a wide variety of molecular techniques have been applied to distinguish *Arcobacter* species from other members of the Campylobacteraceae family. Vandamme *et al.* (1991) used whole cell protein profiling with computer-assisted numerical analysis, which allows a rapid and objective grouping of a large number of strains. This system then enables new isolates to be identified relatively easily and rapidly especially as the distribution of the dense bands in the so-called hypervariable dense band region characterizes protein electrophoretic types within a number of *Campylobacter* species.

Bacterial 16S and 23S rRNA sequences are targets for identification purposes. Ribotyping was first used by Kiehlbauch *et al.* (1991b) to reliably identify *Arcobacter* isolates. The current procedure uses guanidium DNA extraction and hybridisation with non-radioactive digoxigenin-labelled probes, thus making it more acceptable and safer. Years later Suarez *et al.* (1997) designed oligonucleotide primers for the rRNA superfamily VI (Table 1.1). The probes R01 and R05 react with *Campylobacter, Helicobacter* and *Arcobacter* species. Whereas the R02, R04 and R03 primers were designed to be more specific as under high stringency conditions the probe hybridised only with *A. butzleri*. De Oliveria and co-workers (1995) developed a genus specific 16S rRNA-based oligonucleotide DNA probes along with an *A. butzleri* species-specific DNA probe which were able to identify *Arcobacter* species that had been recovered from clinically ill or healthy humans and/or livestock. The 23 and 27 mer probes targeted the genes encoding the 16S rRNA molecule. Using *PVU* II *A. cryaerophilus* 1A and 1B and *A. butzleri* produced different ribotyping patterns thus enabling them to be easily identified. All *A. cryaerophilus* strains had a common ribosomal DNA restriction fragment of 3.2 Kb, whereas DNA group 1B had an additional band at 2.6 Kb. Moreover,

Table 1.1: Primer sequences for *Arcobacter*-specific and *A. butzleri* specific 16S and 23S rRNA based DNA probes. Modified from Mansfield and Forsythe (2000).

Primer	Position	Nucleotide sequence (5'-3')
16S rRNA probes (1, 2, 3, 4, 5)		
Arco I	224-244[a]	AGAGATTAGCCTGTATTGTAT
ArcoII	1426-1446	TAGCATCCCCGCTTCGAATGA
Butz	984-1007	CTTGACATAGTAAGAATGATTTAG
Arco III	269-291	AGTTATGTGTCATAGTCTTGGTA
ARC94	94-111	TGCGCCACTTAGCTGACA
ARC1430	1430-1447	TTAGCATCCCCGCTTCGA
CAH1a	Not given	AATACATGCAAGTCGAACGA
CAH1b	Not given	TTAACCCAACATCTCACGAC
23 mer	Not given	TTTAGCATCCCCGCTTCGAATGA
27 mer	Not given	GATACAATACAGGCTAATCTCTAACAA
23S rRNA probes (6, 7)		
ARCO1	1580-1599[b]	GTCGTGCCAAGAAAAGCCA
ARCO2	1887-1868	TTCGCTTGCGCTGACAT
CRYAE	1375-1395	TAAGTCGAGACTGAAAAGT
BUTZ	1222-1241	CTATTCAGCGTAGAAGATG
SKIR	1466-1483	AGGTCACGGATGGAAGT
N.butz	1174-1199	AGCGTTCTATTCAGCGTAGAAGATGT
N.c1A	1135-1162	ACCGAAGCTTTAGATTCGAATTTATTCG
N.c1B	1713-1736	GGACTTGCTCCAAAAAGCTGAAG
N.ski	1424-1443	CGAGGTCACGGATGGAAGTG
Nested PCR probes (8)		
R01	318	GGAACTGAGACACGGTCCAG
R02	1249	CGTCACCGTATTGCTGCTCT
R03	546	TAAAGAGCGTGTAGGCGGAT
R04	11	TGATCCTGGCTCAGAGTGAA
R05	1185	TGACGTCATCCTCACCTTCC
PCR fingerprinting (9, 10, 11)		
1R	ERIC[c]	ATGTAAGCTCCTGGGGATTCAC
2	ERIC	AAGTAAGTGACTGGGGTGAGCG
1267	Arbitrary primer	GAGCGGCCAAAGGGAGCAGAC

Sources: 1, Harmon & Wesley, 1997; 2, Marshall *et al.*, 1999; 3, Snaidr *et al.*, 1997; 4, Antolin *et al.*, 2001; 5, De Oliveria *et al.*, 1995; 6, Bastyns *et al.*, 1995; 7, Kabeya *et al.*, 2003; 8, Suarez *et al.*, 1997; 9, Vandamme *et al.*, 1993; 10, Atabay *et al.*, 2002; 11, Houf *et al.* 2002b, 2003.

(a) Nucleotide position based on the *A. butzleri* 16S rRNA sequence.

(b) Nucleotide position is based on the *E. coli* 23S rRNA sequence homologous to the 5' and 3'-termini of the primer.

(c) Enterobacterial repetitive intergenic consensus.

most (94%) of the *A. butzleri* strains tested had a unique 3.0 Kb band. The only drawback with this method was that *A. cryaerophilus* and *A. skirrowii* had the same profile, so could not be differentiated (see Table 1.2, p20). Furthermore, Marshall *et al.* (1999) developed a two-step typing scheme based on PCR-RFLP analysis of the 16S rRNA gene. By using *DdeI* and *TaqI* unique fingerprints for *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* were detected.

Due to the inherent specificity, high sensitivity and speed of polymerase chain reaction (PCR), assays have been developed using primers and oligonucleotide probes derived from the sequences of the *Arcobacter* species and *A. butzleri* specific 16S rRNA (Wesley *et al.*, 1995). Gonzalez and co-worker (2000) combined this PCR method with a pre-enrichment step in *Arcobacter* broth supplemented with CAT. It proved to be a rapid and reliable method for assessing *Arcobacter* contamination of poultry meat (see Table 1.2, p20). This method was developed further by Antolin *et al.* (2001) by linking the PCR procedure to an ELISA assay, thus producing a rapid and specific method for the detection (10 cfu/g) and enumeration of *Arcobacter* species from chicken meat. Lau *et al.* (2002) also used an adapted PCR method to identify *A. butzleri* from a patient with acute gangrenous appendicitis.

The 23S rRNA gene has also been used to identify *Arcobacter* species, as it is larger than the 16S rRNA with more variable regions. Therefore, it is more discriminating and specific. The 23S rRNA gene-based PCR-RFLP identification scheme produced unique species-specific banding profiles for *A. butzleri* and *A. nitrofigilis*. Whereas *A. cryaerophilus* and *A. skirrowii* had identical patterns using *Hpa* II, *Cfo* I, and *HinF* I restriction enzymes. This method was evaluated using 21 field strains which were difficult to characterize by phenotypic tests. Most (86%) of the strains were easily identified with this method, but four isolates shared similar patterns and were identified as *Arcobacter*-type strains (Hurtado & Owen, 1997). This method was shown to be reliable, but ribotyping has proven to be both time consuming and heavily reliant on resources. Due to this PCR-mediated detection methods have been developed such as a genus-specific PCR and three species-specific PCR assays which use a variable region of the 23S rRNA as a target sequence. Using ARCO1-ARCO2 primers both reference strains

and field isolates were classified to the genus level. Then using ARCO1 with BUTZ, CRYAE and SKIR (see Table 1.1, p16) species-specific primers the isolates were speciated (Bastyns *et al.*, 1995). Multiple sets of the primers stated above have been used to detect *Arcobacter* species using a multiplex PCR (m-PCR) technique. The amplified products showed that all *Arcobacter* isolates tested produced a 1223 bp band with *A. butzleri* yielding an additional band at 686 bp. Using this technique 108 field strains were analysed which had previously been characterised to the species level by either DNA-DNA hybridisation, dot blot hybridisation, ribotyping or serotyping. All 108 isolates were identified as *Arcobacter* species with 66 strains being identified as *A. butzleri.* These results agreed with those obtained by the other methods (Harmon & Wesley, 1997).

Houf and co-worker (2000) also developed a multiplex PCR assay, which used 5 primers to target sequences in both the 16S and 23S rRNA genes. Thus enabling the simultaneous detection and identification of the three *Arcobacter* species associated with animal food products. The primers amplified a unique product for each *Arcobacter* species (*A. cryaerophilus* = 257 bp, *A. butzleri* = 401 bp and *A. skirrowii* = 641 bp). This PCR assay was combined with an enrichment step in either *Arcobacter* broth (Atabay & Corry, 1998) or ASB (De Boer *et al.*, 1996) to isolate and identify *Arcobacter* from spiked chicken samples. Both *A. butzleri* and *A. cryaerophilus* were detected to a 10^3 cfu/g limit compared to 10^2 cfu/g for *A. skirrowii.* More recently Kabeya *et al.* (2003) developed a one step species-specific PCR assay to identify *Arcobacter* species. Multiple primers (N.butz, N.c1A, N.c1B and N.ski, see Table 1.1, p16) amplified species-specific fragments from reference strains of *A. butzleri, A. cryaerophilus* 1A, 1B and *A. skirrowii* as well as identifying 10 Japanese field isolates. Therefore, this simple assay was found to be a powerful tool for epidemiological surveys.

Winters and Slavik (2000) designed a multiplex PCR technique to detect both *A. butzleri* (1223 bp) and *C. jejuni* (159 bp) from the same specimen in one PCR tube. This method enabled the organisms to be identified from a variety of foods and only took 8 h to perform (see Table 1.2, p20). *Arcobacter* and *Campylobacter* species could also be identified from the same samples by using a PCR-oligohybridization strategy (Al Rashid *et al.*, 2000). Finally, PCR and fluorescent *in*

situ hybridisation (FISH) techniques have been evaluated to detect *Arcobacter* and *Campylobacter* strains in river water and waste water samples. The detection range of PCR and FISH assays in naturally and artificially contaminated samples varied between 1 cell/ml after enrichment to 10 cell/ml without enrichment (Yolanda *et al.*, 2003). These methods have proven to be both species-specific and rapid for the detection of *Arcobacter* species from field samples. Therefore, these techniques may be a useful tool for screening samples to provide valuable information on the occurrence of *Arcobacter* in food, human or environmental specimens.

Lastly, in 2002 Atabay and co workers used randomly amplified polymorphic DNA PCR (RAPD-PCR) to analyse 35 *Arcobacter* isolates from chicken carcasses for genetic variations in enterobacterial repetitive intergenic consensus (ERIC) sequence. The DNA fingerprints of almost all *A. butzleri* isolates had DNA bands at 150 bp, 300 bp and one slightly over 800 bp in common. However, many additional bands were also observed and 11 distinct DNA profiles were obtained from the 35 isolates. These results suggest that there were numerous sources of contamination of the chicken carcasses or that great genotype diversity in *Arcobacter* DNA may exist within poultry flocks. This genetic diversity in *Arcobacter* DNA profiles was also observed by Houf *et al.* (2002b, 2003) who assessed two PCR based typing methods (ERIC-PCR and RAPD-PCR) on *Arcobacter* isolates from poultry products. The authors state that DNA fingerprinting has proven typeability, discriminatory powers and reproducibility which are essential for epidemiological studies.

The advantages and disadvantages of most of the methods discussed above are summarised in the table on the following page (Table 1.2). This table was taken from the Phillips review paper (2001).

Table 1.2: Molecular typing methods used to identify *Arcobacter* (Phillips, 2001).

Method	Samples tested	Advantages	Disadvantages	Reference
Ribotyping	Type & reference strains	Discriminatory between *Arcobacter* strains	Minimum number of cells required	Kiehlbauch *et al.* (1994)
Hybridization with 16S rRNA probes for *Arcobacter* spp. and *A. butzleri*	Field isolates from aborted livestock foetuses; reference strains	No cross reaction of *Arcobacter* spp. probe with *Helicobacter/ Campylobacter* spp. or of *A.butzleri* probe with *A. skirrowii* or *A. cryaerophilus*; differentiates between *A. cryaerophilus* 1A & 1B	Does not distinguish *A. skirrowii*	Wesley *et al.* (1995); De Oliveria *et al.* (1999)
PCR using 16S rRNA DNA probes specific to *Arcobacter* (as above)	Porcine faeces; water	*A. butzleri, A. cryaerophilus* & *A. skirrowii* differentiated; no cross reaction with *Helicobacter/ Campylobacter;* results within 8 h	None described	Harmon & Wesley (1996); Rice *et al.* (1999)
PCR-RFLP analysis 16S rRNA gene	Clinical isolates; reference strains	Differentiates all *Arcobacter* spp.	Does not differentiate between C. *jejuni* and *C. coli*	Marshall *et al.* (1999)
PCR-RFLP analysis 23S rRNA gene	Field isolates; reference strains	Relatively inexpensive; differentiates most *Campylobacter* spp.	Does not distinguish *A. cryaerophilus* & *A. skirrowii*	Hurtado & Owen (1997)
m-PCR using species-specific primers for *A. butzleri* and *C. jejuni*	Artificially contaminated meat, fruit & dairy products	Identifies *C. jejuni* and *A. butzleri* in one assay within 8 h	Not tested for *A. cryaerophilus*	Winters & Slavik (2000)
m-PCR using primers for 16S rRNA genes of *Arcobacter* spp. & a *A. butzleri* specific 23S rRNA gene portion	Reference strains, poultry isolates, porcine isolates	Rapid; specific for *A. butzleri*	Does not differentiate non-*butzleri* species	Harmon & Wesley (1997)
m-PCR-culture using species specific primers	Reference strains, poultry isolates	Sensitive; rapid; distinguishes *A. butzleri, A. cryaerophilus* and *A. skirrowii*	None described	Houf *et al.* (2000)
m-PCR-culture using *Arcobacter*-specific primers	Reference strains and chicken isolates	Sensitive, rapid	Only identifies *Arcobacter* spp. with no differentiation	Gonzalez *et al.* (2000)
PCR-hybridization based on glyA gene	Type & reference strains	Low levels of detection of *A. butzleri* (50 pg DNA or 23, 000 copies)	Only *A. butzleri* or *A. butzleri*-like strains identified	Al Rashid *et al.* (2000)
AFLP profiling	Type, reference & field strains	Highly discriminatory for *A. butzleri* and *A. cryaerophilus*	Large database required; some *A. skirrowii* isolates produce aberrant results.	Scullion *et al.* (2001)

1.5. DESCRIPTION AND DIFFERENTIATION OF *ARCOBACTER* SPECIES.

1.5.1. *Arcobacter butzleri* (formerly *C. cryaerophila* then *C. butzleri*).

A. butzleri was originally identified after an extensive DNA-DNA hybridisation study with phenotypic analysis of 78 aerotolerant *Campylobacter* strains (Vandamme *et al.*, 1991). The results showed the detection of two DNA related groups, some of the strains were members of *C. cryaerophila*, but most were identified as separate species. This new species was named "*Campylobacter butzleri*" (unfortunately the name was never validly published) in honour of Jean-Paul Butzler a Belgium clinician and microbiologist who was one of the first major investigators of *Campylobacter* species. *C. butzleri* was soon renamed *Arcobacter butzleri* as it showed a 40% level of DNA homology with *A. cryaerophilus* (Vandamme *et al.*, 1992a). Also 16S rRNA analysis has shown that *A. butzleri* is most closely related to *A. skirrowii* (Wesley, 1994; Figure 1.1, p7). However, *A. butzleri* does have certain distinguishing features such as they are more temperature resistant as light growth occurs at 42°C, appear to grow more vigorously under optimum conditions, weakly catalase positive, able to reduce nitrate but not nitrite or esculin (see Table 1.3, pp24-25). They can grow also on MacConkey agar, in an 8% glucose solution and in a 1.5% NaCl solution, but not when it is increased to 3.5% (Vandamme *et al.*, 1992a). All strains are resistant to 2.5 μg of cadmium chloride and most strains (72%) are resistant to 20 μg. Therefore, *A. butzleri* in comparison to *A. cryaerophilus* appears to grow more vigorously, was more resistant to heavy metals and has a greater tolerance to higher temperatures and selective agents such as bile (Schroeder-Tucker *et al.*, 1996). *A. butzleri* is believed to be an important pathogen as it was the first aerotolerant '*Campylobacter*' to be linked with human infections. Serotypes 1 and 5 are regarded as the primary pathogenic strains (Lior & Woodward, 1991, see Section 1.6.2). This strain has also been isolated from aborted livestock especially pigs, horses and cattle with enteritis and spoiled meat (Vandamme *et al.*, 1992a).

1.5.2. *A. cryaerophilus* (formerly *C. cryaerophila*).

A. cryaerophilus was named due to its ability to grow at low temperatures (15°C). It is the most diverse species and has been divided into 2 distinct electrophoretic subgroups 1A and 1B, which share 46-69% DNA homology (Vandamme *et al.*, 1992a). These subgroups can be distinguished via many genotypic analyses such as ribotyping, DNA-DNA hybridisation, protein profiles and restriction fragment length (Kiehlbauch *et al.*, 1991b). The most accurate way to differentiate between the two subgroups is by the amounts of the two fatty acid isomers of 16:1. As subgroup 1 has no 16:1 trans 9, but a very high level of 16:1 cis 9 (± 45%). Whereas subgroup 2 has equal amounts of both 16:1 isomers (± 19%) which has also been identified in *A. butzleri* strains (Vandamme *et al.*, 1992a). The subgroups can also be differentiated from other bacteria (Moss, 1990; 1992) as *A. cryaerophilus* contains two fatty acids that have never previously been identified. However, to date no phenotypical tests have been developed to differentiate between the two groups.

Generally this species is described as strongly catalase positive, variable in nitrate reduction with 68% of *A. cryaerophilus* strains not being able to reduce nitrate. Whereas all the other *Arcobacter* species have this ability. Also *A. cryaerophilus* is unable to grow in 1.5% NaCl, 1% glycine or on MacConkey agar (Vandamme *et al.*, 1992a). This particular strain is also very susceptible to cephalothin (30 μg) compared to *A. butzleri.* Only 14 out of 51 *A. cryaerophilus* strains showed resistance to this antibiotic, whereas 14 out of 18 *A. butzleri* strains were resistant (Vandamme *et al.*, 1992a). More importantly *A. cryaerophilus* has been linked to animal abortions especially in cattle and pigs, and recently animal and human enteritis (Vandamme & Goossens, 1992).

1.5.3. *Arcobacter nitrofigilis* (formerly *C. nitrofigilis*).

A. nitrofigilis is the type species and is associated with the roots of *Spartina alterniflora* plants and root-associated sediments in salt marshes (McLung & Patriquin, 1980; McLung *et al.*, 1983). Due to its nitrogenase activity it is able to be a nitrogen-fixing commensal of plants and hence the name 'nitrofigilis'. There have also been reports that *A. nitrofigilis*-like organisms being isolated from sea

sediment (Tharndrup *et al.*, 2000), petroleum contaminated ground-water (Watanable *et al.*, 2000), coral reef (Frias-Lopez *et al.*, 2002) and oil field brine (Gevertz *et al.*, 2000). This particular organism has not been isolated from animals or humans and is therefore not believed to be clinically significant. Nevertheless, this particular species may be able to grow in the animal or human gut due to its ability to grow in 1% bile and it being ureolytic. *A. nitrofigilis* exhibits an 89% 16S rRNA sequence homology with other species of *Arcobacter* (Vandamme *et al.*, 1991). Although it can be differentiated from the other *Arcobacter* strains as it has a completely different fatty acid profile, pigmentation of colonies in the presence of tryptophan and has nitrogenase activity (Vandamme *et al.*, 1992a). Furthermore, due to its natural habitat of salt marshes *A. nitrofigilis* is able to grow in 3.5% NaCl, so it has high salt tolerance and actually requires a certain amount of salt for growth (see Table 1.3, pp24-25). Finally, most reports state that *A. nitrofigilis* is an obligate microaerophile. However, Han (1994) and Park and Han (1997) have reported on the aerotolerance of *A. nitrofigilis*. It can grow under an atmospheric conditions (21% O_2) in brucella media and possesses cytochrome *c* thus enabling this species to obtain its energy by aerobic respiration. Also, as the oxygen concentration is increased, so does the activities of superoxide dismutase (SOD) and catalase which both scavenge toxic forms of oxygen. Therefore, the presence of both of these enzymes enables *A. nitrofigilis* to survive aerobic conditions.

1.5.4. *Arcobacter skirrowii.*

In 1992 Vandamme and co-workers proposed a new aerotolerant *Arcobacter* species isolated from lambs with diarrhoea. They named it *A. skirrowii* in honour of M.B. Skirrow a British microbiologist who was the first to describe a simple isolation technique for *C. jejuni* obtained from stools. *A. skirrowii* has also been isolated from preputial fluids of bulls and from porcine, ovine and bovine abortions (Vandamme *et al.*, 1992a) and duck carcasses (Ridsdale *et al.*, 1998). However, unlike *A. cryaerophilus* and *A. butzleri* it has not been associated with human infections. This organism is slow growing and very susceptible to most antimicrobial agents, compared to the other stains and may have been missed by routine clinical analysis (Mansfield & Forsythe, 2000; Houf *et al.*, 2001a). *A. skirrowii* strains could be differentiated from most other taxa by the presence of an

unknown fatty acid which has an equivalent chain length of 15.276 and it contains a high percentage of 18:1 cis 11. *A. skirrowii* has strong catalase activity, is able to reduce nitrate, but is unable to grow on MacConkey agar or in the presence of 1.5% NaCl (Vandamme *et al.*, 1992a). One of the most important characteristics is that all strains produce alpha-hemolysis on blood agar. This characteristic has only been observed in a few *A. butzleri* strains and is undetectable in *A. cryaerophilus* and *A. nitrofigilis.*

The major differential and biochemical tests for *Arcobacter* species have been summarised in Table 1.3. Table 1.3 also includes the results for *C. jejuni* which can be used for comparative purposes. This Table was modified from the Mansfield and Forsythe review paper (2000).

Table 1.3: Differential growth and biochemical tests. (Modified from Mansfield & Forsythe, 2000).

Test*	*A. butzleri*	*A.cryaerophilus*	*A. skirrowii*	*A.nitrofigilis*	*C. jejuni*
Growth at					
15°C	Positive	Positive	Not determined	Positive	Negative
25°C	Positive	Positive	Positive	Positive	Negative
30°C	Positive	Positive	Positive	Positive	90
37°C, aerobic	Positive	50	Positive	50	Negative
37°C,microaerophillic	Positive	58	Positive	Negative	Positive
37°C, anaerobic	Positive	16-95	Positive	Positive	Negative
42°C, microaerophilic	25-67†	0-18†	11-33†	Negative	Positive
Anaerobic + TMAO	Positive	11	Negative	Positive	Negative
Nutrient agar	Positive	Positive	Positive	Positive	Positive
Buffered charcoal yeast medium	Positive	Positive	Positive	Negative	Positive
Campylobacter charcoal-deoxycholate medium	Positive	95	Positive	Negative	Positive
Campylobacter minimal medium	Positive	Negative	Negative	Negative	10
MacConkey agar	83-100†	16-43†	Negative	Negative	5
Lecithin	75	16	11	Negative	35
Growth in presence of					
Glycine, 1%	53	9-23†	78	Not tested	Growth
NaCl, 2%	92	84	Positive	Positive	Negative
NaCl, 3.5%	42-67	0-33†	61	Positive	Negative
NaCl, 4.0%	Negative	Negative	Positive	Positive	Negative
Bile, 2%	Positive	79	Positive	Negative	80
Glucose, 8%	Positive	45-48	50	Not determined	Not determined
Pteridine 0/129 vibriostat	Positive	Positive	Not determined	Positive	Not determined
Cadmium chloride (2.5 μg disc)	Resistant	Sensitive	Not determined	Not determined	Sensitive
Oxidase	Positive	Positive	Positive	Positive	Positive

Table 1.3 continued

Catalase	33-100†	Positive	Positive	Positive	Positive
Alpha haemolysis	0-50†	Negative	Positive	Negative	95
H_2S production (lead acetate paper)	0-25	0-11	Negative	Negative	Positive
Nitrate reduction	Positive	30-36	Positive	Positive	Positive
Glucose utilization	Negative	Negative	Not determined	Not determined	Negative
Hippurate hydrolysis	Negative	Negative	Negative	Negative	Positive
Urea hydrolysis	Negative	Negative	Negative	Positive	Negative
Dnase activity	0-92	0-72	22-100	100	25-33†
Selenite reduction	Negative	Negative	11	50	70
Alkaline phosphatase	Negative	Negative	Negative	Negative	Negative
Triphenyl-tetrazolium chloride reduction	Positive	95	77	Negative	90
Undoxyl acetase	Positive	Positive	Positive	Positive	Positive
Pyrazinamidase Activity	Negative	Negative	Not determined	Not determined	Positive
Resistance to nalidixic acid (30 μg disc)	14-25†	0-17†	Negative	Negative	Negative
Resistance to cephalotin (30 μg disc)	83-100†	72-100†	Positive	0-22†	95
Metronidazole resistance‡	92	Positive	Positive	Negative	70
Carbenicillin resistance‡	Positive	Positive	Positive	Negative	65
Cefoperazone resistance‡	Positive	Positive	Positive	Negative	Positive
5-fluorouracil resistance‡	Positive	Positive	Positive	Negative	90
G + C content (mol%)	29-31	28-29	29-31	28-29	30-33

Numbers in columns indicate per cent positive. It should be noted that numbers of strains varied between researchers and so per cent values are not necessarily comparable. Sources: On *et al.*, 1996; Schroeder-Tucker *et al.*, 1996; Atabay *et al.*, 1998; Jacob *et al.*, 1993; Barrett *et al.*, 1988; Harrab *et al.*, 1998; Vandamme & DeLey 1991; Vandamme & Goossens, 1992. * = Tests for *Arcobacter* and *Campylobacter* spp. were at 25°C and 37°C respectively unless stated otherwise. † = Variation between sources of information. ‡ = Tested on blood agar medium.

1.6. SOURCES OF TRANSMISSION.

1.6.1. CONSUMPTION OF CONTAMINATED DRINKING WATER.

It is suspected that water sources are the major reservoir for *A. butzleri*, which is the strain that has been most commonly associated with illness. Evidence supporting this theory was provided by Kiehlbauch and co-workers (1991a) who observed that in 63% of patients suffering with *A. butzleri* associated illness all

were linked with the consumption or contact of possibly contaminated water and exposure was usually related to travel. It also appears that *Arcobacter* spp. are extremely common in developing countries due to inadequate water supplies, as 16% of *Campylobacter*-like isolates obtained from Thai children with diarrhoea were identified as *A. butzleri* (Taylor *et al.*, 1991). Furthermore, *A. butzleri* have been isolated from unchlorinated water supplies such as the canal waters of Bangkok (Dhamabutra *et al.*, 1992). From this source 1,666 isolates of *Campylobacter*-like organisms were detected of which 74 were classified as *Arcobacter* spp. and 42 as *A. cryaerophilus*-like organisms. *A. butzleri* along with other faecal bacteria were also isolated from unchlorinated well water which was associated with a gastroenteritis outbreak among girl scouts at a camp in the United States (Rice *et al.*, 1999). Also, drinking water treatment plants have isolated strains of *Arcobacter,* which have the same serotype as human isolates. For example, *A. butzleri* (80%, n = 147) has been detected in drinking water reservoirs (Jacob *et al.*, 1993) and treatment plants (Jacobs *et al.*, 1998) of the former Deutsche democratic republic (DDR). This was further supported by Assanta *et al.* (2002) who observed that *Arcobacter* cells could easily attach to all types of water distribution pipes (i.e. stainless steel, copper and plastic). *A. butzleri* cells were detected on the different piping material after only one hour, but the number of attached cells was dependent upon exposure time and temperature (4°C and 20°C). *A. cryaerophilus* and *A. butzleri* have also been isolated from sewage with average values of 5639 and 188-959 cells/100 ml respectively being detected in incoming raw sewage (Stampi *et al.*, 1993, 1998). The presence of *Arcobacter* species in water should not cause any problems as Rice *et al.* (1999) stated that *Arcobacter* species were sensitive to chlorine inactivation, and showed a greater sensitivity to BrCl and pure oxygen treatment. However, these results contradicted those of Wesley (1996), who showed that *A. butzleri* was capable of surviving and even growing in chlorinated and non-chlorinated distribution water. In addition, some strains showed resistance to ClO_2 (2-4 ppm, Stampi *et al.*, 1993). Therefore, after certain treatments 7-13/100 ml of *A. butzleri* have been detected in outgoing disinfected effluent, suggesting that they may present a serious health risk. Many isolates from river water have been examined for biotype, serotype and putative virulence characteristics (Musmanno *et al.*, 1997). The results showed a phenotypic heterogeneity of *Arcobacter* isolated from

environmental sources and indicated that some strains could be potentially virulent.

Arcobacter species have also been isolated in significant numbers from activated sludge, making up 4% of all the cells examined (Snaidr *et al.*, 1997). This was supported by Stampi and co-worker (1998) who isolated *A. butzleri* in 41% (n = 22) of digested sludge samples and 80% of activated and thickened sludges (see Table 1.4, pp 32-33). Substantially lower (22.7%, n = 22) counts of *Campylobacter* species (*C. jejuni* & *C. coli)* were detected but only in the primary sludge which was exposed to very little treatment. The difference in values may be due to *Arcobacter* being aerotolerant and able to grow at lower temperatures than *Campylobacter.* From these results it was suggested that the application of sludge to the land may provide a means of *Arcobacter* infection in animals and humans via runoff into water courses. This could explain why the predominant serotype in water sources was serotype 1, which corresponds with the serotypes isolated from poultry (Lammerding *et al.*, 1996; Marinescu *et al.*, 1996) and clinical samples (Vandamme *et al.*, 1992b), although serotype 2, 17 and 19 were also isolated (Jacob *et al.*, 1998).

1.6.2. *ARCOBACTER* OCCURRENCE ON POULTRY AND MEAT PRODUCTS.

Arcobacter spp. have also been associated with various food samples especially meat products mainly poultry (Festy *et al.*, 1993; De Boer *et al.*, 1996; Houf *et al.*, 2001b). In France *A. butzleri* was recovered from 81% (n = 201) of poultry carcasses (Marinescu *et al.*, 1996), approximately 50% were serotype 1 which along with serotype 5 are believed to be primarily linked with human infection (Lior & Woodward, 1993). This was also observed in Canada where 97% (n = 125) of carcasses from five different processing plants were contaminated with serotype 1 (Lammerding *et al.*, 1996). As well as this, *A. butzleri* has been cultured from retail purchased whole and ground chicken and turkey samples (Lammerding, 1997). Furthermore, a 100% of chicken carcasses tested by Atabay *et al.* (1998) were infected, with *A. cryaerophilus, A. skirrowii* and *A. butzleri.* This was the first report to isolate *A. skirrowii* from field samples. Many other groups such as Manke *et al.*, 1998; Wesley and Baetz, 1999 and Gonzalez *et al.*, 2000 have investigated the prevalence of *Arcobacter* species on poultry samples. The incidence rate ranges

from 0 to 100%, these variations can be attributed to many facts, such as hygienic conditions on the source farm and processing plant, bias in plant selection or simply due to the sensitivity of the isolation methods (Wesley & Baetz, 1999). These results are summarised in Table 1.4 (pp32-33).

The level of *Arcobacter* contamination on chicken carcasses is extremely high with reports stating several thousand *Arcobacter* cells were isolated per gram of neck skin (Antolin *et al.*, 2001; Houf *et al.*, 2001b, 2003). Although *Arcobacter* species are frequently isolated from chicken carcasses they are rarely recovered from the intestinal contents or faeces of chicken. As Wesley and Baetz (1999) only isolated *Arcobacter* species from 15% (n = 407) of cloacal swab and that experimental infections indicate that *Arcobacter* species were unable to colonise the poultry intestinal tract. These results were supported by Harrab *et al.* (1998) and Houf *et al.* (2002a) who were unable to isolate any *Arcobacter* species from chicken intestinal tract samples (n = 170 & 30 respectively). Thus suggesting that chickens may not be a natural reservoir for *Arcobacter* species especially as poultry have a body temperature of 42°C, which is less favourable for *Arcobacter* growth (Ridsdale *et al.*, 1999). Therefore, Wesley and Baetz, (1999) suggested that carcasses become contaminated during the slaughter process or that the isolation procedures used are not suitable for recovery of this organism from this matrix (Corry & Atabay, 2001).

Subsequently Houf *et al.* (2002a) investigated the occurrence and distribution of *Arcobacter* species in poultry processing. They observed that after evisceration and chilling respectively of 16 broiler flocks, 96.2% and 95% (n = 480) of the samples tested were positive for the presence of *Arcobacter* species. These results compared to 55% and 46% of the samples being positive for the presence of *Campylobacter* species. Thus suggesting that *Arcobacter* species may actually be a greater health risk from contaminated food than *Campylobacter* species which are well-established food-borne pathogens. *Arcobacter* species were also recovered from the surfaces of almost all processing machines, scalder water and transport crates, even after a 3 day period of no slaughter activity. Therefore, *Arcobacter* species were able to survive adequately in this environment. This topic will be discussed in more detail in Section 1.10.1. Due to these results Houf

et al. (2002a) suggested that *Arcobacter* may be indigenous in poultry slaughter houses, causing a constant level of contamination during processing or that these organisms live in and/or on poultry. However, Manke *et al.* (1998) suggested that a turkey processing plant may have multiple contamination sources due to the diversity of DNA patterns detected from the field isolates. This heterogeneity among *Arcobacter* isolates was also shown by Houf *et al.* (2003), who studied *Arcobacter* poultry isolates from a slaughterhouse by molecular characterization. One hundred and fifty nine *A. butzleri* and 139 *A. cryaerophilus* types (n = 1,079 isolates) were isolated on more than one sampling site and day. Unfortunately, this characterization study was unable to clarify the routes of transmission. Nevertheless, this study did show that the slaughter equipment was contaminated with *Arcobacter* before the onset of slaughter, but the authors did not believe that the equipment alone could be responsible for the high levels of *Arcobacter* on the poultry products. Processing water was suggested as causing the contamination of both equipment and carcasses, especially as *Arcobacter* species were present in processing water before the onset of slaughter. However, the types present in water were different from those on the poultry products. Furthermore, no data has been obtained which shows that *Arcobacter* are part of the chickens natural flora (Wesley & Baetz, 1999; Corry & Atabay, 2001; Houf *et al.*, 2002a, 2003). Due to these facts Houf *et al.* (2003) suggested that further studies are required to clarify the roles of processing water and broiler carcasses as potential sources of *Arcobacter* contamination. Therefore, the origin of the contamination still remains unknown and no information is available to date on the natural habitat of the organism. In addition, little is known about the occurrence of *Arcobacter* species at different stages of slaughter or about how the processing procedure may affect their prevalence.

Arcobacter species have also been associated with pork products due to its recovery from healthy pigs, the susceptibility of neonatal piglets to infection and pigs having an internal body temperature of 39°C, which would support the growth of most *Arcobacter* species. Also 0.5% of all pork samples purchased in the Netherlands contained *Arcobacter* species (De Boer *et al.,* 1996) whereas between 5 to 90% of samples tested from slaughter houses in Iowa were contaminated (Collins *et al.*, 1995). Similar prevalence rates of *Arcobacter* in pig

products (0-68%, n = 200) were detected by Ohlendorf and Murano (2002a) when they performed a geographical survey of *Arcobacter* species in ground pork (see Table 1.4, pp32-33). These reports can be compared with a 1.5% prevalence rate of *Campylobacter* reported in ground pork (Palumbo *et al.*, 1986; Norcross *et al.*, 1992), which again shows that *Arcobacter* have considerably higher prevalence rates compared to *Campylobacter*. In addition, Ohlendorf and Murano (2002a) observed that low fat pork (20%, n = 50) had a higher prevalence of *Arcobacter* species compared to high fat pork (6%, n = 50). The contamination is possibly due to the extra handling of the meat and that *Arcobacter* thrive on amino acids which are more abundant in leaner pigs. These results show a potential health risk as current trends demonstrate that consumers are purchasing more lean meats. This high contamination rate in pork products is believed to be due to *Arcobacter* species being increasingly isolated from healthy pigs with Wesley (1997) observing a detection rate of 40% (n = 1102) from faecal samples. Furthermore, a recent farrow-to-finish survey (Hume *et al.*, 2001) showed that incidences of *Arcobacter* positive pigs increased progressively with age, resulting in all the pens in the finishing barn containing *Arcobacter* positive faeces. Prolonged exposure and subsequent re-infection by the organism from contaminated water, handlers, birds, other animals via faecal shedding and transport vehicles have all been suggested by Hume and co-workers (2001) as possible reasons for this occurrence. However, the negative results may not be entirely due to *Arcobacter* negative animals, as difficulties in isolation of *Arcobacter* species and the possibility of intermittent faecal shedding which has been previously reported for *Campylobacter* (Achen *et al.*, 1998) may have occurred.

Arcobacter species have also been isolated from beef, but beef is less contaminated compared to pork and chicken, with only 1 out of 68 mince beef samples (1.5%) being positive for *Arcobacter* (De Boer *et al.*, 1996). However, *Arcobacter* species have been reported to exist in the guts of healthy cattle (Wesley *et al.*, 2000) with 14.3% (n = 1,682) of individual dairy cattle faecal samples being positive for *Arcobacter* species (see Table 1.4, pp32-33). This value compares to a recent survey performed by Golla *et al.* (2002) who detected an incidence rate of 9% (n = 200) from cattle faecal samples.

These reports show that poultry and red meat are considered significant reservoirs of *A. butzleri.* However, to date food has not been directly linked with *Arcobacter* infections in humans, but these bacteria cause disease in animals and are frequently isolated from meat products. Therefore, it is believed that *Arcobacter* species are probable food-borne pathogens, but their role in illness and their prevalence are not yet clear.

1.6.3. PERSON TO PERSON CONTACT.

This type of transmission is believed to be the least common. To date the only reported outbreak was in 1983 at an Italian nursery and primary school, in which recurrent abdominal pains without diarrhoea occurred involving 10 children (Vandamme *et al.*, 1992b). Only *A. butzleri* was isolated from the faeces of all cases, but once the illness had subsided *A. butzleri* strains were no longer recovered. SDS-PAGE of whole cell proteins and cellular fatty acid analysis and DNA hybridisation with *Arcobacter* reference strains were used to confirm the organisms identity (Vandamme *et al.*, 1992b). Furthermore, the results showed that the outbreak strains all belonged to serotype 1 and had identical protein profiles and phenotypic characteristics, thus suggesting an epidemiological relationship. The timing of the cases suggested a person-to-person transmission and it is probable that a teacher was the source of the infection as it was later discovered she suffered with similar symptoms at the time of the outbreak. In addition, it is possible that like *Campylobacter, Arcobacter* species can be contracted from domestic animals especially puppies and kittens suffering with diarrhoea (Skirrow, 1994).

Due to the limited knowledge on *Arcobacter* species and the lack of culture techniques for environmental and animal samples, the precise route of transmission of *Arcobacter* from animals to humans is unknown, as no epidemiological study has been performed to date. However, circumstantial evidence points to both water and meat products as being major reservoirs. It is possible that the number and variety of potential *Arcobacter* sources have not been discovered. Therefore, the opportunities for transmission may be great resulting in many unidentified routes of infection. Nevertheless, with the development of molecular typing techniques, which are able to discriminate

between different strains, the spread of *Arcobacter* and sources of outbreaks may finally be determined.

Table 1.4: Incidence of *Arcobacter* in animals and animal products (Modified from Mansfield and Forsythe, 2000).

Source	Per cent	N	Site	Isolation/Detection method	Reference
Pigs	51	86	Stomach	EMJH P-80 enrichment and selective plating verified by PCR.	Suarez *et al.*, 1997
	0-20	595	Cecal contents	Enrichment & selective plating, Biochemical analysis.	Harvey *et al.*, 1999
	16.8	410	Faeces	Enrichment in EMJH P-80 followed by m-PCR.	McKean *et al.*, 1996
	40.4	952	Faeces	PCR using 16S rRNA DNA probes specific for *Arcobacter* species.	Harmon & Wesley, 1996
	45.7	1102	Faeces	PCR using 16S rRNA DNA probes specific for *Arcobacter* species.	Harmon & Wesley, 1997
	36.4	55	Faeces	Selective enrichment & plating.	Hume *et al.*, 2001
	24.3	74	Preputial fluid, boars	Hybridization with 16S rRNA probes for *Arcobacter* species.	De Oliveira *et al.*, 1999
	82	Not stated	Aborted foetus	EMJH P-80 selective plating.	Ellis *et al.*, 1977
	43	30	Aborted foetus	Direct or filter plating onto selective media confirmed by RFLP.	Schroeder-Tucker *et al.*, 1996
	47	400	Aborted foetus	m-PCR using primers for 16S rRNA genes of *Arcobacter* species.	Wesley, 1997
	41.8	55	Aborted foetus	CAT enrichment & filtered onto non-selective blood agar plates.	On *et al.*, 2002
	22	214	Slaughterhouse foetus	m-PCR using primers for 16S rRNA genes of *Arcobacter* species.	Wesley, 1997
	0-89.9	299	Ground pork	Enrichment & selective plating verified by hybridisation.	Collins *et al.*, 1995; 1996b
	0.5	194	Ground pork	Enrichment & plating onto semi-solid medium.	De Boer *et al.*, 1996
	3.7	27	Ground pork	Enrichment & selective plating.	Zanetti *et al.*, 1996
	0-68	200	Ground pork	JM enrichment & selective plating.	Ohlendorf & Murano, 2002a
Cows	10.52	1236	Faeces	PCR using 16S rRNA DNA probes specific for *Arcobacter* species.	Harmon & Wesley, 1996
	14.3	1682	Faeces	Enrichment in EMJH P-80 followed by m-PCR.	Wesley *et al.*, 2000
	1.5	68	Minced beef	Enrichment & plating onto semi-solid medium.	De Boer *et al.*, 1996
	4.9	61	Minced mixed pork/beef	Enrichment & plating onto semi-solid medium.	De Boer *et al.*, 1996
Poultry	96.8	125	Chicken carcases	Enrichment & filtering onto selective plating.	Lammerding *et al.*, 1994
	81	201	Chicken carcases	EMJH P-80 enrichment & selective plating.	Marinescu *et al.*, 1996
	100	25	Chicken carcases	Enrichment & filtering onto selective plating followed by a probabilistic identification scheme and protein profiling.	Atabay *et al.*, 1998

Table 1.4 continued

	84	50	Chicken carcases	JM enrichment & selective plating.	Johnson & Murano, 1999a
	96.3	480	Chicken carcases	Enrichment & selective plating.	Houf *et al.*, 2002a
	95	44	Chicken	SDS-PAGE.	Atabay, 2003
	0	25	Chicken caeca	Enrichment & filtering onto selective plating followed by a probabilistic identification scheme and protein profiling.	Atabay *et al.*, 1998
	15	407	Chicken cloaca	Enrichment in EMJH P-80 followed by m-PCR.	Wesley & Baetz, 1999
	53	96	Chicken portions	Selective enrichment & plating verified by PCR.	Gonzalez *et al.*, 2000
	90-100	105	Neck skin/ carcasses	Direct plating on selective media.	Houf *et al.*, 2001 & 2002
	24.1	220	Turkey meat	Enrichment & plating onto semi-solid medium.	De Boer *et al.*, 1996
	77	395	Turkey meat	EMJH P-80 enrichment filtered onto BHI media verified by PCR-based fingerprinting.	Manke *et al.*, 1998
	97	125	Poultry Carcasses	Enrichment & filtering onto selective plating.	Lammending *et al.*, 1996
	0	57	Eggs	Enrichment & selective plating.	Zanetti *et al.*, 1996
Ducks	50	10	Carcasses	Selective enrichment & filtered onto mCCDA plating.	Ridsdale *et al.*, 1998
	52	170	Carcasses	Preston enrichment & selective plating.	Harrab *et al.*, 1998
	70	10	Carcasses	m-PCR.	Atabay *et al.*, 2001
	25	4	Caeca	Preston enrichment & selective plating.	Harrab *et al.*, 1998
	0	170	Caeca	Selective plating incubated at 35°C or 42°C, confirmed by ribotyping.	Anderson *et al.*, 1993
Monkeys	14*	268	Faeces	Enrichment and selective plating, most probable number assay and dry weight.	Stampi *et al.*, 1998
Sewage	100	15	Sewage	Filtered onto non-selective plates, confirmed by DNA-DNA hybridisation and biochemical tests.	Taylor *et al.*, 1991
	22.7	88	Primary sludge	Enrichment and selective plating, most probable number assay and dry weight.	Stampi *et al.*, 1993
	40-80	22	Activated, thickened & digested sludge	Enrichment and selective plating, most probable number assay and dry weight.	Stampi *et al.*, 1998
	4	Not stated	Activated sludge	*In situ* hybridisation and PCR using 16S rRNA DNA probes.	Snaidr *et al.*, 1997
Humans	2.4	631	Thai children, faeces	Direct selective plating and filtering onto non-selective agar plates.	Lauwers *et al.*, 1996
	<0.1	21527	Faeces	Selective plating incubated at 35°C or 42°C, confirmed by ribotyping.	Anderson *et al.*, 1993
	0.15	1376	Faeces	Selective plating followed by phenotypic characterization, protein profiling and 16S rRNA PCR.	Engberg *et al.*, 2000
	<0.1	2893	Faeces	Enrichment and selective plating.	Tompkins *et al.*, 1999

* = Tested only for *A. butzleri.*

1.7. CLINICAL ASPECTS.

1.7.1. ANIMAL INFECTIONS RELATED TO *ARCOBACTER* SPECIES.

Since the 1970's *Arcobacter* organisms have been associated with enteritis and abortion of farm animals. *A. cryaerophilus* has been isolated from sporadic cases of ovine abortion (Kirkbride *et al.*, 1985; Vandamme *et al.*, 1992a). This infection is usually caused by ingestion of food contaminated by wild birds and domestic stock, untreated water and other infected ewes and their products of parturition. Once ingested bacteraemia occurs leading to *Arcobacter* colonising the placenta resulting in placentitis. However, no sign of illness is detected in the ewe except some genital discharge may be visible and increased faecal shedding. If infection occurs during the last few weeks of pregnancy the foetus dies, but is not aborted until near the end of gestation period. If abortion occurs near the beginning of the pregnancy, invasion by putrefactive bacteria occurs resulting in uterine sepsis and septicaemia, which usually leads to death of both the ewe and foetus.

In pigs *Arcobacter* associated abortion and stillbirths are very common with *Arcobacter* species being isolated from 43% of all abortions (Ellis *et al.*, 1978; Wesley *et al.*, 1993). This value is comparable to the incidence rate (41.5%, n = 55) detected in the recent survey conducted by On *et al.* (2002) on porcine abortions in Denmark. *A. cryaerophilus* is the species that is most commonly associated with abortions in pigs, with serotype 1A and 1B being equally distributed (De Olivera *et al.*, 1999). Nevertheless, *A. butzleri* and *A. skirrowii* have also been recovered from foetal kidneys and liver (Vandamme *et al.*, 1992a). These results were supported by Wesley and Baetz (1995) who experimentally infected caesarean and colostrum-derived piglets with *A. butzleri*. This organism was shed in the pigs faeces until necropsy occurred, at which time *A. butzleri* was isolated from the ileum, liver, kidneys and brains. It is also plausible that *Arcobacter* species may be connected with gastric ulceration in pigs due to the close relationship with *Helicobacter pylori* which causes gastric ulceration in humans (Wesley *et al.*, 1996; Suarez *et al.*, 1997). The significance of *Arcobacter* is questionable as they have been isolated from aborted (50-83%) and normal porcine (6-18%) foetuses, sows with reproductive problems as well as clinically

healthy specific pathogen-free pigs (Neill *et al.*, 1979; Wesley *et al.*, 1995; Suarez *et al.*, 1997; De Oliveira *et al.*, 1997). Furthermore, *Arcobacter* species can live within the digestive tract of pigs and cattle without causing outward signs of clinical illness or discomfort to the animals (Hume *et al.*, 2001), the only true evidence to support that *Arcobacter* species are pathogenic is based on the fact that they are more frequently recovered from aborted pig litters and infertile sows with vaginal discharge than from healthy animals. Also, due to the antigenic similarity of *Arcobacter* isolates from reproductively impaired and normal sows it is believed that these particular organisms are opportunistic pathogens, which colonise the foetus after placental damage (De Oliveria *et al.*, 1997).

A. cryaerophilus and *A. skirrowii* have also been isolated from aborted bovine fetuses (Vandamme *et al.*, 1992a). However, with cattle the bacteria are usually carried by asymptomatic bulls. *C. fetus* subsp. *venerealis* and *Arcobacter* species have frequently been isolated from the mucous membrane of the prepuce glands, the penis and occasionally from the urethra. In cattle abortion is common, and also results in the cows becoming infertile for approximately 10 months (Ware, 1980). A survey in the USA (1960's) indicated the importance of vibriosis, these infections were later shown to be caused by *Campylobacter* and *Arcobacter* (Skirrow, 1994). Following an infection 4% of cattle failed to reproduce each year resulting in losses of 167 million dollars per year (Bryner, 1975). Even though this data is not recent it demonstrates clearly the importance of these pathogens. From this research it was observed that infected animals produce humoral antibody IgG and IgM in serum and IgA in cervico-vaginal mucus or preputal sections. These immunogenic factors could be used to produce vaccines, which would be effective for the prevention and treatment of infected cows and bulls (Skirrow, 1994).

A. cryaerophilus has also been isolated from naturally infected rainbow trout (Aydin & Engin, 2002). Due to this, Aydin and Engin (2002) undertook experimental infection studies, which caused the trout to die with gross clinical abnormalities. These included exophthalmia, liver damage, bloody hemorrhagic kidney and heart, and swollen intestines. Therefore, the results demonstrate that *A. cryaerophilus* could be a significant pathogen for rainbow trout.

Further studies have isolated *Arcobacter* species from aborted equine fetuses, from bulls and cows with mastitis or diarrhoea as well as clinically healthy cows, poultry, monkeys, raw red meat, ground pork and water. The incidence rates of *Arcobacter* species in animals and meat sources are given in Table 1.4 (see pp32-33). From this evidence it can be suggested that *Arcobacter* species are important veterinary pathogens causing a considerable financial loss to farmers each year due to abortion, infertility and illness.

1.7.2. HUMAN INFECTIONS RELATED TO *ARCOBACTER* SPECIES.

Campylobacter species are the most frequently isolated organism from humans with diarrheal illness (Ketley, 1997; Allos, 2001). This was shown in the infectious intestinal disease survey of England which isolated *Campylobacter* spp. from 12.2% (n = 2893) stool samples, compared to 5.0% and 0.8% for *Salmonella* spp. and *Shigella* spp. respectively (Tompkins *et al.*, 1999). However, because of the morphological and biochemical similarities between *Arcobacter* and *Campylobacter* species, incorrect detection and identification may occur due to the lack of standardised isolation methods for *Arcobacter* species. This leads to a substantial underestimation of the true incidence of *Arcobacter* species in food commodities, animals and human illness (Harrab *et al.*, 1998; Manke *et al.*, 1998). Therefore, it is believed that many *Campylobacter* outbreaks may actually be caused by *Arcobacter* species (Manke *et al.*, 1998). Due to this, it is strongly believed that *Arcobacter* species are human pathogens. *A. butzleri, A. cryaerophilus* and more recently *A. skirrowii* have all been isolated from the faeces of humans and animals with diarrhoeal related diseases (Wesley, 1997). *A. butzleri* is the most commonly reported human *Arcobacter* pathogen especially serotypes 1 and 5 which are most frequently isolated from contaminated water and poultry. Nevertheless, *A. skirrowii* may have been over-looked as an important pathogen due to it being such a slow growing organism (Mansfield & Forsythe, 2000). Yet, this particular species may be very virulent as it has haemolytic capabilities compared to the other *Arcobacter* species. The extent of illness caused by *Arcobacter* species is believed to be significantly underdiagnosed due to inappropriate isolation methods, growth conditions and inconclusive biochemical tests. Furthermore, most clinical laboratories do not routinely identify members of

the Campylobacteraceae family to the species level. Resulting in the true prevalence of *Arcobacter* remaining unknown.

Arcobacter infections in humans are common where such symptoms as persistent diarrhoea with abdominal pains, nausea, vomiting, fever, chills, malaise and occasionally septicaemia, bacteremia, endocarditis and peritonitis have been observed (Vandamme *et al.*, 1992a; Wesley, 1994). This was proven by an epidemiological survey where 29 patients in the US, from whom *A. butzleri* had been isolated, showed that 22 cases had reported severe or prolonged (> 1 month) diarrheal illness, with 51% associated with abdominal pain, 4 patients showed symptoms of bacteremia. Of these 29 patients, 4 people presented severe enough symptoms to be hospitalised. For most of these patients illness was associated with travel and consumption of possibly contaminated water (Kiehlbauch *et al.*, 1991a).

As well as this survey many case studies have observed *Arcobacter* associated illness, such as a 15-month isolation study of stools entering the university hospital in Brussels (Dediste *et al.*, 1998). During this period *Arcobacter* species was isolated from 18 patients (16 *A. butzleri*, 2 *A. cryaerophilus*), 14 of which were suffering from persistent diarrhoea, abdominal pain and nausea. Whereas the other 4 patients were asymptomatic carriers. The patients ranged in age from 22 months to 90 years old, but the highest prevalence was in very young children. Furthermore, for all 16 patients *A. butzleri* was detected as the sole enteric pathogen. An aerotolerant *Campylobacter* possibly *A. butzleri* was recovered from 2.4% of Thai children who were suffering from mild diarrhoea during a survey of paediatric gastro-enteritis (Taylor *et al.*, 1991). These particular strains were also isolated from 4 out of 9 Australian patients suffering with diarrhoea. The only link between these patients was they had all just returned from India or Indonesia. This strain has also been isolated in a similar European study. Thus suggesting that *Arcobacter* may be more common in developing countries (Tee *et al.*, 1987). *A. butzleri* has been isolated from a two-year-old girl (faecal specimen) with gastro-enteritis (Burnens *et al.*, 1992) using *Yersinia* selective CIN agar. During this study they also observed that *A. butzleri* could grow aerobically on primary isolation. In addition, *A. butzleri* has been isolated from a neonate with

bacteraemia (On *et al.*, 1995), but the mother showed no signs of illness at the birth. This is believed to be the first case where *A. butzleri* has been associated with bacteraemia. *A. cryaerophilus* was first isolated as the sole enteric pathogen from a 35-year-old homosexual man with AIDS suffering from loose stools. This species has also been recovered from a stool specimen of a patient suffering from intermittent diarrhoea for 4-6 months (Wesley, 1996) and from the blood of a 72-year-old uremic patient with haematogenous pneumonia (Hsueh *et al.*, 1997). Finally, the most recent case of *Arcobacter* bacteraemia was identified by Lau and co-workers (2002) using 16S rRNA gene sequencing. The 69-year-old female with no predisposing features was admitted to hospital with fever and acute gangrenous appendicitis. *A. butzleri* has previously been isolated from the abdominal contents or peritoneal fluids of three patients with acute appendicitis (Kiehlbauch *et al.*, 1991a). Even though the role of *Arcobacter* species in appendicitis remains unclear, the association of appendicitis with bacterial agents such as *Campylobacter, Yersinia* and *Salmonella* is well documented (Van *et al.*, 1991; Puylaert *et al.*, 1997).

Human enteritis associated with *Arcobacter* species occurs frequently in otherwise healthy people. However, a greater incidence rate is observed in patients with chronic underlying disease such as a 48-year-old male was hospitalised after 12 days of profuse watery diarrhoea up to 15 times a day with severe cramps. The patient had diabetes mellitus type 1 for 25 years with late complications. It is likely that he obtained his *Arcobacter* infection whilst visiting Thailand (Lerner *et al.*, 1994). Also a 52-year-old female was hospitalised after suffering for 3 weeks with fever, malaise, arthralgia and mild diarrhoea with severe abdominal pains. The patient was also suffering from severe alcohol abuse. Symptoms were only relieved by using antibiotics (Lerner *et al.*, 1994). This is expected as these patients were immunocompromised, therefore their immune system was depleted and unable to combat the infection.

Arcobacter species have been isolated from humans and have been associated with illness in both otherwise healthy individuals and patients suffering with a chronic underlying disease (Meng & Doyle, 1998). Nevertheless, information concerning its clinical significance and epidemiology is very limited. Therefore,

challenge studies on non-human primates would be important in determining the enteropathogenicity of these newly recognised organisms.

1.8. PATHOGENICITY FACTORS OF *ARCOBACTER* SPECIES.

Arcobacter species are potential emerging enteropathogens which are associated with enteritis and abortion. Hence, it is plausible that *Arcobacter* have putative virulence factors which enable them to invade their host and cause disease. However, very little information is known about these virulence factors with only 5 papers being published on *Arcobacter* pathogenicity to date.

Figura and co-workers (1993) were one of the first groups to study pathogenicity. They selected several *A. butzleri* river isolates and studied them for cytotonic, cytotoxic and cytolethal distending toxin effects on Vero and CHO cell lines. The results showed that some strains induced elongation of CHO cells and others adhered to cells *in vitro*. Thus suggesting some potential virulent strains. This work was studied further by Musmanno *et al.* (1997). This time 18 river isolates were examined for their ability to produce toxic effects *in vitro.* All the environmental strains except one were cytotoxic, whereby both cell lines (CHO and Vero) became rounded with nuclear pyknosis. Furthermore, 14 strains were able to induce this effect with less than a 1:10 dilution of broth culture filtrates. A cytotonic-like effect was also observed for one strain (8g). This resulted in elongation of CHO cells only. To determine if this elongation was due to a cytotonic or cytolethal distending effect the cells were incubated for a further 96 h, as cytolethal distending toxins (CDT) cause cell death after 3-4 days of exposure (Johnson & Lior, 1988). The cells were still viable after incubation, thus suggesting the presence of a cytotoxin. The production of a cytotoxin by *Vibrio parahaemolyticus* and *E. coli* has been linked with bloody diarrhoea, therefore it is plausible that cytotoxic *Arcobacter* strains may also have this ability.

Musmanno *et al.* (1997) also studied adhesion and invasive properties of these river water isolates using HeLa and INT407 cell lines. No bacteria were detected

inside the cultured cells, suggesting the lack of invasive properties. However, the strain (8g) which produced the cytotonic-like effect was able to associate with the surface of the cells, so may have adhesive mechanisms.

Invasive and toxigenic properties have also been studied by Fernandez and co-workers (1995). They worked on two strains of *A. cryaerophilus* isolated from aborted bovine fetus (strain 7625) and swine faeces (strain 62C) respectively. Hep-2 cell lines were used to test for invasion, whereas a rat ileal loop assay determined the toxic capacity of the strains. Both *A. cryaerophilus* strains caused distension of the ileal loop with fluid accumulation and increased electrolyte concentration; thus suggesting the presence of a toxigenic substance. Similar results were also observed in this model for toxigenic *C. jejuni* strains. In addition, both stains were isolated from inside the Hep-2 cells, suggesting that *A. cryaerophilus* strains have invasive mechanisms. This theory is supported by Wesley *et al.* (1995, 1996) who detected *A. butzleri* colonisation in the intestines of neonate piglets and On *et al.* (2002) who isolated arcobacters from the internal organs of over 40% of pig abortions in which established abortifacient pathogens were absent. Further illustration of the invasive potential of *Arcobacter* was reported by On *et al.* (1995) who described a case of neonatal bacteraemia caused by *A. butzleri* which was maternally acquired and Yan *et al.* (2000) who described an invasive *A. butzleri* diarrheal infection in a 60-year-old patient with liver cirrhosis.

Tsang *et al.* (1996) characterised another possible virulence factor in the form of a haemagglutinin that agglutinated human (blood group A and O), sheep and rabbit erythrocytes. The haemagglutinin is believed to be a 20 kDa protein as its activity was destroyed by heat (80°C) and proteolytic enzyme treatments. It was not present as pili nor fimbriae as in other adhesive bacteria such as *E. coli* or *Salmonella*. Further inhibition tests were performed to understand the nature of the red blood cell receptors. D-galactose inhibited the agglutination reaction, suggesting that *Arcobacter* cells can bind and cross-link to erythrocytes via a glycan receptor, which contains D-galactose as part of its structure (Tsang *et al.*, 1996). From this evidence it can be suggested that *Arcobacter* have virulence factors in the form of adhesive, invasive and toxigenic mechanisms.

Johnson and Murano (2002) investigated the ability of *Arcobacter* species to produce a cytolethal distending toxin (CDT), which is well documented in *Campylobacter* and *Helicobacter* species (Eyigor *et al.*, 1999; Young *et al.*, 2000). Bacterial sonic extracts or cell filtrates were applied to CHO, HeLa and INT407 cell lines. No toxicity was detected on the CHO cells. However, toxicity in the form of cell rounding, rapid death and inhibited growth was observed for INT407 cells and to a lesser extent HeLa cells. For all treatments toxicity was detected within 48 h, but some of the samples produced it in less than 24 h. The toxic effect on INT407 cells was even more pronounced than when these cells were treated with *C. jejuni* extracts. PCR using degenerate primers based on the *cdtA* and *cdtB* subunits was used to screen the *Arcobacter* strains from various sources. No CDT-specific fragments were amplified for any of the 26 *Arcobacter* isolates obtained from humans, poultry, water, and cattle, suggesting that CDT genes were not present in the isolates. These results were supported by the flow cytometry studies which showed that the mammalian cells behaved differently with the 'toxic substance' produced by *Arcobacter* compared to the toxic activity of CDT-producing organisms. Preparations from *C. jejuni* caused $\geq$ 80% of both HeLa and INT407 cells to be blocked in the G_2a phase of mitosis as shown by a higher percentage of cells possessing 4n DNA, which is indicative of cell cycle arrest. In contrast none of the *Arcobacter* isolates displayed the same blocking response. Therefore, these results support the notion that *Arcobacter* species produce a different 'toxic substance' to *C. jejuni* CDT. Additional evidence was provided by fractionation of the toxic component by dialysis with a molecular weight cut off of 10 kDa. This resulted in the loss of toxicity, so the toxic fraction was either smaller than 10 kDa or that endogenous enzymes have degraded the toxin(s). However, further experiments revealed that fractions with a molecular weight less than 10 kDa showed similar toxicity on HeLa and INT407 cells as described earlier. Whereas CDT from *C. jejuni* has a molecular weight greater than 30 kDa. Collectively these results show that *Arcobacter* species have the ability to induce toxicity to different mammalian cell lines. However, the toxic activity, mode of action and relative size is not related to *Campylobacter* CDT.

1.9. *CAMPYLOBACTER* SPECIES.

A comprehensive review of clinical aspects and pathogenicity of the related *Campylobacter* genus is presented as it forms the basis for comparative *Arcobacter* pathogenicity studies.

1.9.1. CLINICAL ASPECTS OF *CAMPYLOBACTER* INFECTIONS.

Over the last decade *Campylobacter* species have been identified as the major cause of bacterial related diarrhoea world wide (Leach, 1997; Wassenaar, 1997; Van Vliet & Ketley, 2001). Thirty thousand cases were reported in 1989 in the UK alone (Griffiths & Park, 1990; Ketley, 1995), which has almost doubled in 2001 to 56,592 cases, with late spring and early autumn showing a seasonal peak. However, recent data has started to show a steady decline with only 46,484 cases being reported in 2002. Nevertheless, *Campylobacter* species are still the most common cause of gastro-enteritis even greater than the number of *Salmonella* (14,607, 2002 data) and *Shigella* (692, 2002 data) cases together. Due to under-reporting it has been suggested that the ratio of *Campylobacter* cases in the community to the cases reaching national surveillance is 7.6:1 (Wheeler *et al.*, 1999). So the actual number of campylobacteriosis cases in 2002 was possibly closer to 350,000. Therefore, *C. jejuni* has proven to be a major public health problem especially in children and young adults, with infection often being derived from a range of food and water based environmental sources (Griffiths & Park, 1990; ACMSF, 1993). However, it is believed that many of these *Campylobacter* outbreaks may actually be caused by *Arcobacter* species (Manke *et al.*, 1998), especially as *Arcobacter* species have similar phenotypic and genotypic characteristics to *Campylobacter*.

C. jejuni enteric disease is very variable causing mild to severe inflammatory diarrhoea. The severe diarrhoea is characteristic of an invasive type of organism such as *Shigella dysenteriae*. This type of illness usually occurs in patients from industrialised nations. Whereas the mild watery diarrhoea which usually last 24 h is seen in developing nations (Butzler & Skirrow, 1979). This type of diarrhoea is

characteristic of an enterotoxin producing organism like *Vibrio cholerae*. The major symptoms caused by *C. jejuni* infection are acute abdominal pains that mimic appendicitis with fever, malaise and often mental confusion. The symptoms develop to profuse diarrhoea that tends to be sticky and often contains blood mucus and faecal leukocytes. Patients often suffer with headaches, muscle pain and nausea with vomiting occurring rarely. These symptoms are similar to those caused by *Arcobacter* species (Section 1.7.2). Bacteraemia especially in the early stages of infection is rarely reported, due to infrequent samples and inappropriate culture techniques, but it is believed to occur at a higher incident rate. The incubation period before symptoms occur is 1-7 days after ingestion of contaminated food or water (Leach, 1997). However, the source of infection and therefore, the exact timing is often difficult to establish (Ketley, 1995). The profuse diarrhoea tends to last 2-3 days, but the other symptoms last for 7-10 days. In approximately 25% of the cases a relapse occurs, but it tends to be less severe than the original attack. Nevertheless, patients may remain culture positive for several weeks after the clinical symptoms have finished (Ketley, 1997). Campylobacterosis is a self-limiting infection which is rarely treated with antibiotics.

Complications due to *Campylobacter* have also been observed which includes haemolytic uremic syndrome, septicaemia infection and reactive arthritis which occurs in 1% of patients 1-2 weeks after the onset of illness. Meningitis, recurrent colitis, acute cholecystitis and even less frequently Guillian-Barré syndrome (Skirrow & Blaser, 1995). This is a neurological complication that causes severe lengthy and occasionally fatal paralysis that has significant association with serological evidence of recent previous infection with *Campylobacter* species (Fujimoto *et al.*, 1992; Mishu & Blaser, 1993). Guillian-Barré syndrome will be discussed in more detail in Section 1.9.4.5.

Even though this organism causes such a high proportion of gastro-enteritis cases and can cause complications, its fatality rate is quite low with only 1 death per 1,000 cases. This is also related to the health of the patients as death rarely occurs in healthy individuals, but in association with immunocompromised individuals like cancer patients, HIV suffers or pregnant ladies (Gerba *et al.*, 1996).

1.9.2. PATHOGENICITY FACTORS OF *CAMPYLOBACTER* SPECIES.

Campylobacter species are considered to be pathogenic with an infectious dose as small as 500 to 800 cells, depending upon strain and host susceptibility (Black *et al.*, 1988). For *Campylobacter* to cause illness they must be ingested and pass through the stomach acid barrier. This usually occurs with food or liquids that act as buffers or are passed quickly through the stomach to the small intestine (e.g. milk or water). Once they reach the intestinal epithelium the initial infection occurs at the jejunum and upper ileum. To cause an infection, *Campylobacter* must first penetrate the protective mucous gel of the epithelium. This is achieved by *C. jejuni* having polar flagella, which provides motility through the viscous mucus causing mucosal colonisation and initiation of the disease. Whereby epithelial cells are damaged which causes a disruption of absorptive capacity of the intestine. In addition, inflammatory changes occur in the mucosa, with the formation of abscesses which have also been detected in *Salmonella* and *Shigella* infection (Skirrow, 1994). This condition may develop into bacteraemia as 0.15% of all cases have recorded the occurrence of this condition. However, the figures may be greater as its been proved that *C. jejuni* are able to translocate across the intestinal cells (Ketley, 1997; Harvey *et al.*, 1999).

Campylobacter species are able to infect and cause disease in the host due to many pathogenic mechanisms. However, the molecular mechanisms involved in pathogenesis are still poorly understood, with only a few pathogenic mechanisms being known or having a proven role. To date four major virulence properties have been described (Leach, 1997; Wassenaar, 1997). These include:-

- Motility
- Adherence
- Invasion
- Toxin production

As these mechanisms are not mutually exclusive any combination may have a role depending on the hosts status and attributes of the infecting strain. Since this project is investigating the cytotoxicity of human *A. butzleri* isolates, only bacterial toxins will be discussed further, with particular reference to toxins produced by *Campylobacter* species.

1.9.2.1. BACTERIAL TOXINS.

Williams and Clarke (1998) defined a toxin as 'any microbial product or substance that is harmful or lethal to cells, tissue culture or organism'. Bacterial toxins are virulence factors which alter the physiology of the host and/or of its defence mechanisms in such away it enables the bacteria to obtain nutrients resulting in bacterial proliferation and/or colonisation of the host. However, in doing so the bacteria cause damage to membranes or act intracellularly. Both of which cause symptoms in the host usually ranging from watery diarrhoea to dysentery with blood and mucus present in the stools. These symptoms are very variable as different strains of the same species often vary in their toxin producing capabilities with between 1-500 mgl^{-1} of toxin being secreted into the surrounding environment. To date, only a few bacterial toxins have been shown experimentally to influence pathogenicity even if they do produce harmful/lethal effects on tissue cultured cells. Only the toxins produced by *Campylobacter* will be discussed further as possible models for *Arcobacter* toxins.

Toxins are divided into 3 classes based on their modes of action to cause infection:

1. **Exotoxins** are produced intracellularly and exported out of the cell enabling it to subvert, alter or destroy host cell functions. The site of action for these secreted proteins is usually quite a distance from the site of infection. Therefore, only low concentrations are required to exert a specific effect (Arbuthnott, 1978; Williams & Clarke, 1998). This group is further subdivided into cytotoxins and enterotoxins.

2. **Cell-associated toxins** remain attached to the cell, such as outer membrane proteins, which are surface exposed. These toxins represent outer membrane structural entities which have key functions in the organism (Stephen & Pletrowski, 1986). This group mainly consists of cytotoxins, but a few enterotoxins have been described as cell-associated toxins.

3. **Endotoxins** are located inside the bacterial cell in the form of lipopolysaccharide (LPS) and are essential components of the outer membrane of Gram-negative bacteria. Due to LPS being anchored to the outer membrane there is very little release of the toxin into the surrounding environment during normal cell growth. Nevertheless, when exposed to phagocytic attack or antimicrobial therapy the cells become lysed and LPS is released. This results in a massive immune response, which can lead to septic shock or even death (Arbuthnott, 1978; Williams & Clarke, 1998). See Section 1.9.4. for more detail.

1.9.3. *CAMPYLOBACTER* SPECIES TOXINS.

Various groups have worked on numerous *Campylobacter* species to identify their toxic potential. However, considerable contradictory evidence has been produced in this area due to different laboratories using a variety of culture techniques, media, strains and tissue culture cells for toxicity testing. Recently, a few groups have attempted to collaborate their results based on the clinical symptoms induced by the isolates. It was suggested that the type of illness observed in the patient corresponds directly with the type of toxin that is being produced (Klipstein *et al.*, 1985; Wassenaar, 1997). Generally invasive cytotoxins are believed to produce inflammatory diarrhoea which usually contains blood and leukocytes. Whereas enterotoxin producing strains tend to produce more watery diarrhoea. Wassenaar (1997) summarized the current knowledge on the toxins produced by *Campylobacter* species and suggested that *C. jejuni* produce at least eight different toxins with one enterotoxin and seven cytotoxins. The toxins, which may relate to those produced by *Arcobacter* species will be discussed in more detail in the following sections.

1.9.3.1. CYTOLETHAL DISTENDING TOXIN (CDT).

Johnson and Lior (1988) first described a distinct and novel cytolethal toxin present in culture filtrates of *Campylobacter* species. It caused progressive distension where the cells become more rounded and appear to disintegrate and a 31% increase in cAMP was detected intracellularly after 24 h. Their membranes appeared to contain small blebs and nuclear staining showed either irregular chromatin condensation or nuclear fragmentation (Whitehouse *et al.*, 1998;

Mooney *et al.*, 2001). After 3-4 days cell death occurred with CHO, Vero, HeLa and Hep-2 and more recently with CaCo-2 cells, as the cells were unable to overcome cell cycle blocks, so they self-destruct. Its effect was only observed on freshly seeded or sparsely seeded cells with no distension or cell death occurring when the toxin was added to confluent cell monolayers. The degree of distension and/or sensitivity of these cell lines varies and a lack of significant distension did not mean that the cells were not sensitive to CDT, as CaCo-2 cells showed very little distension, but could no longer divide due to G_2 blockages. However, it was completely inactive against Y-1 adrenal cells and 3T3 fibroblasts. This new toxin was called 'cytolethal distending toxin' (CDT).

In certain cell lines (i.e. CHO) CDT produced similar morphological changes as the cholera-like enterotoxin, which were indistinguishable after 24 h. During the early experiments the only way to differentiate between the two types of toxin was to apply them to Y-1 cells. Further investigation showed that after 3-4 days the CDT treated cells became progressively distended with some cells (i.e. CHO, Vero, HeLa etc) being five times the length of normal cells and then die with less than 10% of the cells remaining viable. Whereas enterotoxin treated cells just become progressively extended, but did not die (>85% viable). Therefore, an incubation period of 96 h was proposed to discriminate between the cytotonic and cytotoxic activity (Johnson & Lior, 1988).

Johnson and Lior (1988) also observed that CDT production in *Campylobacter* species was very stable and continued to be excreted even after continuous subculture. The toxin was heat sensitive, but the degree of thermal inactivation varied from strain to strain. CDT was however, trypsin sensitive, non dialyzable, only neutralised by homologous rabbit anti toxin and had a molecular weight greater than 30 kDa as it was retained by a PM-30 ultrafiltration membrane.

To date the role in pathogenesis has not been determined, but the activity of CDT suggests that it could contribute to diarrheal disease as CDT is active on CaCo-2 cells (Whitehouse *et al.*, 1998), which are derived from human colonic epithelial cells. Therefore, CDT could have a similar effect on the crypts of the intestines, thus blocking the development of mature epithelial cells needed for absorptive

function, resulting in temporary disruption and erosion and hence watery diarrhoea. CDT may also have an invasive property, as proposed by Purdy *et al.* (2000) using insertional mutations of the *cdtB* gene in *C. jejuni* 81-176 and 11168. These mutant strains were unaffected in enteric colonisation of immunodeficient mice, but their invasiveness in blood, spleen or liver tissue was impaired compared to the control strains. Hence the possible role of CDT in *C. jejuni* invasion of mammalian cells.

1.9.3.2. HAEMOLYSINS.

Haemolysins are a range of bacterial toxins that lyse red blood cells using different modes of action. However, they are also toxic to other cells such as leukocytes, epithelial and connective cells. There are two types of haemolysin visualised on blood agar. The first is α haemolysis which produces green zones around the colonies, where the haemoglobin has been reduced to methemoglobin. Nevertheless, it is rarely observed and appears to be dependent on the pH of the media. Whereas β haemolysis has clear zones as the red blood cells have been lysed completely, which usually disappear after prolonged incubation (Misawa *et al.*, 1995). Haemolysins are split into 3 categories depending on their mechanism of action:

- Enzymatic such as *Clostridium perfringens* α toxin or lecithinase
- Pore-forming including the RTX toxin family
- Surfactant like *Staphylococcus aureus* delta (δ) toxin.

C. jejuni and *C. coli* are generally regarded as non-haemolytic (Smibert, 1984; Walker *et al.*, 1986; Akan *et al.*, 1998), but certain groups have identified specific strains mainly those from humans that were able to produce a haemolysin, which may be related to pathogenicity (Arimi *et al.*, 1990; Misawa *et al.*, 1995; Akan *et al.*, 1998). For example, Akan *et al.* (1998) found that 80% of *C. jejuni* (n = 10) and 60% of *C. coli* (n = 10) strains were haemolytic. Variations in haemolytic activity may be related to different growth conditions, temperature, erythrocyte type and gaseous environment. *Campylobacter* haemolysins may be cell-associated since contact haemolysis assays increased certain *C. jejuni* strains haemolytic ability. Parkhill *et al.* (2000) reported on the presence of contact

haemolysin genes being present in the genome of *C. jejuni* NCTC 11168. Furthermore, culture supernatants or bacterial lysates were always non haemolytic (Wassenaar, 1997). However, secreted haemolytic activities have also been reported to be produced by various *C. jejuni* and *C. coli* strains (Arimi *et al.*, 1990; Pickett *et al.*, 1992; Hossain, 1993; Tay *et al.*, 1995).

It has been suggested that haemolysis is a mechanism for iron acquisition by *C. jejuni* (Wassenaar, 1997). This theory is supported by Istivan *et al.* (1998) who observed that haemolysin was iron-regulated, with the haemolytic titre decreasing when surplus iron was present in the media. However, Pickett *et al.* (1992) detected no iron regulation which suggested that haemolysins are constitutively expressed. Therefore, haemolysins may be involved in other pathogenic mechanisms instead of or as well as iron uptake. Haemolysins may also be involved in survival mechanisms of *C. jejuni* against the hosts immune system. As their ability to lyse leukocytes and phagocytes enables virulent *Campylobacter* to be released into the cytoplasm.

1.9.3.3. 70kDa CYTOTOXIN/VERO-NEGATIVE CYTOTOXIN.

Wong *et al.* (1983) isolated a cytotoxin from culture filtrates of 11 *C. jejuni* isolates obtained from humans with gastro-enteritis. Cytotoxic effects were observed on HeLa, Hep-2, MRC-5 and CHO cells causing the cells to become rounded and die due to disruption of membrane integrity, but showed no reaction against Vero cells or other animal cell lines. Furthermore, toxicity was lost upon subculture (Pang *et al.*, 1987), storage, trypsin treatment and heating to 100°C. This suggested that the toxin was a protein, which may be associated with the bacterial cell membrane and secreted, with cytotoxic activity being detected in the LPS fraction of a bacterial lysate (Kawaguchi *et al.*, 1989). Kawaguchi *et al.* (1989) and Daikoku *et al.* (1990) performed solubilisation studies on this cytotoxin which suggested that the protein was produced intracellularly in the early log phase and as it entered into the stationary phase it was released.

Many other groups such as Goosens *et al.* (1985), Johnson and Lior (1986), Guerrant *et al.*, (1987) and Mahajan and Rodgers (1990) have identified a similar or identical cytotoxin. In most cases using such methods as column

chromatography the molecular weight of this toxin was estimated to be 50-70 kDa. Hence the name '70 kDa' cytotoxin. Several groups also identified that this toxin could not be neutralised by Stx-antitoxin or anti-*Clostridium difficile* toxins. The only down side to these investigations was that not every group performed cytotoxicity assays on Vero cell lines. Therefore, no direct comparisons or solid conclusions could be made about this particular toxin.

1.9.3.4. VERO/HELA CELL CYTOTOXIN/VERO POSITIVE CYTOTOXINS.

Klipstein and co-workers (1985) were the first to report on a toxin that was cytotoxic to Vero cells. Cytotoxicity and highly invasive properties on Vero and to a greater extent on HeLa cell lines were identified from 6 out of 20 *C. jejuni* isolates. This information was correlated with the clinical status of the patients and was observed that all 6 strains were isolated from patients with bloody invasive-type diarrhoea. Only a few other reports have been published on this particular toxin. One such report was by Johnson and Lior (1986) who used a trypan blue exclusion method to study morphological changes of CHO, HeLa and Vero cells after exposure to C. *jejuni* culture supernatant. The basic response of the cells was to become rounded after 24 h and die after a few days. In addition, Johnson and Lior (1986) performed neutralisation experiments using *E. coli* VT antitoxin and *Clostridium difficile* antitoxin, however both tests were unsuccessful. These findings were supported by Florin and Antillon (1992), who isolated 79 *C. jejuni* strains from children with diarrhoea and from chickens. They also observed that the cytotoxin was heat labile after exposure to 100°C for 10 minutes. However, this was contradicted by the work of Lee *et al.* (1998, 2000) who studied a cytotoxin which caused rounding and cell death to CHO, INT407, HeLa, J774 and Vero cells. The cytotoxin in the crude fraction was very stable as it remained toxic after storage at -20°C and -70°C for over 24 months and was heat stable at 100°C for 30 minutes. Nevertheless, cytotoxicity was lost at 40°C after only 4 weeks. It is believed that these cytotoxins were similar to each other, if not identical as heat stability is very variable due to differences in toxin purity (Wassenaar, 1997). Due to the lack of data on this particular toxin very little information is known especially on its modes of action.

1.9.3.5. ADDITIONAL *CAMPYLOBACTER* TOXINS.

A limited number of reports have also shown that shiga-like toxin (Moore *et al.*, 1988), hepatotoxin (Kita *et al.*, 1990, 1992) and cholera-like toxin (Ruiz-Palacios *et al.*, 1983; Klipstein & Engert, 1984a, b) have been produced by *C. jejuni* and some *C. coli* strains. However, inconclusive data and lack of any sequence homology to cholera-like toxin and shiga-like toxin genes in the *C. jejuni* NCTC 11168 genome (Moore *et al.*, 1988; Parkhill, *et al.*, 2000) implies that *Campylobacter* do not produce these particular toxins.

1.9.4. LIPOPOLYSACCHARIDE (LPS).

1.9.4.1. INTRODUCTION.

Approximately a 100 years ago Richard Pfeiffer and Robert Koch were working on *V. cholera*. They discovered a heat stable toxin, which they called endotoxin and subsequently 'lipopolysaccharide' (LPS). LPS form a family of toxic phosphorylated glycolipids found only in the outer membrane of Gram-negative bacteria including many pathogenic organisms such as *Campylobacter* and *Salmonella*. LPS acts as major surface antigens due to the presence of the O-antigen. The presence of the LPS in the outer leaflet of the outer membrane is extremely important, as it is responsible for the low membrane permeability to hydrophobic antibiotics, detergents and hydrophobic dyes. Due to LPS containing only saturated fatty acids the hydrocarbon interior of the monolayer causes the membrane to be less fluid-like than the phospholipid membrane. Hence, a rigid almost crystalline structure of the LPS hydrocarbon portion is produced which makes it very difficult for hydrophobic molecules to penetrate this membrane. Therefore, LPS has a barrier function and is important in protecting the bacteria. LPS has been shown to play a role in bacterial-host cell interactions in other pathogenic bacteria including enteropathogenic *E. coli, Neisseria gonorrhoeae* and *Salmonella enterica* serover Typhi. Furthermore, McSweegan and Walker (1986) suggested that LPS have adhesion properties thus aiding colonisation and infection of *C. jejuni*.

1.9.4.2. LPS HOST INTERACTIONS.

The presence of these glycolipids is extremely important in the host as LPS have antibodies and serum factor binding sites, which are necessary in the recognition

and elimination of bacteria through stimulating an immune response. Low doses of bacterial LPS have beneficial effects on the host resulting in immunostimulation and resistance to the particular infecting strain (Vogel & Hogan, 1990). However, large quantities of LPS are produced during infection. Once the cells die and lyse, usually after application of antibiotics, or by translocation from the gut the LPS may cause various pathophysiological reactions. These include fever, leukopenia, tachycardia, tachyprea, hypotension, disseminated intravascular coagulation and multiorgan failure. This in turn, may culminate in septic shock syndrome due to a massive release of cytokines. This is very dangerous for the host causing approximately 100,000 deaths annually in the U.S. alone (Nogare, 1991).

The host responses to LPS are not induced directly, but are mediated by immune modulator molecules, which are components of the cellular immune system. These include tumor necrosis factor α (TNFα), members of the interleukin family (IL-1, IL-6, IL-8, and IL-12), interferon α, reduced oxygen species and lipids. The immune modulator molecules are mainly released by monocytes/macrophages, but vascular cells, polymorphonuclear cells and T cells also participate in the LPS response. Each individual cell type reacts in a typical way, but in general these reactions are the production of mediators, phagocytosis, proliferation and/or differentiation (El-Samalouti *et al.*, 1999, see Figure 1.3).

For any of these cells to become activated by even minute amounts of LPS, it must first interact with specific LPS binding molecules. These are located on the surface of LPS responsive target cells. Many different LPS binding molecules have been described, but the physiological relevance of only a few have been demonstrated to date. The most prominent cell surface protein which has been shown to be involved in the activation of cells by LPS is the 55 kDa glycoprotein CD14. CD14 exists in two forms, firstly as a glycosyl-phosphatidylinositol (GPI) anchored membrane protein (mCD14) on monocytic cells, polymorphonuclear, leukocytes and on some B lymphocytes (El-Samalouti *et al.*, 1999). The second type is soluble (sCD14), whereby several forms 48, 53 and 55 kDa exist in concentrations of approximately 2-6 μg/ml in serum. This soluble form is either released by monocytes or secreted as GPI-free forms (Bazil & Strominger, 1991;

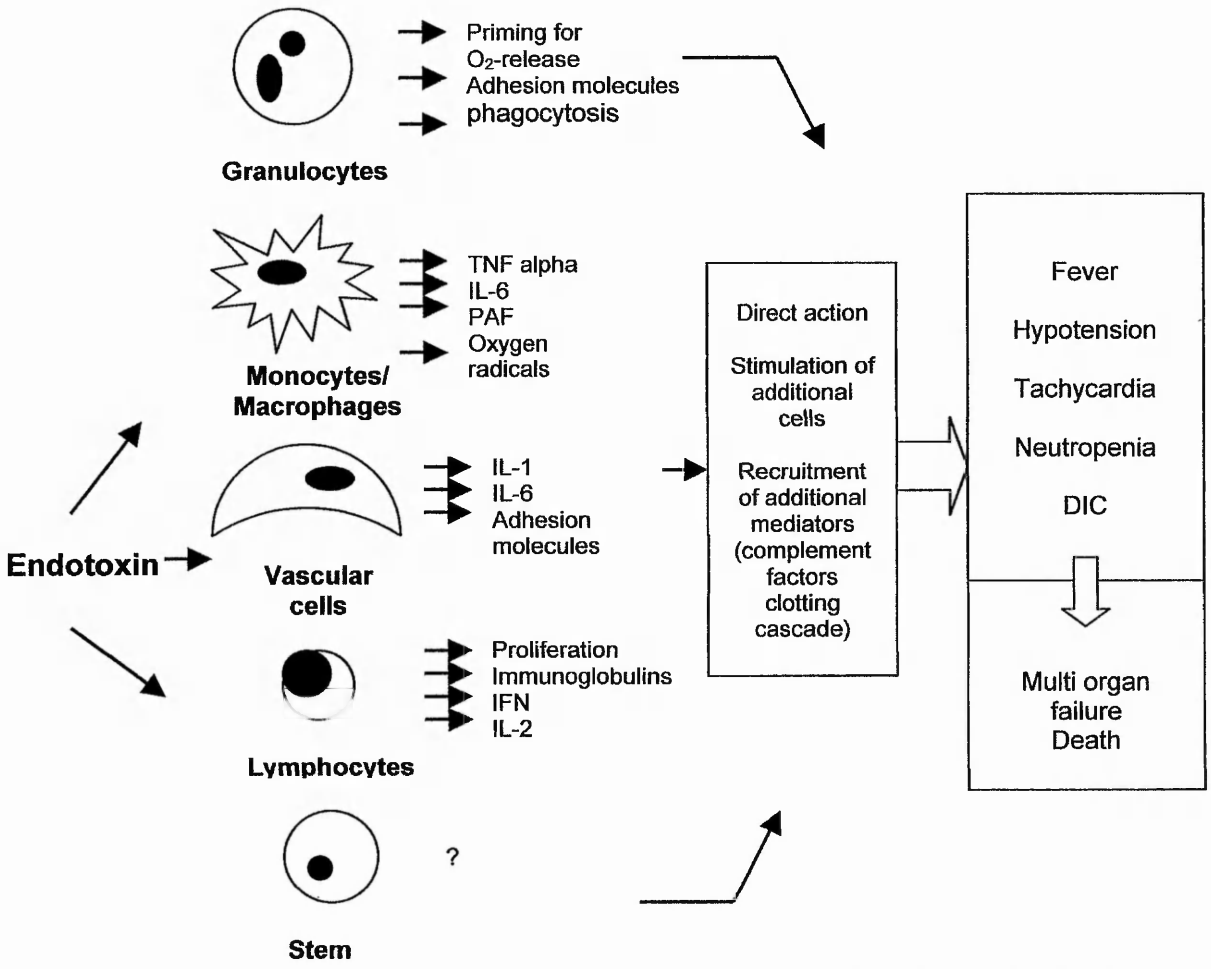


Figure 1.3: Schematic representation of the mode of action of endotoxin in the pathogenesis of septic shock. DIC, disseminated intravasal coagulation. Modified from El-Samalouti *et al.* (1999).

Durieux *et al.*, 1994; Bufler *et al.*, 1995). Binding of LPS to mCD14 on monocytes is very important as it stimulates these cells to produce and release the immune mediators. The binding between LPS and CD14 can be increased further by the presence of a serum component called LPS-binding protein (LBP) (Schumann *et al.*, 1990; Wright *et al.*, 1990). LBP is a 60 kDa glycoprotein that is usually present at a concentration of 5-10 μg/ml, but increases dramatically up to 200 μg/ml during acute phase response (Schumann *et al.*, 1990). This increase is important as LBP reduces the amount of LPS required to activate the monocytes as the LBP-LPS complexes are easily recognised by CD14 receptors.

Even though mCD14 is a key molecule enabling LPS to bind to monocytic cells, the mechanisms involved in cell activation is still unclear (see Figure 1.4). It is known that mCD14 lacks a transmembrane domain, so it is unable to transmit a signal through the membrane like a classical hormone receptor. Also Lee *et al.* (1993) demonstrated that the GPI anchor was not required for the activation of cells via mCD14. Therefore, it is believed that at least one accessory molecule is required to perform the signal transduction thus allowing LPS and mCD14 to function as a receptor. Reports have shown that Toll-like receptor 4 MD-2 complex (TLR4*MD-2) is able to trigger a transmembrane signal transduction response upon recognition of a LBP-LPS complex by mCD14. A yet unknown mechanism leads to the rapid and co-ordinated activation of various intracellular signalling pathways including the activation of the major MAP kinase cascade and translocation of NF-κB. This in turn, causes the cells to produce a large variety of bioactive protein mediators (i.e. TNF α), which initiates an acute phase response, that helps to eliminate the invading micro-organisms (Alexander & Rietschel, 2001). Available data also suggests the existence of direct contact between TLR4 and the lipid A domain of LPS during the course of cellular signal activation (Poltorak *et al.*, 2000, as quoted by Alexander & Rietschel, 2001). This information is supported by Lee *et al.* (1993) who showed that high doses of LPS caused monocyte stimulation to occur without mCD14, also the reaction could not be blocked by anti-CD14 MAbs.

Hailman *et al.* (1994) demonstrated that LPS can bind directly to sCD14, but the generation of LPS-sCD14 complexes are enhanced greatly in the presence of LBP even if it is not involved in the complex itself. LPS-sCD14 complexes enable the activation of some mCD14 cells such as endothelial cells, fibroblasts and smooth muscle cells to produce cytokines (Frey *et al.*, 1992). Therefore, Vita *et al.* (1997) proposed that a specific receptor for the LPS sCD14 complexes may occur on these CD14 negative cells which results in LPS activation. A 216 kDa protein developed by cross-linking experiments on the astrocytoma cell line U373 has been identified as a possible receptor. Nevertheless, it still remains unclear how this protein is involved with LPS signalling.

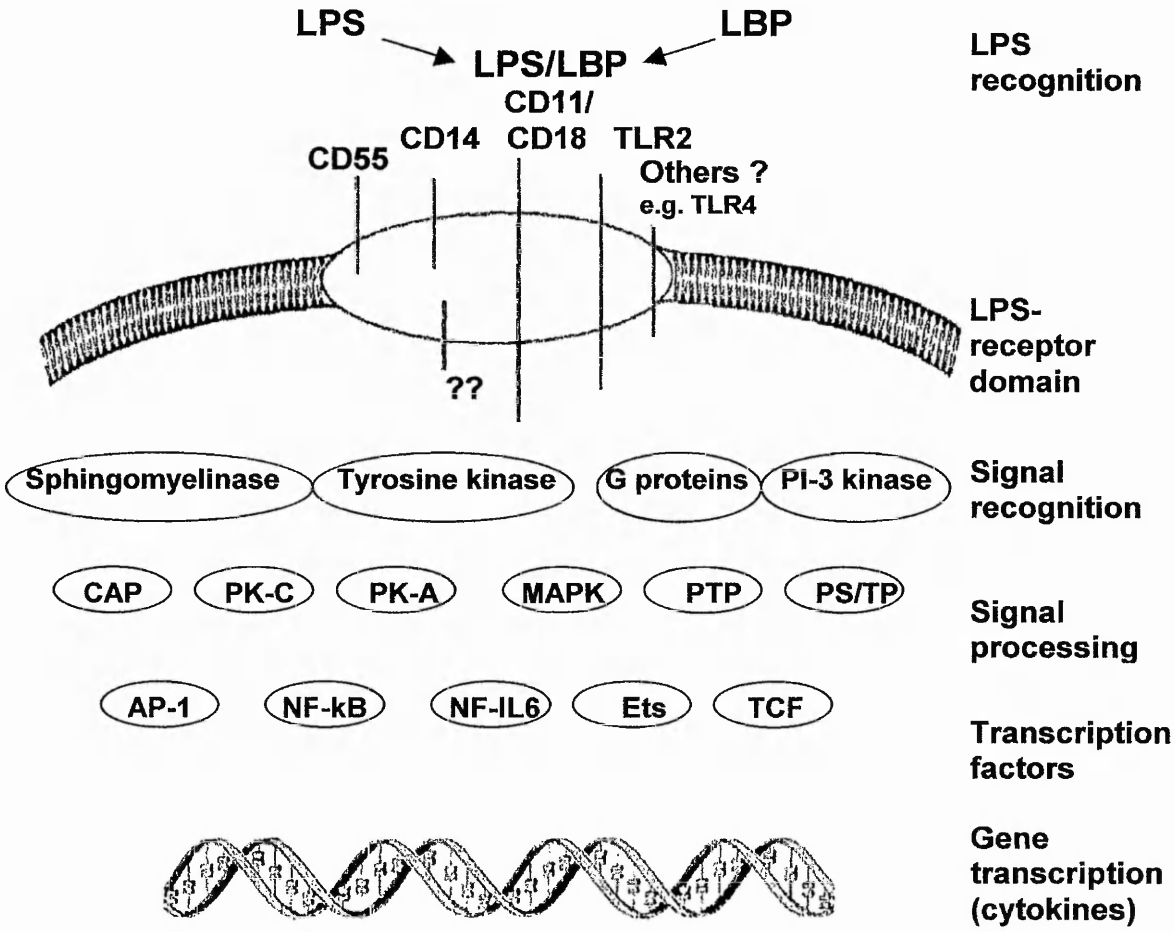


Figure 1.4: Endotoxin recognition and signalling in monocytes/macrophages. Modified from El-Samalouti *et al.* (1999).

From this information it can be stated that CD14 in either the soluble or membrane state plays an important role in LPS recognition in many cells. However, it is not an essential component as additional membrane molecules are required for cell activation. To date many different molecules have been discovered to be involved in LPS recognition. One such molecule is the GPI-linked membrane molecule decay accelerating factor (DAF) CD55. This was identified as the 80 kDa LPS-binding membrane protein (LMP80), which is believed to be an LPS responsive element (Schletter *et al.*, 1995). This was shown when monocyte membranes were incubated with LPS and the CD55 coprecipitated with LPS in the presence of anti-LPS Mabs. Furthermore, CD55 transfected CHO cells are able to respond to

LPS (unpublished observations, as quoted by El-Samalouti *et al.*, 1999). Another possible LPS recognition molecule is the adhesion molecules of the β - integrin family which are phagocytic receptors of leukocytes that are able to recognise LPS moieties of Gram-negative bacteria. The most recently identified LPS signalling receptor is the Toll-like receptor 2 (TLR2), which is believed to be a PG receptor (Yang *et al.*, 1998).

From all this evidence it can be stated that LPS recognition and signalling involves various known and unknown membrane molecules. Therefore, it can be hypothesised that these molecules form a functional LPS receptor domain made up of different subunits. There has been a great deal of progress concerning the interactions between LPS and soluble or cellular receptors, signal transduction pathways that are activated by LPS and the cellular response to LPS. Nevertheless, the mechanisms involved in LPS activity in the host have proven to be very complex and so still remain unclear.

1.9.4.3. LIPOPOLYSACCHARIDE STRUCTURE.

LPS are large complex molecules that contain both hydrophilic carbohydrates and hydrophobic lipid portions, thus producing an amphipathic molecule. The structure varies greatly between bacterial genera, and even species. However, LPS is composed of 3 different regions each with their own structure and function, these are the proximal, hydrophobic lipid A (glycolipid) region, distal hydrophilic O-antigen polysaccharide that projects into the surrounding environment, which are linked together via a core oligosaccharide (See Figure 1.5).

1.9.4.4. LIPID A.

Lipid A from many different Gram-negative bacteria have similar structures. Variations do occur from the type of hexosamine present, degree of phosphorylation to the chain length, number and location of fatty acyl chains (Moran, 1995), but generally lipid A has a very conserved structure. Lipid A of *E. coli* and *Salmonella* contains a hydrophilic backbone of β(1'-6)-linked D-glucosamine (GlcN) disaccharide that is linked to the core via 3-Deoxy-D-manno-2-octulosonic acid (KDO) connected to the O-6' position of the lipid A. This

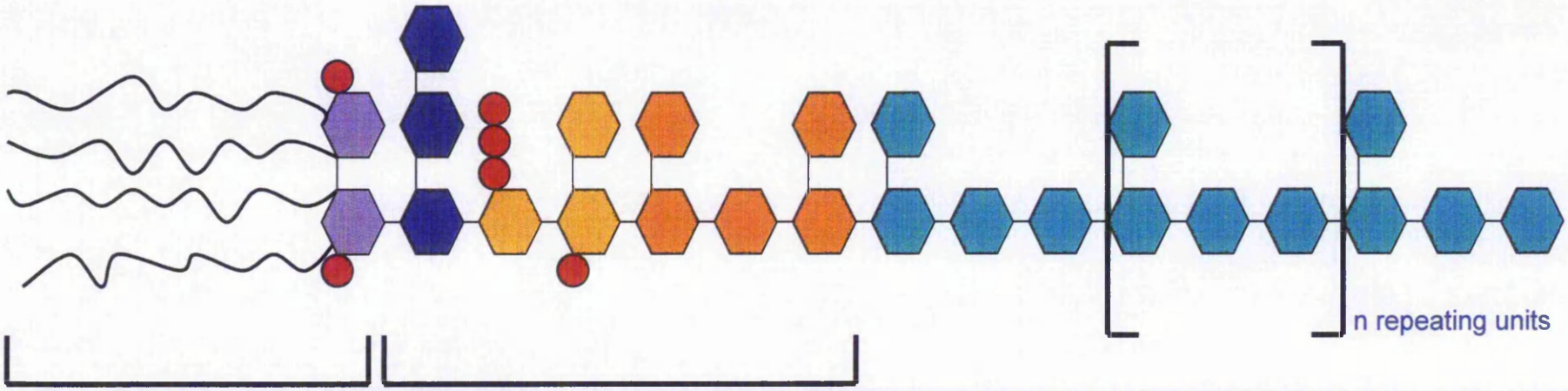


Figure 1.5: The lipopolysaccharide molecule of Gram-negative organisms.

backbone structure is also phosphorylated at position O-1 and O-4 with a 4-amino-4-deoxy L arabinose connected to the O-4 phosphate group. Up to 6 of the remaining positions are substituted by hydrophobic saturated fatty acids (Moran, 1995) that are 12-14 carbons long and either ester or amide linked to the backbone, thus producing an asymmetric distribution. The presence of these fatty acids causes the low fluidity shown by the LPS portion of the outer membrane (Nikaido *et al.*, 1977).

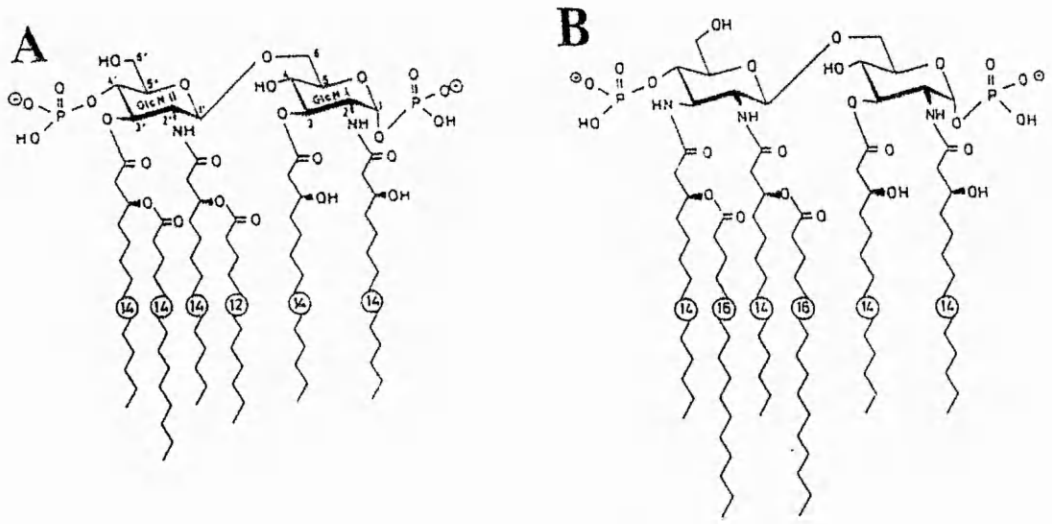
Studies have also shown deviating backbone structures containing GlcN3N monosaccharide, a phosphorylated 2,3 diamino-2,3-dideoxy D-glucose (GlcN3N) disaccharide or a glucosaminuronic acid monosaccharide which all have different properties. A mixed lipid A structure has also been observed containing both GlcN and GlcN3N, which occurs in many *C. jejuni* strains (Moran, 1995). The hybrid backbone of *C. jejuni* HS:2 is a β(1'-6) linked GlcN3N-GlcN (73%) disaccharide with 1' and 4' phosphate groups and 6 substituted fatty acids (Moran *et al.*, 1991b) thus showing a structure similar to that of enterobacterial lipid A (Moran, 1995). Also, a GlcN-GlcN3N disaccharide (12%) and a GlcN3N-GlcN3N disaccharide (15%) which are phosphorylated and acylated as stated above (Moran *et al.*, 1991b) have been observed. All *C. jejuni* serostrains studied have shown the mixed lipid A structure, but the molar ratio of GlcN and GlcN3N exhibit considerable interstrain variations. Therefore, apart from the presence of GlcN3N in the backbone *C. jejuni* lipid A has the same structural principles as enterobacterial lipid A (Moran, 1995) and thus is antigenically the same when tested with antilipid A antibodies. GlcN and GlcN3N have the same gluco-configuration, but GlcN3N have a high proportion of amine-bound 3-OH-14:O, which may have some structural influence.

C. jejuni LPS is also different in that it contains a high proportion of longer non-hydroxylated fatty acids (16:0), which enterobacterial lipid A do not contain. This causes *C. jejuni* LPS to have a decreased, but comparable endotoxin activity to *Enterobacteriaceae* in biological test systems (Moran, 1995). *C. jejuni* LPS possesses 50% lower lethal toxicity, 30-50 fold lower pyrogenicity and 100 fold lower ability to induce TNF secretion than *Salmonella* (Muotiala *et al.*, 1992, as quoted by Moran, 1995). *C. jejuni* LPS and lipid A have a greater phase transition

temperature than *Salmonella,* thus resulting in *C. jejuni* having a lower fluidity at 37°C (Moran, 1995), which affects biological and immunological activity. Also, the acyl chain properties and the presence of a higher proportion of a longer chained fatty acid with GlcN3N may effect the supramolecular structure of *C. jejuni* lipid A causing biological activity modifications.

Lipid A is an essential component of the outer membrane especially as the hydrophobic anchor of LPS. In addition, Neidhardt (1987) showed that lipid A had a pathophysiological importance being responsible for the toxic and immunostimulatory component of LPS with the degree of bioactivity being regulated by the saccharide region of LPS especially the core oligosaccharide (Figure 1.6).

Figure 1.6: Chemical structure of *E. coli* (A) and *C. jejuni* (B) lipid A. Taken from Moran (1997).



The numbers in circles indicate the number of carbon atoms in the fatty acyl chains.

1.9.4.5. CORE OLIGOSACCHARIDE.

The core oligosaccharide is divided into 2 major regions the inner and outer core region, due to different components of each region i.e. hexoses and hexosamines in the outer core whereas heptose and 3-deoxy-D-manno-2-octulosonic acid (KDO) in the inner core. Analysis of the core region was difficult as it is only a

small portion of the LPS molecule, but generally the core contains 8-10 sugar residues in non-repetitive sequences (Aspinall *et al.*, 1993). This region is responsible for mediating binding of activated T-lymphocytes involved in immunodulation and is essential for the permeation properties of the bacterial outer membrane (Rietschel *et al.*, 1990 a, b, 1991).

The core oligosaccharide structure of various bacterial species shows a great deal of variability, but not as great as the O-antigen. Only 5 different core types (R1-R4 and K12) have been observed for more than 100 *E. coli* serotypes with the differences occurring in the outer region structure (Rietschel *et al.*, 1990a). The outer core characteristically contains branched pentasaccharide, which usually consists of 2-acetamido-2-deoxy-D-glucose, glucose and galactose; also it may contain N-acetylglucosamine (Tsang *et al.*, 1990).

The outer core region of many *C. jejuni* serotypes contain terminal oligosaccharide structures that closely resemble human glycospingolipids, due to the presence of common host carbohydrate residues such as N-acetyl-neuraminic acid (Neu5Ac) or L-fucose (L-fuc) (Moran *et al.*, 1991a, 1996). Serotype HS:19 has the same structure as ganglioside GM1, GD1a, GT1a and GD3 (Moran & Malley, 1995). Whereas serotypes HS:1, HS:23 and HS:36 show structural similarity to GM2, but Yuki *et al.* (1994) suggested that *C. jejuni* LPS may express multiple ganglioside mimics. This theory was supported by Linton *et al.* (2000), who observed phase variation in *C. jejuni* NCTC 11168, whereby alternate GM1-like or GM2-like ganglioside mimicking LOS structures were produced. This molecular mimicry causes the host to raise antibodies against these bacterial antigens, but these antibodies may still be present post-infection and attack the human gangliosides, many of which are present on peripheral nerves fibres. Consequently affected persons rapidly develop weakness of the limbs and of the respiratory muscles and areflexia (Jacobs *et al.*, 1997; Nachamkin *et al.*, 1998, Ho & Griffin, 1999) due to inflammatory disruption of peripheral-nerve integrity and function. This neuropathological autoimmune disease is known as Guillain-Barré syndrome (GBS).

The inner core region consists of the sugar L-glycero-D-manno-heptose, 2-keto-3-deoxy-D-manno-octonate (KDO) substituted by polar groups, each forming a branched trisaccharide (Osborn, 1996) and phosphate and ethanolamine. The basic structure of the heptose region is quite uniform with the same structure being observed for *Salmonella* spp. (Naess *et al.*, 1984) and *E. coli* (Rietschel *et al.*, 1991). However, heterogeneity of this region has been observed by nonstoichiometric occurrence of pyrophosphoryethanolomine (Yuki *et al.*, 1992). Even though this structure shows variation it has been determined that LPS of all bacteria has at least 1 KDO residue (or derivative thereof), which is responsible for acid labile linking the core oligosaccharide to the lipid A (Rietschel *et al.*, 1990a,b, 1991). Whereas the O-chain is linked by subterminal glucose II units in the core. The presence of the KDO carboxyl groups causes the LPS to have an acidic character creating an overall negative charge, which has physiological importance. This inner core region may also be responsible for linking the LPS to other cell wall components by divalent cations and ionic linkages, thus creating integrity and stability of the cell wall.

The inner core of various *C. jejuni* strains contains all the major sugar constituents commonly observed in the core region of LPS. However, further analysis by Moran *et al.* (1991a) showed that D-glucuronic acid and N-acetyl-neuraminic acid (Neu5Ac) were also present. The inner core oligosaccharides of 8 *C. jejuni* serotypes (HS:1, HS:2, HS:3, HS:4, HS:10, HS:19, HS:23 and HS:36) contain a trisaccharide L,D-Hep-α(1,3)-L,D-Hepα(1,5)-KDO (Aspinall *et al.*, 1993). Also, the heptose adjacent to KDO is replaced by D-glucose in β(1-4) linkage, so a common tetrasaccharide in *C. jejuni* is observed. Serotypes HS:23 and HS:36 contain the tetrasaccharide Glc-α-L, D-Hep-α(1,3)-L,D-Hep-α(1,5)-KDO, which is structurally similar to the tetrasaccharide in enterobacterial LPS. Other serotypes have a tetrasaccharide Gal-α(1,3)-L,D-Hep-α(1,3)-L,D-Hep-α(1,5)-KDO in which D-galactose substitutes D-glucose (Moran, 1997). Therefore, *C. jejuni* strains have a common, but unique tetrasaccharide in the inner core and a trisaccharide which has been observed in other bacterial species inner core. From this it may be possible to isolate antibodies that react with *C. jejuni* LPS only, thus aiding diagnosis.

1.9.4.6. O-antigen (O side chain).

O-antigens are long hydrophobic carbohydrate chains up to 40 oligosaccharide repeated units long (Robbins & Uchida, 1962). Each individual unit contains up to 7 different sugars including linear trisaccharide, pentasaccharide, branched oligosaccharide polymers consisting of 4 to 6 sugars and even monosaccharides such as neutral sugar, amino sugars and unusual sugars like 6-deoxyhexoses or 3,6-dideoxyhexoses.

Each parental bacterial strain contains its' own characteristic LPS structure in which the nature, ring form, anomeric configuration and type of substitution of individual monosaccharide residues and their sequence of a repeating unit is unique for particular strains. Due to these specific sugars of the O-antigen varying greatly between different bacterial strains it enables them to be classified. These structures contain immunodeterminants against which the anti-O antibodies formed during infection or on immunisation are detected, thus providing each strain with an unique O antigenicity that is immunologically distinct. Therefore, this O-antigen can be used for serotyping of strains (Penner & Hennessy, 1980).

O-antigens have a special function enabling bacteria to escape phagocytosis and possibly death by the antibody complementation system in a host organism, thus contributing to the virulence of bacterial infections (Llang-Takasaki *et al.*, 1982). Most pathogenic organisms are able to produce novel types of O-antigens, which cause a minimal immune response as the level of antibodies for these particular antigens are extremely low. This selective pressure has resulted in the tremendous diversification in O-antigen structure and methods of reversible modification to avoid detection by the host's immune response. For example Brooks *et al.* (2001) reported the ability of *C. fetus* serotype A strains to undergo variation in the expression of two serologically (acetylated and non-acetylated forms) distinct O-antigen epitopes. Therefore, a given strain can be structurally modified both chemically and serologically allowing new strains to be distinct from the parental strain.

1.9.4.7. LIPOOLIGOSACCHARIDE (LOS).

In many Gram-negative bacteria such as *Neisseria* and *Haemophilus* species complete LPS molecules are not expressed (Mandrell & Apicella, 1993). Instead they produce an unique glycolipid called lipooligosaccharide (LOS), which have similar lipid A and core oligosaccharide structure to LPS except it only possesses a limited number (up to 10) of saccharide units (see Figure 1.7). Furthermore, the genes responsible for the assembly of the core regions of LOS are not clustered together in operons as in enteric bacteria, which produce LPS, thus providing an explanation why these organisms produce LOS instead of LPS (Fleischmann *et al.*, 1995). Even though differences between LPS and LOS occur both structurally and genetically they both share a similar array of functional activities including serum resistance, ganglioside molecular mimicry, phase variation and endocytoxicity. Also, the importance of LOS acting as a ligand of human receptors promoting adhesion and invasion of host cells has been observed by many different groups (Cope *et al.*, 1991; Porat *et al.*, 1995; Schwan *et al.* 1995 as quoted by Preston *et al.*, 1996).

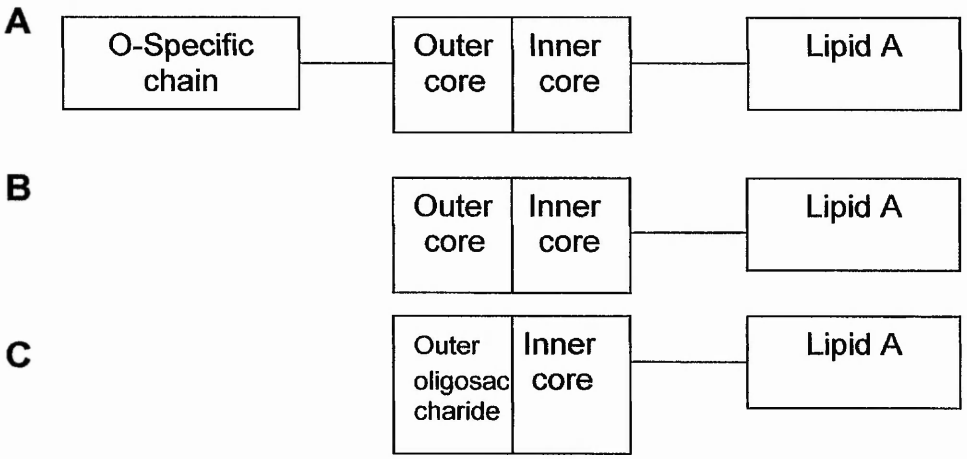


Figure 1.7: General architecture of high-M_r LPS (A), low-M_r LPS (B) and LOS (C). Taken from Moran *et al.* (2000).

The term LOS has not been universally accepted due to the lack of evidence to prove if LOS are distinct molecules or are rough versions of 'Enterobacterial LPS'. However, current reports are proving that they are not simply rough molecules as SDS-PAGE and immunochemical analysis of LOS with monoclonal and polyclonal

antibodies have shown some distinct differences. Genetic organisation between the two types of molecule provides further evidence that these structures have undergone evolutionary change instead of merely being defective LPS molecules (Schneider *et al.*, 1984 as quoted by Preston *et al.*, 1996).

1.9.4.8. *C. JEJUNI* LIPOPOLYSACCHARIDE.

There has been significant controversy concerning the structure of *Campylobacter* LPS. Early studies of phenol water extracts and proteinase K treated whole cell lysates identified the presence of low molecular weight LPS which usually fell into the 4.5-5.0 kDa range (Preston & Penner, 1987). However, by immunoblotting these extracts with serotyping antisera, two distinctly different types of LPS were detected. From this study it was observed that all serostrains have low molecular weight LPS that resolve into a small number of bands, which have a similar banding pattern to rough LPS from Enterobacteriaceae or LOS which is characteristic of *Neisseria* and *Haemophilus* strains (Preston *et al.*, 1996). As well as the low molecular weight LPS some serostrains possess high molecular weight molecules which form a ladder-like pattern characteristic of Enterobacterial smooth form LPS, which have an attached O-specific polysaccharide (Preston & Penner, 1987; Moran & Penner, 1999). Evidence to support these findings was detected by Perez-Perez *et al.*, (1986) who showed that LPS of *Campylobacter* spp. are immunologically related to LPS structures in Enterobacteriaceae. Also, immunoblotting has shown that *Campylobacter* spp. possess a core LPS structure (Aspinall *et al.*, 1993; Chart *et al.*, 1996). Preston and Penner (1987) showed that a third of all *C. jejuni* tested had high molecular LPS, with 10 out of 34 antisera revealing a ladder-like pattern. Whereas the remaining serostrains produced low molecular weight LPS only. Furthermore, Moran *et al.*, (2000) quoted that 18 serostrains of *C. coli* showed both low and high molecular weight molecules. From which they stated that the presence of both LPS and LOS-like molecules in the same species was a novel feature of *Campylobacter* amongst Gram-negative bacteria.

Blake and Russell (1993) used different LPS extraction and detection methods to compare the merits and limitations of each technique. No LPS/LOS was detected after silver staining when the phenol-chloroform method with proteinase-K

treatment was used. Yet, immunoblotting of the same samples showed low molecular weight bands indicative of LOS. Whereas the Al-Hendy *et al.* (1991) extraction method showed additional bands similar to semi rough to smooth LPS consisting of core oligosaccharides with O polysaccharide chains. Therefore, extraction methods, growth conditions and staining methods can influence the type of LPS or LOS observed. It has also been suggested that *Campylobacter* is able to vary the antigenicity of its cell surface. This phenomenon has been detected both in the laboratory when *C. coli* strains were maintained for long periods of time under static culture conditions and in a host during the course of infection (Mills *et al.*, 1992). Consequently, *Campylobacter* strains may be able to demonstrate a high degree of variation in genetic expression and hence its structure, which is triggered by the host *in vivo.* This is very important for *C. jejuni* virulence enabling it to switch on/off genes providing different antigenic expression, thus avoiding an attack by the host's immune response.

At present only a limited number of genes involved in *C. jejuni* LPS/LOS biosynthesis have been characterised. These include *WaaC, pg1*, OH4384 and OH4382 LOS gene clusters (Gilbert *et al.*, 2000). Parkhill *et al.* (2000) also observed polymorphic regions, the majority of which were homopolymeric tracts of either G or C residues were largely clustered in 3 regions of the genome involved in capsular polysaccharide biosynthesis, flagellum modification and LOS biosynthesis. Furthermore, no orthologues to *wzx*, *wzy* and *wzz* genes were identified, which are essential for heteropolymeric O chain biosynthesis. Additionally, Wood *et al.* (1999) observed that *C. jejuni* strains produced LPS whereas others produced LOS. These strains shared 6 predicted open reading frames (orfs), but an additional orf (*orfE*) of unknown function was identified in the LOS producing strains. Further investigation showed that insertion of *orfE* into an LPS producing *orfE* negative strain resulted in the loss of the O chain. Therefore, the production of either LPS or LOS in *C. jejuni* strains may be due to the presence or absence of this particular orf.

Reports have also suggested that *C. jejuni* produces a capsular polysaccharide. As Chart *et al.* (1996) observed that the heat stable antigens on *C. jejuni* were more likely to be capsular than long chained LPS. These findings were supported

by Karlystev *et al.* (2000) who discovered that *C. jejuni* 11168 had similar genes to those involved in the biogenesis of the K1 and K5 capsular polysaccharide of *E. coli*. Due to this information various *C. jejuni* strains were studied with high molecular weight LPS being extracted from all the strains. This LPS is unrelated to LOS, but has similarities to capsular polysaccharide. This led to the realisation that what was once believed to be high molecular weight LPS was actually capsule and the capsule was the main serodeterminant of the Penner typing scheme (Karlyshev *et al.*, 2000; Bacon *et al.*, 2001) Furthermore, the previously described O-antigen of *C. jejuni* was shown to be a capsular polysaccharide as *C. jejuni kps*M, *kps*S, and *kps*C (involved in the transport of the sugar polymers) mutants were unable to produce an O-antigen (Whitfield & Roberts, 1999; Karlyshev *et al.*, 2000; Bacon *et al.*, 2001). Due to this new information and that the only LOS genes were detected by Parkhill *et al.* (2000) in the complete *C. jejuni* NCTC 11168 genome, it is believed that only capsular polysaccharide and LOS are produced by *Campylobacter* spp. and are responsible for vital roles in *C. jejuni* virulence and survival mechanisms (Bacon *et al.*, 2001; Guerry, 2002).

1.10. SURVIVAL MECHANISMS.

1.10.1. SURVIVAL AND CONTROL OF *ARCOBACTER* SPECIES.

In order for *Arcobacter* species to cause disease they must survive many harsh conditions both in the environment and in the host. Due to *Arcobacter* species being isolated from many food products and water sources it is essential that the survival capabilities of this organism be established, enabling the effectiveness of existing and newly designed treatments for eliminating *Arcobacter* to be determined. To date very little information is known concerning *Arcobacter* survival abilities. *Arcobacter* species are sensitive to temperatures greater than 42°C, which suggests that they are unlikely to survive cooking processes. However, unlike *Campylobacter* species, *Arcobacter* are both aerotolerant and able to survive at low temperatures (5°C) (Phillips & Long, 2001). Both these qualities enabled *Arcobacter* species to survive aerobic digestion of sewage (see Section 1.6.1). In addition, Rice and co-workers (1999) showed that *A. butzleri* was able to survive in ground water for up to 16 days with less than a 0.5 $\log_{10}$

reduction, whereas *C. jejuni* and *C. coli* were culturally detectable for only 11-14 days in surface water, with an approximate decline of 6-7 $\log_{10}$ units (Korhonen & Martikainen, 1991).

A. butzleri survival ability in water was also reported by Assanta *et al.* (2002). They observed that *A. butzleri* was able to produce extracellular polymeric substances (EPS), which enabled them to form attached communities on stainless steel surfaces such as water distribution pipes. Various groups in the past (Costerton *et al.*, 1978; Krovacek *et al.*, 1992; Mafu *et al.*, 1998) have reported that EPS enabled other bacteria (i.e. *Aeromonas* spp.) to survive harsh conditions by providing nutritional advantages and helping to protect them against outside stresses such as cold temperature, disinfectants and other antimicrobial agents. This could provide some explanation to why *Arcobacter* species were isolated from the surfaces of almost all the processing machines and transport crates even after a 3-day period of no slaughter activity (see Section 1.6.2, Houf *et al.*, 2002a).

Houf and co-workers (2002a, 2003) also reported on the survival ability of *Arcobacter* species with them being successfully isolated from the poultry carcasses after evisceration. Therefore, washing and chilling alone were unable to significantly reduce or eliminate these organisms. Due to this, Phillips and Duggan (2001, 2002) investigated other treatments such as organic acid sprays and the incorporation of nisin and/or sodium lactate to decontaminate poultry and meat products. Citric acid and lactic acid at 0.5% to 2.0% (w/v) effectively eliminated *A. butzleri* growth. Sodium lactate and nisin had very little effect in reducing the numbers of *A. butzleri* in pure culture (Phillips, 1999). Whereas, nisin was even less effective in killing *Arcobacter* on poultry products, as it bound to the carcass with less available to inhibit *Arcobacter* growth. Phillips and Duggan (2001) also observed that a combination of treatments did reduce the number of *Arcobacter* on poultry carcasses. Short term treatment (8 h) with both trisodium phosphate (TSP, 0.5 mM) or EDTA (1mM) were effective in reducing *A. butzleri* in pure culture. After 8 h, recovery of the *A. butzleri* started to occur, but using nisin at 4°C after TSP or EDTA treatment prevented sublethal recovery ensuring that the initial inhibition was maintained.

Gamma radiation is also used in the food industry to eliminate spoilage and pathogenic bacteria. Collins *et al.* (1996a) studied the effect of irradiation on both *A. butzleri* and *C. jejuni.* The results stated that *A. butzleri* (D_{10}, 0.27 kGy) was 1.4x more resistant to irradiation than *C. jejuni* (D_{10}, 0.19 kGy), suggesting that *Arcobacter* are more resilient organisms than *Campylobacter* species. However, for both organisms the recommended levels (0.3-1 kGy) of irradiation in the food industry are adequate to eliminate them. Otth *et al.* (2001) also observed that *A. butzleri* showed greater resistance to desiccation compared to *C. jejuni* strains, with 55% (n = 11) of *A. butzleri* stains resisting desiccation for 12 h or more compared to 2-10 h for *C. jejuni.*

Finally, many groups such as Kiehlbauch *et al.* (1992), Atabay and Aydin (2001) and Houf *et al.* (2001) have investigated the susceptibility of *Arcobacter* species to antimicrobial agents. *Arcobacter* are susceptible and resistant to a variety of antibiotics (see Section 1.2). Nevertheless, it appears that *Arcobacter* are resistant to antimicrobial agents such as erythromycin, tetracycline and chloramphenicol, which are commonly used to treat *Campylobacter* infections.

1.10.2. SUBLETHAL INJURY.

A variety of environmental changes from 'normal' conditions for a micro-organism is stressful and may injure the organism. Injury is either classed as lethal or sublethal injury (Figure 1.8). The term lethal is used when the cells are irreversibly injured and are unable to produce progeny under any condition or after any resuscitation procedure. Whereas sublethal injury implies damage to structures within the cells the expression of which entails some loss or alteration of cellular functions, susceptibility to selective agents and antimicrobial leakage of intracellular material (Ray & Speck, 1973). Sublethal injury is reversible by repair if the cells are exposed to favourable resuscitation conditions, such as a non-selective nutrient rich medium under optimal growth conditions (Ray & Speck, 1973).

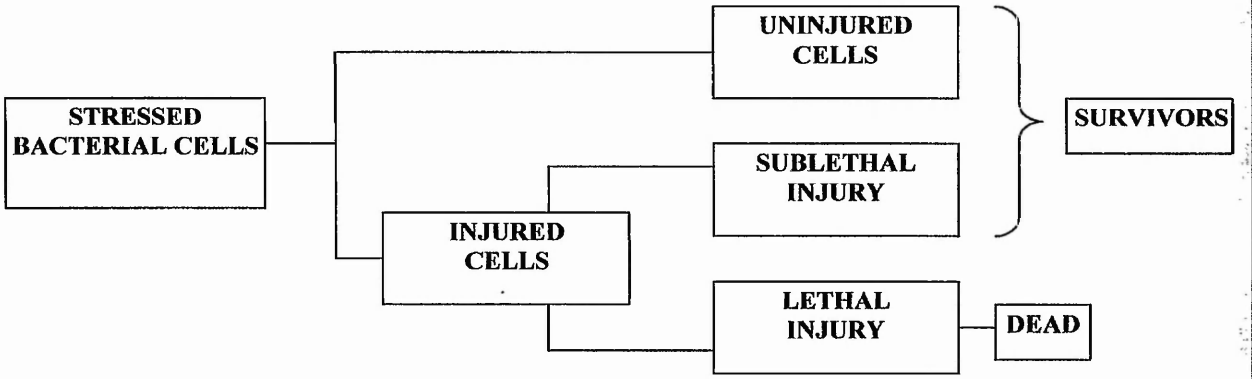


Figure 1.8: Flow diagram to show the effects of stressful conditions (i.e. drying or freezing) upon bacterial cell. Modified from Ray & Speck (1973)

In food microbiology the phenomenon of 'injury' may present problems, since many of the physical treatments (drying, freezing, osmotic activity) and chemicals (preservatives and disinfectants) used in food processing factories are designed to kill microbes. Then again, if the treatment is not severe enough the surviving microbes are likely to be injured. Thus viable counts may be low, when in fact the sample contains a high number of injured cells. The injured cells may remain undetected as selective media usually contain ingredients such as salt, deoxycholate, lauryl sulphate, bile salts, detergents, dyes and antibiotics, which prevent the growth or reproduction of injured cells.

However, in food and beverage products once the stress causing injury is removed cellular repair enables the cells to regain their normal capabilities, which includes pathogenic and enterotoxin-production properties. Therefore, important food poisoning organisms may be undetected causing the food products to be incorrectly declared 'safe'. At present samples are often incubated for an hour or more in a non-inhibitory medium prior to analysis. For example *Salmonella* is incubated in buffer peptone water for 18 h, thus allowing injured cells to be recovered.

1.10.3. COLD & HEAT SHOCK RESPONSE.

As bacteria are not aggregated into tissue like plant and animal cells, they must respond to their environment individually. Harsh environmental conditions can have a major effect on proteins, which require a specific intracellular environment to maintain proper structure, which dictates their function. For these reasons bacteria must have survival mechanisms which enable them to adapt to constantly changing environments. Such mechanisms include the cold and heat shock response, which protects cells and repairs damaged components.

1.10.3.1. HEAT SHOCK PROTEINS

Heat shock proteins (HSPs) are induced as a survival mechanism, when cells undergo stressful conditions. These conditions include physico-chemical factors (temperature upshifts, pH, osmolarity changes and irradiation) and metabolically harmful substances (ethanol, antibiotics and heavy metals) (Bukau, 1993). However, these inducers vary with respect to the kinetics, induction levels and production of specific subsets of HSPs.

HSP are a group of highly conserved proteins, which are grouped into 3 main classes according to their molecular weight. These include 85-110 kDa (e.g. HSP90 & HSP100), 60-80 kDa (e.g. HSP60, HSP70 & Lon) and <50 kDa (small HSPs like HSP27 & GrpE). Some HSP are expressed constitutively at low levels as they aid the folding of particular proteins, whereas others are expressed at an increased level during or after exposure to stress (Morimoto *et al.*, 1994). Long-term over-expression of HSPs can be lethal, therefore a strict control mechanism is in operation to maintain the balance between synthesis and degradation. The rate of HSP synthesis is rapid starting within seconds after a temperature shift, until it reaches a peak after 5-10 min, where the individual HSP synthesis has increased between 5 and 10 fold. However, after an adaptation period synthesis decreases to a new steady state level, which is greater than at the lower temperatures (Herendeen *et al.*, 1979; Yamamori & Yura, 1980). An overview of the heat shock response is given below in Figure 1.9. This diagram shows that environmental stresses (i.e. heat) cause damaged or unstable proteins (denatured, improperly folded or modified), which rapidly accumulate within the

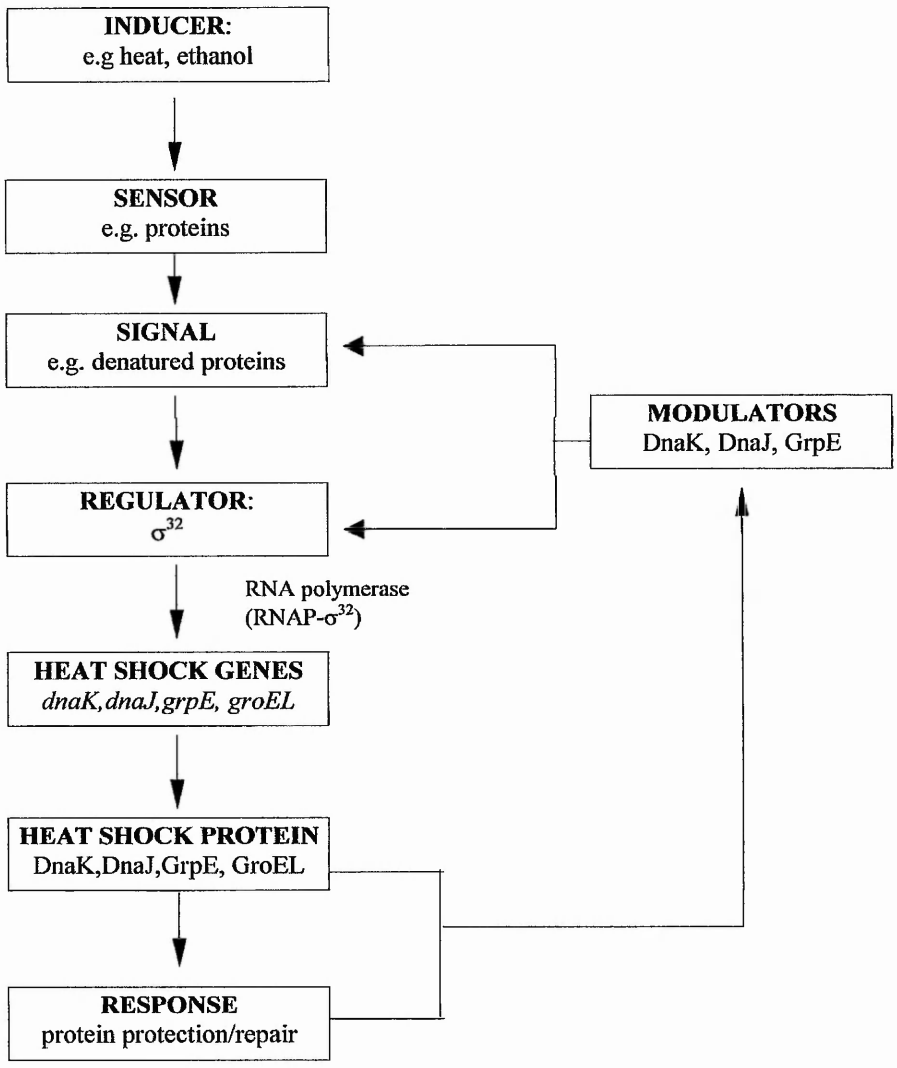


Figure 1.9: Overview of the heat shock response. Modified from Bukau, (1993).

cell (Parasell & lindquist, 1995). The presence of these abnormal proteins triggers the induction of a heat shock response, which prevents protein aggregation and further damaged (Feder *et al.*, 1995). It is believed that DnaK, DnaJ and GrpE are negative modulators that form a 3 part chaperone system, which regulates the heat shock response. Bukau (1993) proposed that DnaJ was a key regulator that dissociates from σ^{32} upon detection of damaged protein. After repair DnaJ accumulates and causes σ^{32} to re-associate with DnaJ, DnaK or GrpE, resulting in the heat shock response being shut off. Therefore, DnaJ provides a direct link between σ^{32} dependent transcription and damaged proteins (Bukau, 1993).

1.10.3.2. COLD SHOCK RESPONSE

Temperature seems to have a considerable effect on bacteria, mainly due to it affecting virtually all chemical reactions, which in turn affects the physiological state of microbial cells. Therefore, exposure to temperatures which are lower than their optimum, results in numerous changes in the bacteria such as the rate of enzymatic reactions and the uptake/transport system decreases. Also, the requirement for organic substrates increases (Nedwell & Rutter, 1994) all of which effect the growth rate of the bacteria. The initiation of translation is also affected by low temperatures (Broeze *et al.*, 1978). Furthermore, the fluidity of the cellular membrane is affected by temperature as normal cell function requires a fluid-like lipid bilayer. However, as temperature decreases the fatty acid chains of the membrane lipids undergo a change of state to a more ordered crystalline array of fatty acid chains, which makes the membrane rigid. Bacteria respond to this change by incorporating low-melting point fatty acids into the membrane, thus maintaining fluidity and function. It has also been observed by Ray *et al.* (1994) that lipopolysaccharide phosphorylation is dependent on temperature with less phosporylation occurring at lower temperatures than at higher ones. It is believed that this change may serve to modulate the outer membrane in Gram-negative bacteria. This in turn enables the bacteria to adapt to cold temperature growth.

Bacteria are able to survive due to adaptation, via the expression of different classes of proteins. Proteins that are synthesised at higher levels at low temperature are known as cold acclimatisation proteins (CAPs) and proteins synthesised at a higher rate after a sudden decrease in temperature are called cold shock proteins (CSPs). Nevertheless, these two classes of proteins can overlap. Compared to the heat shock response very little information is known about the cold shock response. It was initially discovered in *E. coli* by Jones *et al.* (1987) and was believed to be an adaptive response, which enabled *E. coli* grown at 37°C to survive a shift to 10°C. At 10°C growth halts for approximately 4 h before exponential growth is resumed (Jones *et al.*, 1987; Nystrom *et al.*, 1992). During this lag period the vast majority of intracellular proteins are switched off, but the synthesis of 16 small acidic proteins is induced greatly. These proteins are cold shock proteins (CSPs) and are approximately 7.5 kDa in size and share a sequence homology of over 60%.

1.11. AIMS.

The major aims of this project were to study the physiology and cytotoxicology of the emerging food-borne pathogen *Arcobacter* primarily using the type strain *A. butzleri* NCTC 12481, which was originally isolated from a case of human gastroenteritis.

(i) Physiological aspects of *A. butzleri* NCTC 12481.

- To investigate the physiological parameters (pH, temperature growth ranges, nitrate/nitrate reduction) of *A. butzleri* by using impedance microbiology.
- To study survival characteristics of *A. butzleri* NCTC 12481 such as the effects of sublethal temperatures (*D* & *z* values and cold storage) and sites in injury (structural and metabolic). This work will be complemented by looking at the heat and cold adaptation of *A. butzleri.*

(ii) Cytotoxicity studies.

- To determine if four human *A. butzleri* isolates (NCTC 12481, Rigs 1714, Rigs 16799 & Rigs 15342) produce any cytotoxic effects on different mammalian cell lines (N2a, CHO & ECV) *in vitro*. This will involve screening samples for cytotoxicity using methyl tetrazolium thiazolyl blue (MTT) assay and morphological studies.
- If cytotoxicity was identified it would be characterised regarding the type (i.e. heat stable or labile) and location of the toxin(s) (i.e. membrane associated) and if growth conditions and age of the culture affect cytotoxin(s) production.
- To establish the cytotoxicity of lipopolysaccharide (LPS) extracted from *A. butzleri* NCTC 12481. Furthermore, determine the presence of CD14 (LPS receptor) on the surface of different mammalian cells, using such techniques as Western blotting, ELISA and flow cytometry.

CHAPTER TWO

MATERIALS AND METHODS

2.0: MATERIALS AND METHODS.

2.1. MATERIALS.

Unless otherwise stated in the text all the chemicals used for buffers, reagents and tissue culture media were of the highest grade and were purchased from Sigma-Aldrich Company Ltd. (Poole, UK) and Fisher Scientific (Leicester, UK). All diluents, liquid and solid media for bacterial culture work were purchased from Oxoid Ltd. (Basingstoke, UK). All sterile plastic ware used for tissue culture were of the highest quality and were obtained from SLS Ltd. (Nottingham, UK).

2.2. PREPARATION OF MEDIA, REAGENTS AND AGAR PLATES.

All media was prepared according to the suppliers' instructions. Media and reagents for bacterial experimental work in general were sterilised by autoclaving at 121°C, for 15 min (Astell-Hearson autoclave). Media was always allowed to cool before being used and liquid media was usually stored at room temperature. Where sterile supplements were added to agar, the agar was cooled to 48°C, the supplements added and then poured. Agar plates and broths were incubated at 37°C (LEEC incubator, LEEC limited, Nottingham, UK) for 24 h before use, to dry the plates and induce any contaminants. The agar plates were stored at +4°C for the maximum of 2 weeks.

2.2.1. BLOOD AGAR.

Fourteen grams of blood base No 2 agar (CM271) were rehydrated in 300 ml distilled water, autoclaved and supplemented with 7% (v/v) defibrinated horse blood (SR0050B, final pH 7.4 ± 0.2). From this volume approximately 12 agar plates were produced.

2.2.2. NITRATE AND NITRITE BRAIN HEART INFUSION (BHI) BROTH.

Eighteen and half grams of BHI broth base (CM225) were rehydrated in 500 ml of distilled water (final pH 7.3 ± 0.2). Unless otherwise stated, 50 ml and 10 ml volumes of the dissolved media were decanted into 250 ml conical flasks and 50 ml conical flasks or Universal bottles respectively and autoclaved. The BHI broth was also used as a base to produce nitrate and nitrite broths (Section 2.16), whereby 0.1 ml or 1 ml of 10 mM and 50 mM nitrate or nitrite stock solutions (filter sterilised with 0.2 μM Acrodisc syringe filters, Gelman Sciences, USA) were added to BHI broth (9.9 ml or 9.0 ml respectively) to produce 0.1 mM, 1 mM and 5 mM nitrate and nitrite BHI based broths.

2.2.3. BRUCELLA BROTH WITH FBP SUPPLEMENT.

Fourteen grams of brucella base (CM169) were rehydrated in 500 ml of distilled water and autoclaved (final pH 7.0 ± 0.2). The cooled media was supplemented with 15 ml of FBP supplement (0.9 mM iron (II) sulphate, 1.3 mM sodium metabisulphite and 2.3 mM pyruvic acid), all ingredients were dissolved in distilled water and filter sterilised (0.2 μM Acrodisc syringe filters, Gelman Sciences, USA). Unless otherwise stated, 50 ml and 10 ml volumes of the supplemented media were aseptically decanted into sterile 250 ml conical flasks and sterile 50 ml conical flasks or Universal bottles respectively.

2.2.4. TRYPTONE SOYA BROTH (TSB).

Twenty grams of TSB base (CM129) were rehydrated in 500 ml of distilled water (final pH 7.3 ± 0.2). Unless otherwise stated, 50 ml and 10 ml volumes of the dissolved media were decanted into 250 ml conical flasks and 50 ml conical flasks or Universal bottles respectively and autoclaved.

2.2.5. BRILLIANT GREEN BILE AGAR (BGBA).

Twenty grams of brilliant green 2% bile broth (CM31, final pH 6.9 ± 0.2), 10 g bacteriological agar No 1 (L11), and 2.5 g NaCl were dissolved in 500 ml distilled water and autoclaved. From this volume approximately 20 agar plates were produced.

2.2.6. CEFOPERAZONE, AMPHOTERICIN B & TEICOPLANIN (CAT) AGAR.

Twelve grams of *Arcobacter* broth (CM965) and 10 g bacteriological agar No 1 (L11) were rehydrated in 500 ml distilled water, autoclaved and supplemented with 4 ml of sterile CAT supplement (SR174, cefoperazone 8 mg/L, teicoplanin 4 mg/L and amphotericin B 10 mg/L). The CAT supplement was rehydrated in 4 ml sterile distilled water. From this volume approximately 20 agar plates were produced.

2.2.7. CHARCOAL CEFOPERAZONE DEOXYCHOLATE AGAR (CCDA).

Twelve grams of *Arcobacter* broth (CM965) and 10 g bacteriological agar (L11) No 1 were rehydrated in 500 ml distilled, autoclaved and supplemented with 2 ml of sterile CCDA supplement (SR155E, cefoperazone 32 mg/L and amphotericin B 10 mg/L). The CCDA supplement was rehydrated in 2 ml sterile distilled water. From this volume approximately 20 agar plates were produced.

2.3. BACTERIAL STRAINS AND STOCK CULTURE GROWTH CONDITIONS.

A. butzleri NCTC 12481 type strain was originally isolated from a patient with diarrhoea (Kiehlbauch *et al.*, 1991a) and was obtained from the National Collection of Type Cultures (NCTC, PHLS, Colindale, London, UK). Three *Arcobacter* strains Rigs 1714, Rigs 16799 and Rigs 15342, were isolated from humans and kindly donated by Dr. S.L.W. On (Danish Veterinary Laboratory, Copenhagen, Denmark). The cultures were aseptically streaked over a pre-dried blood agar plate (Section 2.2.1). The plates were incubated at 37°C, under microaerophilic conditions, in a modified anaerobic cabinet (Compact M, Don Whitley Scientific Ltd, UK) with 10% (v/v) carbon dioxide, 5% (v/v) oxygen and 85% (v/v) nitrogen gas mixture for 48 h. The cultures were maintained by re-streaking them onto blood agar under the conditions stated above. This process was repeated every 48-72 h thus enabling a constant stock of the four *Arcobacter* strains to be kept for experimental purposes. The purity of the cultures were regularly checked by performing a Gram stain to determine the presence of small Gram-negative rods only.

2.4. STORAGE OF BACTERIAL CULTURES.

Stock cultures of bacterial strains were stored on Protect Bacterial Preserver Beads (STC Technical Service Consultants Ltd., UK). Colonies (24 h old) were picked off and aseptically transferred to a vial of protect beads. Several loopfuls were required to produce a thick suspension in the cryopreservation fluid. The vial was capped, inverted 6 times and left for at least 30 seconds. As much cryopreservation fluid as possible was removed using a sterile pipette and disposed of using normal methods for infectious material. The vial was recapped, details recorded on its side and stored at -20°C. This procedure was repeated every 3-4 months.

2.5. RECOVERY OF BACTERIAL CULTURES.

Continual streaking of the cultures may cause phenotypical variations in the strains. Therefore, after every 10-15 passages of each culture a single preserver bead from the stock culture vial was removed under aseptic conditions, using a sterile needle. The bead was streaked over a blood agar plate (Section 2.2.1) and grown under the conditions stated in Section 2.3.

Precaution :- Due to the cultures being frozen, they were subcultured twice before being used for experimentation. This was necessary as freezing may affect the expected phenotypical traits of a particular strain.

2.6. PRELIMINARY *A. butzleri* NCTC 12481 GROWTH EXPERIMENTS.

2.6.1. DETERMINATION OF THE OPTIMAL GROWTH MEDIA.

A series of experiments was set up to determine the optimum growth conditions for *A. butzleri* NCTC 12481. Three types of media were tested; Brucella broth with FBP supplement, BHI and TSB (Section 2.2).

One 250 ml conical flask (50 ml media) and one Universal bottle (10 ml media) of each broth was inoculated with a colony of a 24-48 h growing plate culture (Section 2.3). The inoculated conical flasks were shaken (100 rpm, Grant Shaking Water bath, Grant Instruments Ltd., Cambridge, UK) aerobically at 30°C and 37°C for 24 h. The Universal bottles were incubated under microaerophilic conditions at 30°C and 37°C for 24 h. After the incubation period the optical density (650 nm, PU8625 UV/Vis, Phillips), purity streak plates, enumeration using the Miles & Misra technique (10^{-1} to 10^{-5}, in 0.85% saline) on blood agar, and Gram stains were performed.

Note:- From these preliminary experiments, BHI broth was chosen as the standard liquid medium and hence is the only one referred to in the rest of this section.

2.6.2. DETERMINATION OF THE TEMPERATURE GROWTH RANGE OF *A. butzleri* NCTC 12481.

It was also important to determine the temperature growth range of *A. butzleri* NCTC 12481. This was achieved by using the Rapid Automated Bacterial Impedance Technique (RABIT) with the indirect method (Firstenberg-Eden and Eden, 1984; Silley and Forsythe, 1996). An *A. butzleri* NCTC 12481 preculture was produced by aseptically inoculating a loopful of culture from a 24-48 h blood agar plate into 10 ml of BHI broth in a 50 ml conical flask. The flask was left statically in a water bath (Grant Shaking Water bath, Grant Instruments Ltd., Cambridge, UK) at various temperatures (25-40°C) for 24 h. After which time the purity of the samples was checked by an aliquot (1 ml) of the culture being taken aseptically and the optical density (650 nm) and a Gram stain being performed. A pure *Arcobacter* culture normally has an optical density (650 nm) no greater than 0.6 and small Gram-negative rod morphology. A 2% (200 µl) volume of the preculture was used to inoculate a 10 ml BHI broth in a 50 ml conical flask. This flask was left static in a water bath at the appropriate temperature (25-42°C) for 24 h. Before the culture was used for experimental purposes the purity was re-checked.

The 24 h cultures (1 ml) were serially diluted from 10^{-1} to 10^{-8} in sterile phosphate buffered saline (PBS, 9 ml). Aliquots (0.1 ml) of the 10^{-5} to 10^{-8} dilutions were spread onto duplicate pre-dried blood agar. The plates were incubated under standard conditions (Section 2.3). This method would obtain the actual number of viable cells present, which will be compared to the detection times given by the RABIT. The tubes for the RABIT experiment were set up by aseptically transferring 0.3 ml aliquots of the neat culture and the 10^{-1} to 10^{-5} dilutions into triplicate small test tubes (3" by ½") containing 3 ml of BHI broth. Triplicate sterile control tubes were also set up containing uninoculated BHI (3.3 ml). The tubes were inserted into a RABIT conductance cell (Don Whitley Scientific, UK). Each cell contained a total volume of 0.75 ml of fresh 1% potassium hydroxide (Merck, UK) mixed in a 1:1 ratio with 2% agar No 1, which was allowed to set prior to the insertion of the small test tubes. The conductance cells were sealed with silicone bungs to prevent the loss of carbon dioxide and inserted into preheated temperature blocks. Once connected to the instrument the detection parameter were adjusted as follows: temperature according to the growth temperature, stabilisation period of 30 min, resolution time of 6 min, duration time of 48 h and detection criterion of -20μS/6 min. Then the assay was left to run for 48 h after which time the data was collected, analysed and a doubling time calculated for each temperature.

Example doubling time (t_d) calculation using the 32°C data (Section 3.2):

From the calibration curve (average time to detection (TTD) vs. log cfu ml^{-1}) created for each growth temperature a two log difference was calculated.

log 8 = 6.1 h

log 6 = 15 h

To determine the 2 log difference, the log 6 value was subtracted from the log 8 value.

15 – 6.1 = 8.9 h

To calculate the doubling time of *A. butzleri* at 32°C the following equation was used (Firstenberg-Eden & Eden, 1984).

t_d = 2 log difference in detection time x correction factor (0.15)

8.9 x 0.15 = 1.34 h

Therefore the doubling time (t_d) at 32°C was 1.34 h.

The doubling time was then used to determine the specific growth rate (μ',h^{-1}).

$\mu' = \ln 2/t_d$

$0.693/1.34 = 0.52\ h^{-1}$

Therefore the specific growth rate of *A. butzleri* NCTC at 32°C was 0.52 h^{-1}.

Following the termination of the test, the tubes were removed and the contents of each triplicate set were pooled into a sterile Universal bottle. The pH and the optical density (650 nm) were taken for each dilution to establish if the turbidity (growth) of the micro-organism was comparable to the detection times recorded by the RABIT. It was also to check the purity of the cultures. The terminal pH was measured to determine if *A. butzleri* strains fermented their growth medium.

Due to the temperature block connected to the RABIT apparatus not being able to stabilise below 25°C, conventional methods were used to measure the growth rate at lower temperatures. This was achieved by producing a pre-culture as in the RABIT experiment. This 24 h culture (0.3 ml) was used to inoculate BHI broths (3 ml) in small test tubes (3" by ½"). As with the RABIT experiment a set of uninoculated tubes (3.3 ml) were used as sterile control. The tubes were then incubated at 15°C and 22°C (Gallenkamp illuminated cool incubator, Sanyo Gallenkamp Plc. UK). At hourly intervals the optical density (650 nm, blanked against BHI broth) was taken and at 3 h intervals Miles & Misra plate counts for each sample were performed. A 1 ml aliquot was serially diluted in 0.1% peptone water (Difco, UK) to an appropriate dilution (10^{-1} to 10^{-10}). Aliquots (0.02 ml) of each dilution were dropped onto the surface of triplicate blood agar plates using the Miles & Misra technique. The plates were incubated at 37°C under microaerophilic conditions for 48 h. For each dilution only 3 to 30 colonies were counted to obtain the cfu ml^{-1} (cfu ml^{-1} = colony count x 50 x dilution factor) as values above and below this range are considered to be statically inaccurate. The cultures were incubated for a total of 32 h.

2.6.3. EFFECT OF pH ON THE GROWTH OF *A. butzleri* NCTC 12481.

The pH of the BHI broth used was altered to various pH values (pH 4.5 - pH 8.5) using 0.1 M hydrochloric acid and 0.1 M sodium hydroxide. The pH altered broths were sterilised before being aliquoted into the small test tubes. Since sterilisation

of these broths can affect the pH slightly, the pH before and after sterilisation was recorded. Thus checking that the pH had not drifted from the desired values. If the pH had altered greater than 0.5 of a pH unit then the broth was discarded and fresh broths produced.

Two cultures, one incubated at 30°C and the other at 37°C and RABIT conductance cells were prepared as in Section 2.6.2. The two cultures (1 ml) were diluted 10 fold in sterile PBS (9 ml). Aliquots (0.3 ml) of the 10^{-1} diluted cultures were aseptically inoculated into triplicate tubes of BHI at a particular pH. The samples were analysed as in Section 2.6.2. This experiment was repeated using different dilutions (neat, 10^{-1} & 10^{-2}) thus enabling the doubling time at each pH to be calculated.

2.6.4. GROWTH CURVE OF *A. butzleri* NCTC 12481.

From the growth rate experiments (Section 2.6.2 and 2.6.3) it was decided that all further experiments should be done at 37°C, as it is human body temperature and is therefore, more significant to this study in relating the toxicity of *Arcobacter* spp. to food poisoning in humans. For this reason, the growth pattern of *A. butzleri* NCTC 12481 at this temperature was studied. The growth curve was constructed using the optical density (650 nm) with the cell count (cfu ml^{-1}) at set intervals (hourly intervals for 9 h then at 24 h and 31 h). A 10 ml preculture was produced as in Section 2.6.2 and a 2 ml volume was used to inoculate a fresh 100 ml BHI broth in a 250 ml conical flask. The flask was incubated with shaking (100 rpm) at 37°C in a water bath. Aliquots (2 ml) were aseptically removed at set time intervals. One 1 ml aliquot was used to measure the optical density at 650 nm. The other 1 ml aliquot was serially diluted in 0.1% peptone water (Difco, UK) to an appropriate dilution (10^{-1} to 10^{-6}). Aliquots (0.02 ml) of each dilution were pipetted onto the surface of triplicate blood agar plates using the Miles & Misra technique (Section 2.6.2).

2.7. STANDARDISED GROWTH CONDITIONS FOR *ARCOBACTER* STRAINS.

2.7.1. EXPONENTIAL AND STATIONARY PHASE CULTURES.

From preliminary experiments (Section 2.6) it was determined that to produce an exponential or stationary phase broth culture of *Arcobacter* strains a preculture must first be produced under the following conditions. A loopful of 48 h culture was removed from a blood agar plate and aseptically inoculated into a 10 ml BHI broth in a 50 ml conical flask. The flask was shaken (100 rpm, Grant Shaking Water bath, Grant Instruments Ltd., Cambridge, UK) in a water bath overnight (18-24 h) at 37°C. A large volume culture was produced using a 2% (2 ml) inoculum of the preculture into a fresh 100 ml BHI broth in a 250 ml conical flask. The flask was shaken (100 rpm) at 37°C for 9 h producing an optical density value at 650 nm (blanked against sterile BHI broth, Spectrophotometer, PU8625 UV/Vis, Philips) of 0.2-0.3 for an exponential phase culture. Whereas for a stationary phase culture the flask was shaken for 24 h producing an optical density value of 0.4-0.5 (650 nm). Before any broth cultures were used for experimentation their purity was checked as in Section 2.6.2.

2.7.2. HARVESTING BACTERIAL CULTURES.

The 100 ml stationary and exponential phase broth cultures were aseptically decanted into sterile 50 ml centrifuge tubes and centrifuged at 3500 g for 20 min (Centaur 1, MSE, UK). The supernatant was removed and for the cytotoxicity assay (Section 2.17.1) only the supernatant was aliquoted into suitable working volumes and stored at -20°C. However, for most experiments the supernatant was disposed of using normal methods for infectious material. The pellet was washed in 20 ml of 0.1 M potassium phosphate buffer (0.1 M di-potassium hydrogen orthophosphate anhydrous and 0.1 M potassium di-hydrogen orthophosphate, mixed to produce a pH 7.3 solution, Merck, UK) and re-centrifuged at 3500 g for 20 min. The final pellet was resuspended in 2.5 ml of 0.1 M potassium phosphate buffer for stationary phase cells (20 fold concentration) and in 1.25 ml of 0.1 M potassium phosphate buffer for exponential phase cells (40 fold concentration). The washed cell suspensions where then standardised to a cell density of

6 mg wet weight ml^{-1} using 0.1 M potassium phosphate buffer, thus producing cultures with comparable biomass.

2.8. PRELIMINARY HEAT TREATMENT EXPERIMENTS.

Stationary and exponential phase cultures were grown and harvested as in Section 2.7. Aliquots (2ml) of the cell suspension were aseptically transferred to sterile capped small test tubes. An additional tube containing 2 ml of 0.1 M potassium phosphate buffer and a thermometer was used to monitor the temperature. The tubes were submerged into water baths (Grant Instruments Ltd., Cambridge, UK) at various temperatures (40-70°C in 5°C intervals). Once the control tube had reached the appropriate temperature (approximately 2 min) the samples were exposed for a further 3 min. One tube was left untreated at room temperature to act as a control to determine the original number of micro-organisms present. Once all the tubes had been removed the samples were serially diluted in 0.1% peptone water (Difco, UK) from 10^{-1} to 10^{-7}. Only the 10^{-3} to 10^{-7} dilutions were plated onto triplicate blood agar (non-selective) and in earlier experiments brilliant green bile agar (selective, BGBA) using the Miles & Misra technique. BGBA was later replaced by commercially available *Arcobacter* species selective media cefoperazone, amphotericin B and teicoplanin (CAT, SR174) and *A. butzleri* specific selective media charcoal cefoperazone, deoxycholate agar (CCDA, SR155E). The plates were incubated at 37°C for 48 h under microaerophilic conditions.

The results in this section concern the recovery of cells after exposure to heat. In order to fully understand these results a series of definitions are required. The definitions have been taken from Ray & Speck (1973) who studied cold shocked bacteria.

2.8.1. DEFINITION OF TERMS (Ray & Speck, 1973)

Dead cells: Nonviable, lethally or irreversibly injured or damaged. After stress, these cells lose their ability to multiply and form colonies on a non-selective complete agar medium. This can be expressed as

$$\% \text{ Dead} = \left(1 - \left[\frac{\text{Colony count after stress (heating)}}{\text{Colony count before stress (heating)}} \right] \right) \times 100$$

Surviving cells: After stress (heating), these cells retain their ability to multiply and form colonies on a non-selective agar medium. This can be expressed as

$$\% \text{ Survivors} = \left[\frac{\text{Colony count after stress (heating)}}{\text{Colony count before stress (heating)}} \right] \times 100$$

Metabolically (Sublethal) injured: Those survivors that are able to multiply and form colonies on a non-selective complete agar medium, but not on minimal agar medium.

$$\% \text{ Metabolically injured} = \left(1 - \left[\frac{\text{Colony count on minimal medium}}{\text{Colony count on complete medium}} \right] \right) \times 100$$

Structurally (Sublethal) injured: Those survivors that are able to multiply and form colonies on a non-selective complete agar medium, but not on a selective agar medium.

$$\% \text{ Structurally injured} = \left(1 - \left[\frac{\text{Colony count on selective medium}}{\text{Colony count on non-selective medium}} \right] \right) \times 100$$

Uninjured cells: Unharmed, undamaged, normal. These survivors are able to multiply and form colonies equally well on complete and minimal or on non-selective agar media.

% Uninjured = % Survivors – % Sublethal injury (Metabolic + Structural)

2.9. TIME EXPOSURE TO DETERMINE *D* AND *z* VALUES.

In the food industry heat is a commonly used method of reducing microbial numbers. The Decimal reduction time or *D* value (*D*) is the time required to kill 90% (= 1 log reduction) of a specific organism in a specific substrate at a particular temperature. The *D* value can then be used to determine the *z* value which is an increase in temperature required to cause a 10 fold (90%) decrease in the *D* value. In order to determine *D* and *z* values for *A. butzleri* NCTC 12481 time exposure experiments at different temperatures were necessary.

Stationary and exponential phase cultures were produced and harvested (Sections 2.7.1 & 2.7.2). The purity of the cultures were checked (Section 2.6.2) and aliquots (2 ml) were transferred to small test tubes. For each temperature a control tube was set up which contained 2 ml of potassium phosphate buffer with a thermometer to monitor temperature. The tubes were submerged in water baths at various temperatures (47°C, 50°C, 52°C & 55°C), as soon as the control tube had reached the desired temperature, the tubes were incubated for set time periods (usually either 2 or 3 min intervals). Once the tubes had been removed the samples were serially diluted in 0.1% peptone water from 10^{-1} to 10^{-7}. Only 10^{-3} to 10^{-7} dilutions were spread onto triplicate blood, CAT and CCDA or BGBA agar plates. The plates were incubated at 37°C for 48 h under microaerophilic conditions. The same procedure was used for the untreated control sample.

2.10. HEAT TREATMENT OF *A. butzleri* NCTC 12481.

From these preliminary experiments it was determined that a temperature of 55°C with an exposure time of 3 min produced the best results for heat treating *A. butzleri* NCTC 12481 cultures (for certain preliminary experiments a temperature range of 45°C to 60°C was used to determine the heat shock response). Therefore, the following methodology was standardised. An appropriate volume (usually 2 ml) of each culture was aseptically transferred to a sterile capped small test tube (3" by ½"). An additional tube contained 2 ml of

buffer with a thermometer to monitor temperature. The tubes were submerged into a water bath at 55°C. Once the control tube had reached the desired temperature (approximately 2 min), the tubes were incubated for a further 3 min period. A tube was left untreated at room temperature to act as a control to determine the original number of micro-organisms present. For certain experiments such as ethidium bromide staining, untreated and treated controls were required (Section 2.11). The treated control was produced in the same way as above except it was exposed to 100°C for a total of 5 min. During initial experiments the tubes were placed directly onto ice after heating, but latterly the samples were immediately diluted (10^{-1}) at room temperature.

2.11. STAINING WITH FLUORESCENT DYES.

Stationary and exponential cultures and heat shocked samples were prepared (Sections 2.7.1, 2.7.2 & 2.10). Aliquots (1 ml) of neat, 10^{-1} to 10^{-3} dilutions of untreated, heat-shocked samples and the boiled control were transferred into sterile Eppendorf tubes. To each tube 4 µl/ml of 10 mg/ml of ethidium bromide or 6.5 µl/ml of 0.3 mg/ml propidium iodide was added and left for 10 min. After which time the samples were centrifuged in a microcentrifuge (Micro Centaur, MSE, UK) at 14,000 g for 5 min. The supernatant was removed and the pellet washed with 1 ml of 0.1 M potassium phosphate buffer (pH 7.3) which removed any unbound ethidium bromide and prevented any background fluorescence, which may have affected the results. The samples were centrifuged (14,000 g, 5 min), the supernatant was removed and the pellet was resuspended in 1 ml of 0.1 M potassium phosphate buffer.

The fluorescence and the optical density value of each sample was recorded. The fluorescence was measured by a 6200 fluorimeter (Jenway, UK) with 610 nm emission filter and 530 nm excitation filter. The machine was blanked against 0.1 M potassium phosphate buffer and the control was set at 100.00 by using the neat boiled control, which emitted the most fluorescence. The fluorescence was read from a digital readout using 1 ml plastic cuvettes (1.5 ml grooved ps cuvettes

with a spectral range: 340 to 800 nm, Sigma-Aldrich). The optical density was read on a spectrophotometer (PU8625 UV/Vis, Philips) set at 650 nm and blanked against 0.1 M potassium phosphate buffer.

2.12. LEAKAGE OF 260 NM AND 280 NM ABSORBING MATERIAL.

Leakage of 260 nm (DNA) and 280 nm (protein) absorbing material is a sign of damage to the cell membrane. This material was detected by heat treating aliquots (2 ml) of stationary phase and exponential phase (Sections 2.7.1 & 2.7.2) *A. butzleri* NCTC 12481 cultures at 45°C, 50°C, and 55°C for 3 min (Section 2.10). Untreated and boiled controls were also tested. Aliquots (1 ml) were aseptically transferred to prelabelled Eppendorf tubes and centrifuged (MicroCentaurs, MSE, UK) at 14,000 g for 5 min. After which the supernatant was transferred to a 1 ml quartz cuvette and the optical density at 260 nm and 280 nm read on an UV/Vis spectrophotometer (PU8625 UV/Vis, Philips) preblanked against 0.1 M potassium phosphate buffer.

2.13. COLD STORAGE OF *A. butzleri* NCTC 12481.

The effect of cold storage (+4°C and -20°C) in BHI medium and phosphate buffer was studied. One millilitre of stationary and exponential phase cultures (Section 2.7.1) were diluted to 10^{-1} in PBS (9 ml), to dilute any nutrient carry over from the original BHI broth culture. After which 1 ml aliquots were aseptically transferred to 9 ml of BHI broth and 9 ml of 0.1 M potassium phosphate buffer (pH 7.3), thus producing 10^{-2} dilutions. The 10^{-2} dilution in BHI and phosphate buffer were aliquoted (2 ml) into duplicate sterile 15 ml centrifuge tubes. One set of tubes was placed at +4°C and the other set at -20°C. Samples were removed after 1, 3, 7, 14 and 21 days. The frozen samples were submerged in a water bath at 30°C for 5 min until the samples had thawed. The cold stored samples (1 ml) were then serially diluted in 0.1% peptone water (9 ml, Difco) from 10^{-1} to 10^{-10}. Aliquots (0.1 ml) of each dilution were spread over triplicate plates of selective (BGBA, CAT &

CCDA) and non-selective media (blood agar). The plates were incubated at 37°C for 48 h under microaerophilic conditions. After the appropriate incubation period between 30 and 300 colonies were counted. This plate count procedure was also used with the original untreated culture to compare with the cold stored samples.

2.14. ADAPTATION EXPERIMENTS.

2.14.1. HEAT ADAPTATION OF *A. butzleri* NCTC 12481.

Aliquots (2 ml) of stationary and exponential phase cultures (Sections 2.7.1 & 2.7.2) were aseptically transferred into five sterile capped small test tubes. A tube was left untreated at room temperature to act as a control to determine the original number of bacteria present. The second tube was heat treated at 55°C for 3 min (Section 2.10). The other 3 tubes were placed at 40°C for various time intervals (1-3 h) before being heat-treated (55°C, 3 min). After all the various treatments, the cultures were diluted from 10^{-1} to 10^{-10} in 0.1% peptone water and 200 µl of each dilution were inoculated in triplicate onto non-selective (blood agar) and selective agar plates (CAT & CCDA) using the Miles & Misra technique. Once the plates had dried they were incubated under the appropriate conditions (Section 2.3). This experiment was repeated using different exposure times (5, 10, 20, 30, 45 min) at 40°C, prior to heat treatment at 55°C. These experiments were replicated three times.

For exponential phase cells (Section 2.7.1) only, the experiment was repeated (three times), however the culture (2 ml aliquots) was pre-exposed to 40°C for 5, 30 and 45 min. Control tubes were set up whereby the *A. butzleri* NCTC 12481 cultures were pre-exposed to 40°C for equivalent periods of time, but were not heat-treated at 55°C afterwards. The samples were plated out and incubated under the same conditions as stated above.

2.14.2. COLD ADAPTATION OF *A. butzleri* NCTC 12481.

Stationary and exponential phase cultures were produced as in Section 2.7.1. The cultures (1 ml) were diluted 10 fold in BHI (9 ml) and 2 ml volumes were aliquoted

into nine sterile capped small test tubes (3" by ½"). One tube was left untreated at room temperature to act as the control, so that the original bacterial concentration could be determined. The second tube was put directly at +4°C and another directly at -20°C. The other 6 tubes were placed in an incubator (LEEC incubator, LEEC limited, Nottingham, UK) at 25°C for 1-3 h. At hourly intervals, 1 tube was place at +4°C and another at -20°C. All the tubes except the untreated control tube were left at the relevant temperatures for 24 h. After which the samples were serially diluted in 0.1% peptone water (10^{-1} to 10^{-10}). The samples were plated out and incubated under the same conditions as stated above (Section 2.14.1).

This experiment was repeated except the aliquots of the *A. butzleri* NCTC 12481 culture were incubated at 15°C (Gallenkamp illuminated cool incubator, Sanyo Gallenkamp Plc. UK) instead of 25°C. Also the samples were left for 30 min, 1, 3 and 5 h. The same untreated and cold shock controls were used as previously described. Both these experiments were replicated three times.

2.15. TETRAZOLIUM DYE ASSAY TO STUDY METABOLIC STATUS.

Stationary and exponential phase cultures were produced as in Section 2.7.1 and heat-treated as in Section 2.10. *E. coli* 0157:H7 (NCTC, PHLS, Colindale, London, UK) was used as a comparative organism. The *E.coli* cultures for this experiment were produced in the same way as *A. butzleri* NCTC 12481 (Section 2.7.1 & 2.10), except *E. coli* was grown in TSB at 37°C under aerobic conditions for 4 h for exponential phase (OD650 nm = 0.5) and 12 h for stationary phase cultures (OD650 nm = 1.0). Aliquots (0.3 ml) of untreated culture (control) or heat-treated culture were pipetted into duplicate sterile small test tubes (3" by ½") containing 0.01 M Hepes buffer (2.7 ml) and 0.1% tetrazolium dye (1 ml). A blank tube was set up containing 0.3 ml of untreated culture and 3.7 ml of 0.01 M Hepes buffer. Hepes buffer was stored at +4°C. Tetrazolium dye (2,3,5-triphenyltetrazolium chloride, 0.1% stock solution) was stored at +4°C in a dark container.

For experiments requiring more than five reading time points the volume in the tubes above was doubled. Prior to the heat treatment assays, control experiments were set up using different volumes of cell suspensions to ascertain the most appropriate volume for subsequent assays.

The tubes were placed into a water bath at 37°C for at least five minutes to allow the solution to temperature equilibrate. The assay reaction was started by adding 1ml of glucose solution (0.02 M) at 30 s intervals. At various timed intervals (10, 20, 30, 45, & 60 min) 1 ml samples were removed and the optical density value at 490 nm was read. The tube which did not contain tetrazolium dye was used to blank the spectrophotometer (PU8625 UV/Vis, Philips). Therefore, the turbidity of the solution did not affect the readings. This method was modified from Steward *et al.* (1991).

For later experiments this method was modified to determine if the decreasing volume or the introduction of oxygen to the samples affected the readings. The experiment was performed by leaving one assay tube in the spectrophotometer and taking readings every 10 s for between 10 and 60 min. Similar assays were performed except only 0.2 ml of glucose (0.1 M stock solution) was added instead of 1 ml (0.02 M stock solution). The difference in volume was corrected by increasing the volume of Hepes buffer from 2.7 ml to 3.5 ml.

For all experiments the optical density (650 nm) of each tube was measured, with the spectrophotometer being blanked against Hepes buffer. Thus enabling the microbial biomass of each tube to be determined.

2.16. NITRATE AND NITRITE REDUCTION BY *A. butzleri* SPECIES.

Precultures of all 4 *Arcobacter* strains were produced as in Section 2.7.1. The precultures were used to inoculate (2% inoculum) 0.1 mM nitrate-BHI based broths (Section 2.2.2). After 24 h incubation at 37°C the optical density (650 nm) and Gram stain were performed to check the purity of the cultures. Using a

spotting tile the broth cultures were tested for the presence of nitrate or nitrite by adding a drop of 1% sulphanilamide in 1 M HCl and 0.02% naphthylene diamine in 1 M HCl solutions to a drop of broth culture. If nitrite was present a deep pink or red colour developed and hence nitrate had been reduced. In the absence of a red colour a small amount of zinc dust was added. Zinc reduces nitrate to nitrite, which produced a red colour and hence determines if nitrate had been reduced further than nitrite. The 0.1 mM nitrate-BHI based broth cultures were used to inoculate (2%) 10 ml BHI broths containing 1 mM of nitrate. After 24 h at 37°C the purity was checked as before and the spot test was performed to determine the presence of nitrite or nitrate. These cultures were in turn used to inoculate (2%) 10 ml BHI broths containing 5 mM nitrate. Following the 24 h incubation period at 37°C, purity and spot tests were performed.

In later experiments, cultures grown in 1 mM nitrate-BHI based broths were also serially diluted in 0.1% peptone water (10^{-1} to 10^{-8}). Aliquots (0.1 ml) of 10^{-5} to 10^{-8} dilutions were spread onto duplicate pre-dried blood agar plates and incubated as in Section 2.3. This method determined the actual number of viable cells present, for comparison with the detection times given by the RABIT method. Neat and diluted cultures (10^{-1} to 10^{-5}) were used to inoculate (0.3 ml) triplicate sterile small test tubes (3 ml) of BHI broth (control), BHI broth with nitrite (1 mM) or with nitrate (5 mM). The tubes were inserted into RABIT conductance cells (Don Whitley Scientific, UK). The indirect conductance method was used, as described in Section 2.6.2. The tubes were inserted into temperature blocks preheated at 37°C and analysed using the same detection criteria as given in Section 2.6.2. After 48 h the cells were removed and the optical density (650 nm) of each tube containing the 10^{-1} dilution was measured to determine if the turbidity of the culture was comparable to the detection times recorded by the RABIT. It was also to check the purity of the cultures as pure *Arcobacter* cultures rarely have an optical density greater than 0.6. Also the nitrate or nitrite spot test was performed on the 10^{-1} dilution to determine if any nitrate or nitrite remained in the broths.

2.17. PREPARATIONS OF SAMPLES FOR CYTOTOXICITY SCREENING.

2.17.1. UNTREATED AND HEAT TREATED SAMPLES.

Stationary and exponential phase broth cultures were produced and harvested as in Sections 2.7.1 and 2.7.2. These cell suspensions were aliquoted (2 ml) into sterile small test tubes and heat-treated in a water bath at 45-60°C (5°C increments) for 3 minutes once the sample had reached the correct temperature (Section 2.10). An untreated control was also prepared. Only *A. butzleri* NCTC 12481 was tested in these preliminary toxicity experiments. From these experiments it was decided that only the untreated control and a 55°C heat-treated sample were required for future experiments. The next step was to determine the conditions, which optimised toxicity detection.

Using all 4 strains of *Arcobacter*, 10 ml aliquots were removed from 100 ml stationary or exponential phase broth cultures prior to harvesting the bacterial cells. It was divided into 2 ml volumes and heat-treated at 55°C for 3 min (Section 2.10). Aliquots (1 ml) were transferred into 1.5 ml Eppendorf tubes and centrifuged at 14,000 g for 5 min (MicroCentaur, MSE, UK). The BHI supernatant was removed, transferred to Eppendorf tubes labelled 'heat treated spent media' (spent media refers to the BHI broth in which *Arcobacter* cells had grown in, but the cells have been removed by centrifugation) and stored at -20°C until required. During the harvesting step (Section 2.7.2) the spent media was removed and stored at -20°C in Eppendorf tubes labelled 'untreated spent media'. The washed cells in 0.1 M potassium phosphate buffer were split into 5 ml samples. The first aliquot was left untreated, but the other aliquot was heat treated at 55°C as previously stated (Section 2.10). Both the heat-treated sample and untreated control were aliquoted into 1.5 ml Eppendorf tubes and centrifuged at 14,000 g for 5 min (MicroCentaur, MSE, UK). The supernatant was removed and transferred to Eppendorf tubes labelled 'untreated' or 'heat-treated' cell-free extract. The pellet was resuspended in 0.1 M potassium phosphate buffer (1 ml) and vortexed. The samples were bath sonicated (QH Kerry, Kerry Ultrasonics Ltd) on ice for 15 min and centrifuged at 8,000 g for 30 min (MicroCentaur, MSE, UK). The supernatant

was removed to Eppendorf tubes labelled 'untreated' and 'heat-treated' cell sonicate. Both the cell sonicates and the pellets were stored at -20°C. However, the pellets were not used in cytoxicity testing (see Figure 2.1, inserted in the front of this thesis). Before the samples could be used in the cytotoxicity assay they were thawed quickly and filter sterilised through 0.2 μm hollow fibre syringe filter (Dynagard, Microgon USA) to remove any cell debris and bacterial contaminants from the samples. A protein estimation assay (see Section 2.20.1) was also performed on the test samples before use, enabling them to be adjusted accordingly with 0.1 M phosphate buffer to obtain a protein concentration of approximately 0.3 mg ml^{-1}.

As well as fractionating the samples it was also important to determine if growth conditions had any effect on the detectable cytotoxicity levels of the type strain *A. butzleri* NCTC 12481. Therefore stationary and exponential phase cultures were grown under microaerophilic and aerobic conditions at 30°C and 37°C.

2.17.2. EFFECT OF HEAT AND PROTEOLYTIC ENZYMES.

To determine whether any cytotoxins were heat resistant or proteinaceous, the bacterial cell-free extracts were heat treated and exposed to proteolytic enzymes. Exponential phase cultures were produced and harvested as in Sections 2.7.1 and 2.7.2. Aliquots (4 ml) of the cell suspension were either left untreated or heat-treated at 55°C as in Section 2.10. The untreated and heat-treated samples were aliquoted (1 ml) into sterile capped small test tubes for further treatment. A tube of the untreated and a tube of the heat-treated samples were left without any further treatment. These samples acted as the control to determine the levels of cytotoxicity produced by each particular batch of *A. butzleri* NCTC 12481. One set of tubes were submerged into a boiling (100°C) water bath for 10 min. A proteolytic enzyme (0.1% (v/v) trypsin or 0.25% (v/v) pronase, both from Sigma-Aldrich Ltd, UK) was added to another set of tubes and incubated at 37°C for 30 min. After which time the samples were boiled (100°C) in a water bath for 10 min to de-activate the proteolytic enzymes. The last set of tubes was treated as above by exposing the sample to either of the proteolytic enzymes. However, after the 30 min incubation period the enzymes were neutralised by diluting (1:5) the sample with Dulbecco's Modified Eagles Medium (DMEM, see Section 2.18.2)

Figure 2.1: Preparation of untreated and heat treated samples for cytotoxicity screening.

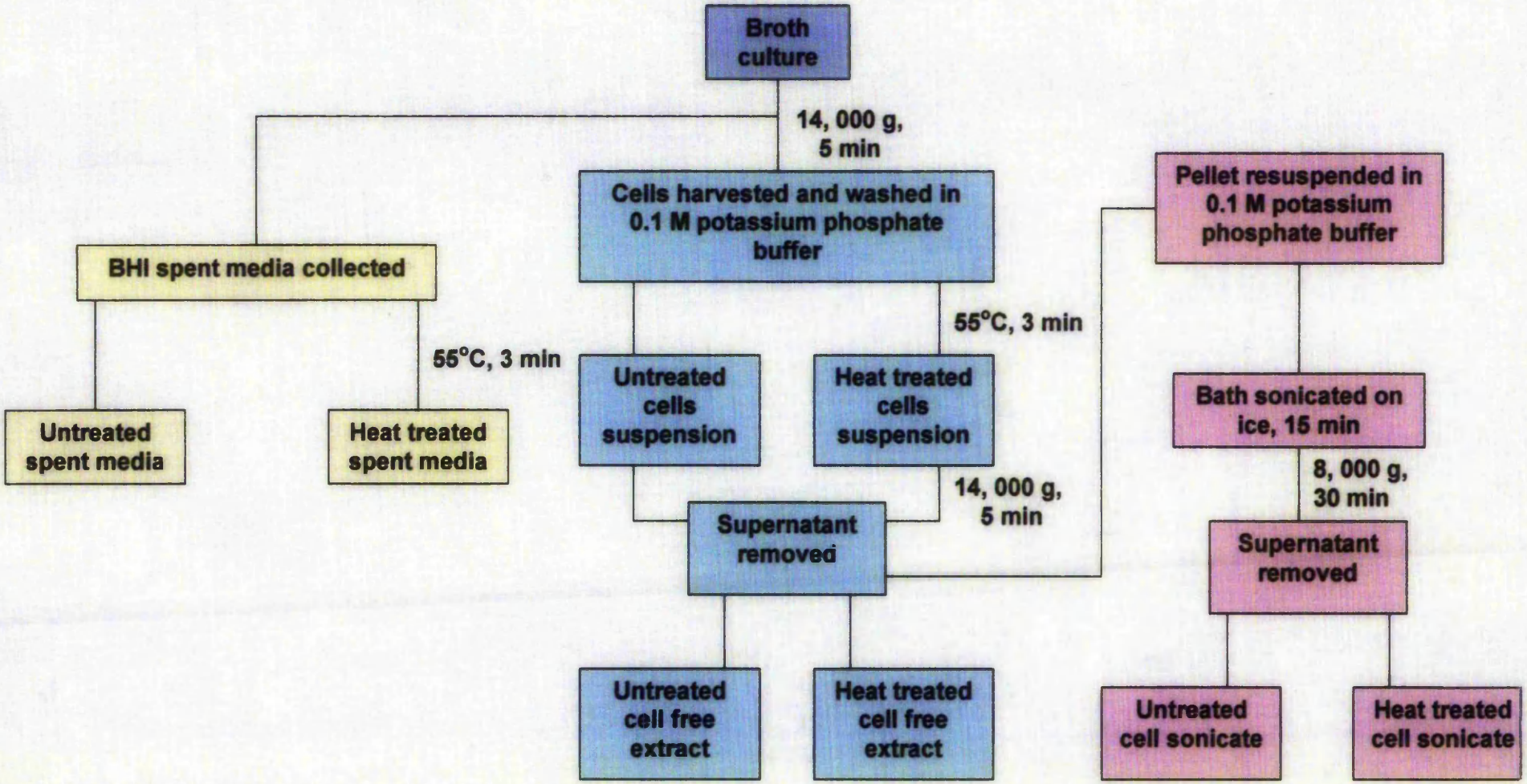
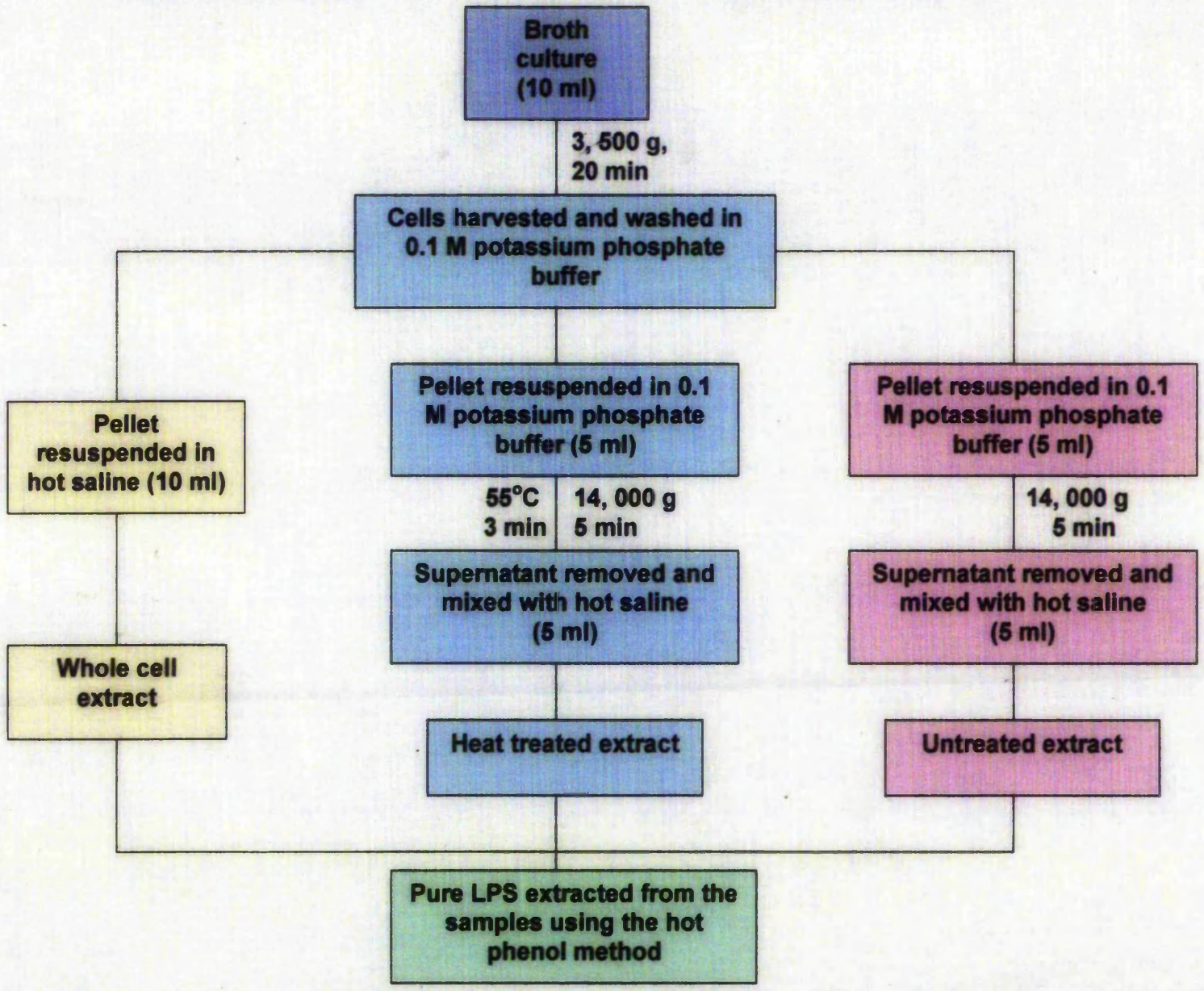


Figure 2.2: Preparation of samples for the extraction of pure LPS.



containing 10% foetal calf serum. All the extracts were allowed to cool for 30 min and filter sterilised (0.2 μm, Dynagard syringe filters) before being applied to mammalian cell cultures for cytotoxity assays. Potassium phosphate buffer (0.1 M) was used as a control to determine any cytotoxic effect of this buffer to the mammalian cells and was treated as per the samples (i.e. heat, trypsin).

2.17.3. EXTRACTION AND PURIFICATION OF LPS.

An exponential phase culture was produced as in Section 2.7.1. The culture was split into three 10 ml aliquots. All 3 samples were centrifuged at 3,500 g for 20 min (Centaur 1, MSE, R.J. Jenning, Nottingham, UK). The supernatant was removed and disposed of in the usual manner for infectious material. The pellets were resuspended in 20 ml of 0.1 M potassium phosphate buffer and recentrifuged (3,500 g, 20 min). The pellet of one aliquot was resuspended in 10 ml of hot (65-68°C) 0.85% saline. This sample was referred to as 'whole cell extract'. The other two aliquots pellets were resuspended in 0.1 M potassium phosphate buffer (5 ml). One aliquot was heat treated at 55°C for 3 min (Section 2.10). Once heat-treated this sample and the untreated sample were transferred (1 ml aliquots) into prelabelled 1.5 ml Eppendorf tubes and centrifuged 14,000 g for 5 min (MicroCentaur, MSE, UK). After this time the supernatant was removed and transferred into two tubes containing 5 ml of hot (65-68°C) 0.85% saline. One tube was termed 'heat-treated extract' the other was referred to as 'untreated extract'. To extract pure LPS from the samples the hot phenol method by Luderitz *et al.* (1982) was used (see Figure 2.2, inserted in the front of this thesis). An equal volume (10 ml) of hot 90% phenol (65-68°C, Merck, UK) was added to all three tubes, mixed vigorously and incubated for 15 min in a water bath at 65-68°C. The samples were centrifuged at 10,000 g for 15 min (Europa 24M, MSE, UK) to produce two layers. The upper aqueous phase was carefully removed. The phenol phase was mixed with an equal volume of hot saline (approximately 10 ml) and treated as stated above. The aqueous phases were combined and transferred to dialysis tubing (cellulose membrane dialysis tubing retaining all molecules weighing 12 kDa and greater, Sigma-Aldrich Ltd, UK). The tubing had been cut to length and boiled in distilled water with a few granules of EDTA for 1 h before use. The samples were dialysed in deionised distilled water (2 L) for 24 h at +4°C with gentle stirring (Magnetic stirrer, Analytical supplies Ltd, Derby). The

water was changed several times during this period. After this period of time the samples were freeze-dried (Edwards freeze-dryer, UK). The dried extracts were weighed and resuspended in 0.1 M potassium phosphate buffer (10 ml) to be used for cytotoxicity assays and analysed by gel electrophoresis.

2.18. CELL LINE MAINTENANCE AND STORAGE.

2.18.1. CELL LINES.

Mouse N2a neuroblastoma, Chinese Hamster Ovary (CHO) cells and Human Umbilical cord endothelial cells (ECV 304) cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK).

2.18.2. THAWING THE CELL LINE FROM LIQUID NITROGEN STORE.

Ten millilitres of fresh growth medium (500 ml Dulbecco's Modified Eagles Medium with sodium bicarbonate, 110 mg/L sodium pyruvate and 4500 mg/L glucose (DMEM), 50 ml foetal bovine serum (FBS, 10%), 5 ml glutamine, penicillin G and streptomycin (each 2 mM) was added to a sterile 50 ml tube. This tube was warmed at 37°C in an incubator. A vial of N2a, ECV, and CHO cell lines were retrieved from the liquid nitrogen store and thawed quickly in a water bath at 37°C. The entire contents of the vial was added to the fresh warmed growth medium and centrifuged for 7 min at 250 g (Labofuge 400e, Heraeus Instruments, Germany). The supernatant was carefully removed and the pellet was gently resuspended in 1 ml of fresh growth medium by 20 passes through a sterile pipette. All the resuspended cells were added to a T-25 vented flask containing approximately 5 ml of fresh growth medium. The cells were incubated for 24 h at 37°C in a humidified atmosphere of 95% (v/v) air: 5% (v/v) carbon dioxide in a Jouan IG150 incubator. After this time the growth medium was decanted from the upper surface of the flask to avoid detaching cells, and 5 ml of fresh growth medium was added again along the upper surface of the flask. The cells were incubated again under the same conditions until the cells were 60-80% confluent. At which stage the cells needed to be passaged (Section 2.18.3). After a single passage the cells were deemed fit for experimentation. A class II (Gelaire BSB 4) Laminar flow hood

was used at all times to ensure sterility of all procedures conducted with the cell lines.

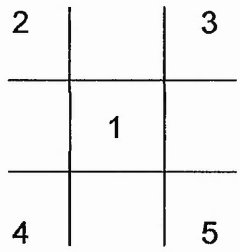
2.18.3. MAINTENANCE AND GROWTH OF THE CELL LINES.

ECV and CHO cell lines needed to be trypsin treated to remove the cells from the flasks. Trypsin-EDTA (10x, 200 mM) stock was diluted 1:10 in sterile PBS to produce a 1x solution. The growth medium (DMEM) was decanted from the upper surface of the flask and 1 ml of 1x Trypsin-EDTA was added to a T-25 vented flask (3 ml was used for T-75 vented flasks) to rinse any serum from the cells. The 1x Trypsin-EDTA was removed and 2 ml of fresh 1x Trypsin-EDTA was added to the flask (6 ml was used for T-75 vented flasks) and left for 5 min at 37°C or until the cells had detached from the surface of the flask. ECV cell lines may be more resistant to the effect of trypsin and hence the flask was gently tapped to release the cells. The reaction was blocked by adding 10 ml (30 ml for T-75 flask) of fresh growth medium (2-3 min), which neutralised the effect of the trypsin. The contents of the flask were transferred to a sterile 50 ml centrifuge tube and centrifuged at 250 g (Labofuge 400e, Heraeus Instruments, Germany) for 7 min. The supernatant was decanted into a waste beaker and the pellet was resuspended in 1 ml of fresh growth medium by 20 passes through a pipette. Care was taken not to froth the cell suspension. A 1/5 (1:40 dilution) of the cell suspension was transferred to a new flask, which contained either 5 ml (T-25) or 20 ml (T-75) of growth medium. The flasks were again incubated at 37°C in a Jouan IG150 incubator with a humidified atmosphere of 95% (v/v) air: 5% (v/v) carbon dioxide until the cells were 60-80% confluent (approximately 3-5 days) thus maintaining the cells in a logarithmic phase of growth. The same procedure was used for N2a cell lines except the cells were detached from the flask by jets of growth medium from a pipette over the surface of the cells instead of 1x Trypsin-EDTA. Care was taken not to scrape the surface of the flask.

2.18.4. PLATING OUT TISSUE CULTURE CELLS.

Cell lines passaged as described above, were enumerated by resuspending the cells in 1 ml of growth medium. The sample was diluted 10 fold in 0.4% trypan blue solution (180 μl trypan blue: 20 μl cell suspension) in an Eppendorf tube. The suspension was mixed well, so that the cells were evenly distributed. Ten

microlitres of the diluted cells was pipetted into a haemocytometer counting chamber (Neubauer haemocytometer 1/400 mm^2 (B.S.748)) and the number of cells within 5 large areas were counted, as shown below:-



Only viable (clear) cells were counted, any that had taken up the blue stain were considered to be dead. However, the cells were counted within 10 min of adding the cells to trypan blue, because after this time viable cells may begin to take up the dye. From these values, the volume of cell suspension required per plate can be calculated, using the following equation:-

Cell density/ml = Average per square x 10^4 x dilution factor.

10^4 = Conversion factor from mm^2 to cm^3, as each square has a volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equal to 1 ml the subsequent cell concentration per ml can be determined.

24-well plates were seeded with the following cell number:-

N2a cells = 50 000 cells/ml (25 000 cells/well)*
ECV cells = 25 000 cells/ml (12 500 cells/well)
CHO cells = 25 000 cells/ml (12 500 cells/well)

*A higher concentration of N2a cells was required as these cells were easily detached from the well surface when the growth media was changed.

Therefore, if 12 ml of fresh growth medium was prepared, with 600,000 (12 x 50,000) cells and 0.5 ml of this cell density was applied to a 24-well plate, then each well will contain 25,000 cells.

In a typical experiment in which 24 wells were plated out, a surplus of cells was normally prepared. Therefore, the volume required in 12 ml of growth medium would be as follows:-

Haemocytometer count of 5 squares = 406 cells

= 81.2 cells/square

Cell density = $81.2 \times 10^4 \times 10 = 8.12 \times 10^6$ cells/ml

= 8120 cells/μl

Volume required = 600 000 ÷ 8120 = 73.9μl in 12 ml

The required volume was added to the fresh growth medium. The cells and medium were immediately mixed to prevent clumping and then 0.5 ml volumes were added to each well of a 24-well plate. The plates were incubated at 37°C for 24 h as before to allow the cells to attach and recover. The growth medium in each well was carefully removed and 0.5 ml of fresh medium was added to each well. The cytotoxicity samples (Section 2.17.1 to 2.17.3) were applied to the cell lines. A concentration range of 10, 5 and 2.5% of the total volume per well was used i.e. 50 μl of the sample was applied to each well to produce a 10% applied concentration. The appropriate control was used according to what the samples were suspended in, i.e. sterile BHI for spent media samples and filter-sterilised 0.1 M potassium phosphate buffer for the other samples. The controls were applied at the same concentrations as the samples. These 24-well plates were re-incubated for a further 48 h.

The cells not being used in the experiment were subcultured by passaging 1/5 of the cell suspension into 5 ml in a T-25 flask, which was incubated as before.

2.18.5. MTT ASSAY.

The cytotoxicity of these samples was determined by using a MTT (methyl tetrazolium thiazolyl blue) assay (Sladowski *et al.*, 1993). This method assesses cell viability by a dye reduction assay, in which MTT dye intracellularly

accumulates in viable cells, where it is reduced primarily by mitochondrial enzymes from a yellow to a blue formazan product. Therefore, the lighter the blue colour the fewer viable cells that were present and hence the more toxic the sample was.

A 5 mg/ml stock of MTT was freshly prepared in sterile distilled water, which could be stored at -20°C for up to 2 weeks. MTT (50 μl) was added to each well and reincubated under standard conditions for 1 h. After 1 h the media was carefully removed without disturbing any cells, 1 ml of dimethyl sulphoxide (DMSO) was then pipetted into each well and the samples were agitated gently for approximately 2 min on a Titertek shaker (Flow Laboratories, UK). Care was taken not to leave the samples too long as the colour deepens with time resulting in inaccurate values. Aliquots (150 μl) from each well were transferred to a 96-well plate and the optical density was read at 570 nm using the Specta fluor (Tecan, UK Ltd Reading, UK) (Mosmann, 1983). The values were averaged and calculated as a percentage of the control sample to determine the level of toxicity.

2.18.6. FREEZING CELL LINES.

The cell monolayers were gently detached by using 1x Trypsin-EDTA or 20 passes through a sterile pipette, as stated in Section 2.18.3. The cell suspensions were transferred to sterile 50 ml centrifuge tubes and centrifuged for 7 min at 250 g (Labofuge 400e, Heraeus Instruments, Germany). The supernatant was decanted and the pellet was resuspended in 1 ml of freezing medium (65% (v/v) DMEM containing L-glutamine, penicillin G and streptomycin as stated before, 25% (v/v) foetal bovin serum & 10% (v/v) DMSO) by 3 or 4 passes through a sterile pipette. Care was taken not to froth the cell suspension. The resuspended cells were transferred to a cryovial, which was wrapped in tissue paper and placed at -70°C for 24 h. After which time the vial was transferred to a liquid nitrogen container, where it was stored until required.

2.18.7. MORPHOLOGY OF MAMMALIAN CELLS AFTER EXPOSURE TO *A. butzleri* NCTC 12481 CELL-FREE EXTRACT.

N2a, ECV and CHO cell lines were seeded into a 24-well plate (Section 2.18.4) and were allowed to recover for a period of 24 h at 37°C in a Jouan IG150 incubator with a humidified atmosphere of 95% (v/v) air: 5% (v/v) carbon dioxide. The growth medium was then replaced with 0.5 ml of fresh DMEM.

The untreated and heated cell-free extracts were applied to the cell lines at concentrations of 2.5 and 10% (v/v) of the total volume per well. Duplicate plates were re-incubated for a further 24 h and 48 h, after which time an MTT assay was performed on half of the plate (Section 2.18.5). The growth medium from the other half of the plate was discarded carefully to avoid disrupting the cell monolayer and the cells were fixed at -20°C for a minimum of 30 min in a fixing solution containing 90% (v/v) methanol in PBS. The fixed cells were then stained for 2 minutes at room temperature with Coomassie blue stain (1.25 g Coomassie blue-R250, 10% (v/v) glacial acetic acid, 40% (v/v) methanol 50% (v/v) distilled water) washed three times with distilled water and allowed to air dry overnight.

The total number of cells were determined in each of five random counting chamber fields (100 cells per field) per well, with a total of 3 wells per plate being counted using an inverted light microscope (Olympus Optical Company Ltd, London, UK). For each cell line different criteria was used depending on cell shape. For both CHO and ECV the cells were spilt into 3 categories, round, flat and elongated cells. Round cells were typically round in shape and had a smooth morphology, whereas flat cells were more flattened with an irregular morphology. Finally, elongated cells tended to be flattened with an extended process, which was twice the length of the cell body. This enabled the presence of any cytolethal distending or cytolethal rounding toxin to be identified in the culture extracts. For N2a cells the number of cells with axon-like processes which were greater than 2 cell body diameters in length were counted out of the total number of cells per field. These results were then averaged and converted into percentages of the total and a t test was performed to measure the significance. This experiment was replicated three times.

2.19. LIPOPOLYSACCHARIDE GEL ELECTROPHORESIS.

2.19.1. BUFFERS AND REAGENTS FOR THE ISOLATION OF LPS.

Lipopolysaccharide sample buffer 2x concentration.

Glycerol	4 ml
0.5 M Tris(hydroxymethyl)aminomethane	5 ml
10% Sodium dodecyl sulphate	8 ml
Deionised distilled water	2.6 ml
2-Mercaptoethanol	400 µl
Bromophenol Blue powder	10 mg

All reagents were mixed together except the 2-mercaptoethanol, approximately 10 mg bromophenol blue powder was added to the solution and dissolved by shaking. The buffer was dispensed into 2.5 ml aliquots and stored at -20°C. Before use 50 µl of 2-mercaptoethanol was added to each 2.5 ml aliquot whilst working in the fume cupboard. Once the 2-mercaptoethanol had been added, the remainder of the solution was stored at +4°C and used within a week.

PROTEINASE-K SOLUTION.

Proteinase-K	2.5 mg
1x LPS sample buffer	1 ml

1 ml of 2x LPS sample buffer was mixed with 1 ml of deionised distilled water to produce a 1x concentration. The Proteinase-K was dissolved in the 1x LPS sample buffer.

ISOLATION OF LPS FROM UNTREATED AND HEAT SHOCKED CELL-FREE EXTRACTS.

Untreated and heat treated cell-free extracts which had already been tested for cytotoxicity, were analysed further to determine both their LPS and protein content (Section 2.20). To isolate pure LPS from these samples Proteinase-K digestion was used (Hitchcock & Brown, 1983). To 1 ml of sample a 100 µl of 2x sample buffer was added and boiled for 10 min, 1.5 ml Eppendorf tubes were used which

had a hole pricked in the top. After which 100 μl of deionised distilled water was added together with 40 μl of Proteinase-K in 1x sample buffer. The sample was mixed thoroughly and incubated at 60°C for 3 h. Samples were then cooled to room temperature, mixed and aliquoted in 100 μl volumes and stored at -20°C until required. The whole procedure was carried out in a fume cupboard as the 2x and 1x sample buffers both contain 2-mercaptoethanol, which is toxic. Frozen samples were allowed to thaw to room temperature, then before analysis the samples were boiled and mixed as freezing causes LPS dimerization and micelle formation.

LIPOPOLYSACCHARIDE SOLUBILISATION BUFFER FOR PURIFIED LIPOPOLYSACCHARIDE.

Tris(hydroxymethyl)aminomethane	0.757 g
Glycerol	10 ml
2-Mercaptoethanol	5 ml
Sodium dodecyl sulphate	3 g
Bromophenol Blue	0.01%
Deionised distilled water	up to 100 ml

Tris(hydroxymethyl)aminomethane was dissolved in approximately 80 ml deionised distilled water, adjusted to pH 6.8 with 0.1 M HCl and the volume made up to 100 ml. In a fume cupboard, glycerol, sodium dodecyl sulphate, 2-mercaptoethanol and 0.01% bromophenol blue was dissolved in 50 ml of Tris(hydroxymethyl)aminomethane solution and then made up to 100 ml with Tris(hydroxymethyl)aminomethane solution. The solubilisation buffer was aliquoted into 10 ml volumes, with the aliquot in current use being stored at +4°C and the remaining aliquots at -20°C.

PREPARATION OF PURIFIED LPS.

To ensure the electrophoresis and staining procedures were working purified *Salmonella* Typhimurium LPS (extracted by the hot phenol water method) was purchased from Sigma-Aldrich Chemicals. The purified LPS was dissolved in deionised distilled water to a 1 mg/ml concentration. Once dissolved it was mixed

with an equal volume of solubilisation buffer and boiled for 5 min in 1.5 ml Eppendorf tubes with holes pricked into the top. The samples were allowed to cool to room temperature and aliquoted into 20 μl volumes. Samples were stored at -20°C until required.

2.19.2. SDS-POLYACRYLAMIDE ELECTROPHORESIS FOR LPS ANALYSIS.

LOWER GEL BUFFER FOR SDS POLYACRYLAMIDE GEL.

Tris(hydroxymethyl)aminomethane (90.87 g) was dissolved in approximately 400 ml deionised distilled water, and adjusted to pH 8.8 with concentrated HCl. The solution was made up to 500 ml with deionised distilled water and stored at +4°C.

12.5% RESOLVING GEL (LOWER GEL).

Lower gel buffer	5 ml
30% Acrylamide:bisacrylamide	8.3 ml
Deionised distilled water	6.7 ml
TEMED	10 μl
Fresh 10% ammonium persulphate	60 μl

All the reagents were mixed together except TEMED and ammonium persulphate. The mixture was de-gased for approximately 5 min, then TEMED and ammonium persulphate were added in the fume cupboard. The mixture was swirled gently and used immediately.

UPPER GEL BUFFER FOR SDS POLYACRYLAMIDE GEL.

Tris(hydroxymethyl)aminomethane (30.29 g) was dissolved in approximately 400 ml deionised distilled water, and adjusted to pH 6.8 with concentrated HCl. The solution was made up to 500 ml with deionised distilled water and stored at +4°C.

4.5% STACKING GEL (UPPER GEL).

Upper gel buffer	2.5 ml
30% Acrylamide:bisacrylamide	1.5 ml
Deionised distilled water	6.0 ml
TEMED	10 µl
Fresh 10% ammonium persulphate	30 µl

All the reagents were mixed together except TEMED and ammonium persulphate. The mixture was de-gased for approximately 5 min, then TEMED and ammonium persulphate were added in the fume cupboard. The mixture was swirled gently and used immediately.

RUNNING BUFFER NUMBER 1.

Tris(hydroxymethyl)aminomethane	6.06 g
Glycine	28.80 g
Sodium dodecyl sulphate	2.00 g

Tris(hydroxymethyl)aminomethane, glycine and sodium dodecyl sulphate were dissolved in 1.8 L of deionised distilled water, then made up to 2 L with deionised distilled water. Adjusted to pH 8.3 if required with 0.1 M HCl, but it was not usually necessary and stored at +4°C for several weeks.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS PAGE) FOR LPS.

One-dimensional SDS-PAGE was performed with a BIORAD miniprotean® II electrophoresis cell kit (Bio-Rad laboratories, UK). The gel plates were cleaned with detergent and tap water, rinsed with distilled water and ethanol prior to assembly. Once assembled (according to manufacturer's instructions) and clamped onto the gel casting stand the 12.5% (w/v) acrylamide resolving gel was prepared as stated above and pipetted between the 2 glass plates (to within 1 cm of the bottom of the well comb). Care was taken not to introduce air into the mixture. The gel was covered with a layer of water saturated butan-2-ol to prevent shrinkage. Once the resolving gel had polymerised (approximately 40 min) the layer of butan-2-ol was rinsed off using deionised distilled water. Any excess

water was absorbed with filter paper (Whatman number 1). The resolving gel was then overlaid with a 4.5% (w/v) stacking gel and a 10 toothed comb placed between the plates to produce the sample wells. The combs were removed after approximately 20 minutes once the stacking gel had polymerised.

The polymerised gels and clamp were removed from the casting stand and loaded into the inner cooling core of the electrophoresis apparatus and then into the running buffer tank. The gels were totally submerged in running buffer making sure that the upper buffer chamber between the 2 gels was completely full. Before the samples, pure LPS control and molecular markers were loaded they were mixed with an equal volume of Laemmli sample buffer (2x concentration, Sigma-Aldrich, Poole, UK), boiled for 10 min, and vortexed. Five microlitre of the molecular markers (Silver stain markers 6.5 kDa-180 kDa, Sigma-Aldrich, Poole, UK) and 10 μl of the purified LPS and sample LPS were required to produce a banding profile. The gels were run at 50 mA per gel until the dye front had just reached the bottom of the gel (approximately 35 min) using a PowerPac 300 (Bio-Rad laboratories, UK).

One of the duplicate gels was stained with Coomassie brilliant blue (Section 2.20.4) to determine the effectiveness of the Proteinase-K digestion to denature the proteins in the sample. The other gel was silver stained (Section 2.19.3) to visualise the LPS profiles.

2.19.3. SILVER STAINING TECHNIQUE.

LPS profiles were detected by a modified Tsai & Frasch (1982) carbohydrate silver staining procedure (PHLS Colindale, London UK).

SILVER STAIN FIXING SOLUTION.

Methanol	200 ml
Glacial acetic acid	25 ml
Deionised distilled water	275 ml

All the reagents were mixed together and stored in a dark reagent bottle for 2 days at room temperature.

SILVER STAIN OXIDISING SOLUTION.

Periodic acid (0.75 g) was dissolved in 100 ml fixing solution. This reagent was always made up fresh in a dark reagent bottle.

SILVER STAINING SOLUTION.

Silver nitrate in 5 ml deionised distilled water	1 g
0.1 M sodium hydroxide	28 ml
(0.2 g in 50 ml deionised distilled water)	
Ammonium hydroxide	2 ml

In a fume cupboard all the ingredients were pre-measured and weighed out and then stored at +4°C until they were ready to be used. Immediately prior to making the solution the deionised distilled water (5 ml) was added to the silver nitrate in a dark reagent bottle and dissolved by gentle shaking. Sodium hydroxide was added to a 250 ml dark reagent bottle containing a magnetic stirring bar. Whilst mixing the ammonium hydroxide and the silver nitrate solution were added in a dropwise manner. To the solution 115 ml of deionised water was added, mixed and used within 5 min of preparation. The solution should be clear and was not used if a dark brown precipitate formed.

SILVER STAIN DEVELOPING SOLUTION.

Citric acid	50 mg
Formalin	0.5 ml
Deionised distilled water	1000 ml

The citric acid was dissolved in the deionised distilled water in a dark reagent bottle. The formalin was added and mixed well. A fresh solution was prepared every time.

LIPOPOLYSACCHARIDE CARBOHYDRATE SILVER STAINING.

1. A polyacrylamide gel was removed from the clamps and placed into a large clean polystyrene weigh boat (14 x 14 cm, Fisher Scientific, UK). Fixing solution (50 ml) was poured over the gel and another inverted weigh boat was applied as a lid. The gel was shaken gently on a flat bed shaker (IKA labortechnik, Germany) at room temperature overnight (18 h).
2. The fixing solution was discarded, 50 ml oxidising solution was added and gently mixed on a shaker for 5 min.
3. The oxidising solution was removed and the gel was washed 3 times, in 80 ml deionised distilled water whilst shaking gently, 15 min for each wash. Making sure that the gel was fully immersed.
4. During the last washing step, the silver stain was prepared and 50 ml poured into another clean weigh boat. The gel was carefully placed into the staining solution and mixed gently on the shaker for 10 min.
5. The staining solution was discarded and the gel was washed 3 times, in 80 ml deionised distilled water, 10 min for each wash.
6. During the last washing step, the developing solution was prepared and 80 ml placed into a clean weigh boat. The gel was transferred to the developing solution and gently rocked whilst being held over a light box (Whatman, UK). The bands would appear within 2-5 min.
7. The reaction was stopped with copious amounts of deionised distilled water.

LPS profiles were recorded, with Grab-it version 2.5 (Windows 95/NT) software or by photography.

2.20. SDS- POLYACRYLAMIDE ELECTROPHORESIS FOR PROTEIN ANALYSIS.

2.20.1. PROTEIN ESTIMATION.

Protein estimation was performed using a Bio-Rad DC protein estimation assay which is a colourimetric assay following detergent solubilisation. The reaction is equivalent to the well-documented Lowry *et al.* (1951) assay. A calibration curve

was produced by preparing bovine serum albumin (BSA) standards in 0.1 M potassium phosphate buffer (preliminary experiments determined that potassium phosphate buffer was compatible with the reagents in the assay). From a BSA 1.4 mg/ml stock solution, 1 mg/ml to 0.2 mg/ml (0.2 mg/ml increments) standards were produced. Potassium phosphate buffer (pH 7.3) was used as the control. Aliquots (5 μl) of the standards and the samples were pipetted into triplicate wells of a 96-well flat bottom plate (Falcon Flexible assay plate). Reagent A, an alkaline copper tartrate solution (25 μl) was added to each well followed by Reagent B, a dilute Folin Reagent (200 μl). Care was taken whilst pipetting to avoid cross-contamination of the sample wells. The 96-well plate was agitated gently on a flat bed shaker (IKA labortechnik, Germany) for 5 min to mix the reagents. After a further 10 min development time the samples were read at 750 nm (3 light flashes) using the Specta fluor (Tecan, UK Ltd Reading, UK). The calibration curve was plotted (OD 750 nm vs. mg/ml protein) using the Microsoft Excel package. The equation of the line was calculated using linear regression analysis. Subsequently the protein concentration (mg/ml) was determined.

2.20.2. ACETONE PRECIPITATION.

Occasionally the amount of protein was too low to apply the required 10 μg. In these cases an acetone precipitation was needed to increase the protein concentration by 10 fold. A 100 μl of sample was mixed with 900 μl of acetone in a 1.5 ml Eppendorf tube and incubated at -20°C overnight. The samples were centrifuged at 14,000 g for 20 min (MicroCentaur, MSE, UK). The supernatant was carefully decanted, so as not to disturb the pellet of precipitated proteins. The pellets were air dried for 30 min to remove any residual acetone. The pellets were resuspended in 20 μl of SDS-PAGE Laemmli sample buffer (Sigma-Aldrich, Poole. UK) boiled for 10 min and applied to a gel.

2.20.3. BUFFERS AND REAGENTS FOR PROTEIN GEL ELECTROPHORESIS.

LOWER GEL BUFFER.

Tris(hydroxymethyl)aminomethane (90.86 g) was dissolved in approximately 700 ml of deionised distilled water and adjusted to pH 8.8 with 1 M HCl, before adding sodium dodecyl sulphate (2.0 g). Once dissolved the solution was made up to 1 L with deionised distilled water and stored at +4°C.

10% RESOLVING GEL (LOWER GEL).

Lower gel buffer	17.5 ml
30% Acrylamide:bisacrylamide	11.6 ml
Deionised distilled water	4.0 ml
TEMED	50 µl
Fresh 10% ammonium persulphate	175 µl

All the reagents were mixed together except TEMED and ammonium persulphate. The mixture was de-gased for approximately 5 min, then TEMED and ammonium persulphate were added in the fume cupboard. The mixture was swirled gently and used immediately.

UPPER GEL BUFFER.

Tris(hydroxymethyl)aminomethane (15.14 g) was dissolved in 300 ml of deionised distilled water and adjusted to pH 6.8 with 1 M HCl, before adding sodium dodecyl sulphate (1.0 g). Once dissolved the solution was made up to 500 ml with deionised distilled water and stored at +4°C.

4% STACKING GEL (UPPER GEL)

Upper gel buffer	5.0 ml
30% Acrylamide:bisacrylamide	1.3 ml
Deionised distilled water	3.2 ml
TEMED	20 µl
Fresh 10% ammonium persulphate	50 µl

All the reagents were mixed together except TEMED and ammonium persulphate. The mixture was de-gased for approximately 5 min, then TEMED and ammonium persulphate were added in the fume cupboard. The mixture was swirled gently and used immediately.

PROTEIN GEL ELECTROPHORESIS.

See Section 2.19.2. except a 4% acrylamide stacking gel and a 10% acrylamide resolving gel were used.

2.20.4. COOMASSIE BLUE STAINING TECHNIQUE.

COOMASSIE BLUE FIXING AND STAINING SOLUTION.

Coomassie Brilliant Blue stain	0.25 g
Methanol	500 ml
Glacial acetic acid	25 ml
Deionised distilled water	up to 450 ml

The stain was dissolved in the methanol, glacial acetic acid added and made up to 1 L with deionised distilled water. Stored at room temperature for several weeks.

COOMASSIE BLUE DESTAIN SOLUTION.

Glacial acetic acid	100 ml
Deionised distilled water	900 ml

All the reagents were mixed together in a fume cupboard and stored at room temperature for several weeks.

COOMASSIE BLUE PROTEIN STAIN

The polyacrylamide gel was removed from the clamps and placed into a large polystyrene weigh boat (14 x 14 cm, Fisher Scientific, UK) containing 100 ml Coomassie blue stain as stated above. The gel was gently agitated (IKA Labortechnik, K250 basic shaker) at room temperature for 1 h, to allow the gel to take up the stain. After 1 h the protein bands were visualised on a light box

(Whatman, UK). In order to remove background colour the gel was placed into a destaining solution on a shaker overnight (18 h)

The gels were recorded, with Grab-it version 2.5 (Windows 95/NT) software or by photography.

2.21. THE DETECTION OF CD14 RECEPTORS ON THE SURFACE OF N2A, ECV AND CHO CELLS.

2.21.1. GEL ELECTROPHORESIS AND WESTERN BLOTTING FOR THE DETECTION OF CD14.

2.21.1.1. PREPARATION OF CELL LINES EXTRACTS FOR GEL ELECTROPHORESIS AND WESTERN BLOTTING.

All 3 cell lines (N2a, ECV & CHO) were plated out at an initial density of 50,000 cells/ml into sterile T-25 vented tissue culture flask (Section 2.18.4). The cells were incubated for 48 h at 37°C in a Jouan IG150 incubator with a humidified atmosphere of 95% (v/v) air: 5% (v/v) carbon dioxide. The cell monolayers were rinsed gently with ice cold DMEM, care was taken not to disrupt the cells which were then resuspended in 1 ml of 0.1% (w/v) sodium dodecyl sulphate in PBS, boiled for 1 min and placed in an Eppendorf tube. The cell suspension was vortexed for 1 min and 5 μl volumes were taken for protein estimation (Section 2.20.1). The protein content of the human blood and serum controls were also calculated.

Once the protein content had been determined the samples were mixed with an equal volume of Laemmli sample buffer (100 μl, 2x concentration, Sigma-Aldrich, Poole, UK), boiled for 10 min, and centrifuged for 1 min at 10,000 g (MicroCentaur, MSE, UK). Denatured proteins were stored at -20°C until required.

2.21.1.2. SEPARATION OF CELL EXTRACT PROTEINS BY GEL ELECTROPHORESIS.

BUFFERS AND REAGENTS FOR PROTEIN GEL ELECTROPHORESIS.

7.5% RESOLVING GEL (LOWER GEL).

1.5 M Tris-HCl pH 8.8	2.50 ml
40% Acrylamide:bisacrylamide	1.88 ml
Deionised distilled water	5.52 ml
10% Sodium dodecyl sulphate	100 µl
TEMED	10 µl
Fresh 10% ammonium persulphate	100 µl

All the reagents were mixed together except TEMED and ammonium persulphate. The mixture was de-gased for approximately 5 min, then TEMED and ammonium persulphate were added in the fume cupboard. The mixture was swirled gently and used immediately.

4% STACKING GEL (UPPER GEL).

0.5 M Tris-HCl pH 6.9	2.5 ml
40% Acrylamide:bisacrylamide	1.0 ml
Deionised distilled water	6.4 ml
10% Sodium dodceyl sulphate	100 µl
TEMED	10 µl
Fresh 10% ammonium persulphate	100 µl

All the reagents were mixed together except TEMED and ammonium persulphate. The mixture was de-gased for approximately 5 min, then TEMED and ammonium persulphate were added in the fume cupboard. The mixture was swirled gently and used immediately.

One-dimensional SDS-PAGE was performed as in Section 2.19.2 except a 7.5% (w/v) resolving gel and a 4% (w/v) stacking gel (Laemmli, 1970) were used.

Before separation by SDS-PAGE, the previously prepared cell extracts, controls and molecular markers (pre-stained molecular weight standards ranging from 30 – 120 kDa) were boiled for 10 min, vortexed and loaded into the stacking gel. For the cell line samples 50 μg of protein was loaded, whereas for the human blood and serum controls only 10 μg was required to produce ladder-like banding profiles. The gels were run at 40 mA per gel (approximately 45 min) until the dye front had reached about 1 cm from the bottom of the gel, using the Bio-Rad PowerPac 300 (Bio-Rad Laboratories, UK). Duplicate gels were produced, one gel was Coomassie blue stained (Section 2.20.4) to visualise the protein banding profiles, whereas the other gel was used to electrophoretically transfer the proteins to a nitrocellulose membrane.

2.21.1.3. WESTERN BLOTTING OF CELL EXTRACTS SEPARATED BY SDS-PAGE.

PROTEIN ELECTROBLOTTING BUFFER.

Tris(hydroxymethyl)aminomethane (5.81 g) was dissolved in a mixture of methanol (200 ml) and deionised distilled water (800 ml). To this solution 2.89 g of glycine and 0.315 g of sodium dodecyl sulphate were added.

THE ELECTROBLOTTING PROTOCOL FOR PROTEIN.

Electroblotting was performed using a Trans-Blot® SD semi-dry transfer cell (Bio-Rad Laboratories, UK) with a Mode 200/2.0 power supply power pack (Bio-Rad Laboratories, UK). The graphite electrode of the transfer unit was soaked with distilled water and the excess was removed with tissue paper. A total of 12 sheets of filter paper (3MM Chr chromatography paper, Whatman International Ltd., UK), cut slightly larger than the gel (8 cm x 9 cm) were required for the transfer. All the sheets of filter paper were soaked in the electroblotting buffer and 6 sheets were transferred one at a time to the anode electrode. Air bubbles were removed by gently rolling a glass rod over the surface of the filter paper. A sheet of nitrocellulose membrane (Cellulose nitrate, 0.45 μM ba85, Schleicher and Schull, Anderman, UK) cut to the size of the gel was soaked in transfer buffer and placed on top of the pieces of filter paper. Any bubbles were removed and the

electrophoresed gel (pre-soaked in electroblotting buffer for 5 min) was placed onto the nitrocellulose membrane. Another 6 pre-soaked pieces of filter paper were carefully placed on top of the gel and any air bubbles removed. The cathode was placed on top and connected to the power supply and the components were transblotted at 45 mA constant current for 1.5 h. To determine the efficiency of the transfer of proteins from the gel to the nitrocellulose, the Western blot was submerged in copper phthalocyanine 3,4',4",4"' tetrasulphonic acid (tetrasodium salt) staining solution (0.05% (w/v) copper phthalocyanine 3,4',4",4"' tetrasulphonic acid in 12 mM hydrochloric acid in distilled water) for 2 min, after which time the protein bands on the nitrocellulose membrane would have taken up the stain. The blot was destained by submerging it into a 12 mM sodium hydroxide solution (Bickar & Reid, 1992).

IMMUNOPROBING OF WESTERN BLOTS

The unbound sites on the resultant Western blot were blocked by incubating at room temperature in 80 ml of 3% (w/v) bovine serum albumin (BSA) in a TBS solution (BSA/TBS, 5 mM Tris, 20 mM altered to pH 7.4 with 1 M HCl) for at least 1 h. The blot was then incubated in 40 ml of CD14 (M-305) rabbit polyclonal IgG primary antibody (1:500 dilution in BSA/TBS, Autogen Bioclear, UK Ltd.). The blot was incubated at +4°C overnight (18 h). To remove any unbound primary antibody the blot was washed 6 times, for 10 min each in 40 ml of a TBS/Tween solution (0.05% (v/v) Tween-20 in 1x TBS). The blot was then probed in 40 ml of an anti-rabbit IgG whole molecule alkaline phosphatase conjugate (antibody developed in goat, 1:1000 in BSA/TBS, Sigma-Aldrich, Poole, UK) at room temperature for 2 h. To remove any unbound secondary antibody the blot was washed (6 x 10 min) in 40 ml of TBS/Tween. The blot was allowed to equilibrate in 80 ml substrate buffer (0.75 M Tris-base, pH 8.5) for 2 min, after which time the developer was added (33 µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) [50 mg BCIP in dimethyl formamide (DMF), and 44 µl nitro blue tetrazolium (NBT) [75 mg NBT in 70% (v/v) DMF] added to 20 ml of substrate buffer). Colour development of any antibody-antigen binding was visualised after approximately 30 min. The reaction was stopped by excessive amounts of deionised distilled water and the blot was dried between 2 pieces of clean filter paper overnight (18 h). The blots

were recorded by Grab-it version 2.5 (Windows 95/NT) software or by photography.

2.21.2. DECTECTION OF CD14 RECEPTORS ON N2A, ECV AND CHO CELL LINES USING THE ELISA TECHNIQUE.

N2a, ECV and CHO cells were plated out at an initial density of 50,000 cells/ml in a sterile 24-well plate (Section 2.18.4) and grown for 48 h at 37°C in a Jouan IG150 incubator with a humidified atmosphere of 95% (v/v) air: 5% (v/v) carbon dioxide. After which time the growth media was carefully removed, to avoid disturbing the cell monolayer and fixed in 4% (v/v) formalin in PBS for 10 min. The cells were then washed 3 times with PBS. The mammalian cells act as an antigen layer and so each well was blocked with 800 μl of blocking solution (3% (v/v) BSA in PBS) at 37°C for 3 h. The wells were aspirated and the plates dried by inversion on absorbent paper. Aliquots (200 μl) of the CD14 (M-305) rabbit polyclonal IgG primary antibody (1:200 dilution in BSA/PBS, Autogen Bioclear, UK Ltd.) were pipetted into triplicate wells and the plate was incubated overnight (18 h) at +4°C. Controls wells were also set up on the plate whereby both or either of the primary or secondary antibody were absent and replaced by blocking solution. The wells were washed out thrice with PBS/Tween (0.05% (v/v) Tween-20 in PBS) followed by a further 3 washes with deionised distilled water. The plates were dried by inversion onto absorbent paper.

Aliquots (200 μl) of anti-rabbit IgG whole molecule alkaline phosphatase conjugate (antibody developed in goat, 1:1000 in BSA/PBS, Sigma-Aldrich, Poole, UK) were pipetted into the appropriate wells and the plate was incubated at room temperature for 2 h whilst shaking on a flat bed shaker (IKA labortechnik, Germany). The wells were again washed thrice with PBS/Tween followed by a further 3 washes with deionised distilled water. The plate was dried by inversion onto absorbent paper. A 400 μl aliquot of the substrate solution (1 mg/ml paranitrophenyl phosphate in 0.1 M glycine buffer [0.1 M glycine, 1 mM MgCl, 1 mM ZnCl, pH 10.4 with 1 M NaoH]) was pipetted into each well. It took 1 h for the yellow colour to develop. The reaction was stopped with 3 M sodium hydroxide

(200 μl). Aliquots (150 μl) were transferred to a 96-well plate and the optical density was read at 405 nm using the Specta fluor (Tecan, UK Ltd Reading, UK).

The above method was repeated, except goat anti-rabbit IgG conjugated to horseradish peroxidase conjugated secondary antibody (1:1000 dilution in BSA/PBS, Dako, P0448) was used. This meant that a horseradish peroxidase substrate solution (20 ml 0.1 M sodium acetate buffer [pH 6.0 using 0.1 M acetic acid], 150 μl TMB [10 mg 3,3',5,5'-tetramethylbenzidine (TMB) in 1 ml dimethylsulphoxide (DMSO) stored at +4°C in the dark] and 8 μl, 30% (v/v) hydrogen peroxide) was required to develop the antigen-antibody binding. It took 1 h for the colour to develop. The reaction was stopped by adding 200 μl of 2.5 M sulphuric acid, which turns the samples yellow. Aliquots (150 μl) were transferred to a 96-well plate and the optical density was read at 450 nm using the Specta fluor (Tecan, UK Ltd Reading, UK).

Once the optical density had been read. The triplicate values were averaged and the controls added together and subtracted from the samples to remove any background colour due to non-specific binding of the antibodies.

2.21.3. FLOW CYTOMETRY TO DETERMINE THE LEVELS OF CD14 RECEPTORS ON THE SURFACE OF N2A, ECV, AND CHO CELLS.

N2a, ECV and CHO cells were plated out at an initial density of 50,000 cells/ml into sterile T-25 vented culture flasks (Section 2.18.4). The cells were grown for 48 h at 37°C in a Jouan IG150 incubator with a humidified atmosphere of 95% (v/v) air: 5% (v/v) carbon dioxide. Using a sterile cell scraper the cells were scraped from the surface of the flask, transferred to a sterile 50 ml centrifuge tube and centrifuged at 500 g (Mistral 2000R, MSE, UK) for 3 min at +4°C. The cells were washed twice in a 1x PBS solution (PBS with 0.1% (w/v) BSA, 0.02% (w/v) NaN_3) and resuspended in 1 ml 1x PBS solution. Twenty microlitres of each cell sample was diluted in 180 μl of 0.4% trypan blue solution and the cell number was counted in a haemocytometer counting chamber (Neubauer haemocytometer 1/400 mm^2 (B.S.748)).

Aliquots containing 2 x 10^5 cells were pipetted into polystyrene round bottomed culture tubes (12 x 75 mm, Elkay Laboratory Products (UK) Ltd.). Several control tubes were set up, whereby the mammalian cells were not exposed to a primary or secondary antibody. These tubes were centrifuged (500 g, 3 min, Mistral 2000R, MSE, UK) and the pellets were resuspended in 200 µl Sheath fixing solution (0.5% (v/v) formaldehyde in deionised distilled water). This control acts as a negative control as it will not produce any fluorescence. A positive control was set up, whereby monocytes from fresh blood (kindly donated by Professor R. Rees, The Nottingham Trent University) were used instead of the sample cell lines. This was an ideal positive control as CD14 receptors are found on the surface of monocytes (El-Samalouti *et al.*, 1999). Therefore, if the antibodies were able to bind to an appropriate receptor a high level of fluorescence would be detected. Two further controls were used to determine if any non-specific binding was occurring with either the primary antibody or secondary antibody. These controls were set up by substituting 1x PBS solution or an isotype control (normal rabbit IgG, Autogen Bioclear, UK Ltd) for the primary antibody, but the secondary was used at the normal dilutions. To the remaining tubes a 100 µl of CD14 (M-305) rabbit polyclonal IgG primary antibody (Autogen Bioclear, UK Ltd.) at various dilutions (1:100, 1:200, 1:500, and 1:100 in 1x PBS solution) was added to the cells. Following the 20 min incubation period at room temperature the cells were washed twice by adding 500 µl of 1x PBS solution and centrifuging in the culture tubes at 500 g for 3 min (Mistral 2000R, MSE, UK) at +4°C. After the last centrifuge step the supernatant was removed and the cells were resuspended in 100 µl of goat anti-rabbit IgG-FITC secondary antibody (containing 2.5% rabbit serum, Autogen Bioclear, UK Ltd) at various dilutions (1:50, 1:100 and 1:200 in 1x PBS solution) and incubated for 20 min on ice whilst under dark conditions. The cells were again washed twice in 500 µl of 1x PBS solution. After the last washing step the supernatant was removed and the cells resuspended in 200 µl of Sheath fixing solution.

The samples were run on a Beckman Coulter Epics XL flow cytometer by Steve Reader. This experiment was repeated, but the MAS072 monoclonal mouse antihuman CD14 primary antibody (Harlan Sera Lab), purified IgG2bK monoclonal

immunoglobulin isotype (Pharmagen International) and anti-mouse IgG-FITC conjugate (Sigma-Aldrich) were used. These antibodies were kindly donated by Professor R. Rees, The Nottingham Trent University.

CHAPTER THREE

RESULTS

3.0: RESULTS.

3.1. DETERMINATION OF OPTIMAL GROWTH CONDITIONS.

Due to the limited knowledge on *Arcobacter* spp. especially regarding the culturing conditions of this organism it was necessary to perform a series of preliminary experiments concerning growth media and optimal growth temperature.

Table 3.1: Optical density (650 nm) of *Arcobacter butzleri* NCTC 12481 after being grown under different growth conditions for forty eight hours.

Growth media	Optical density (650 nm)			
	Aerobic		Microaerophilic	
	30°C	37°C	30°C	37°C
TSB	0.34	0.38	0.37	0.33
Brucella + FBP	0.31	0.34	0.19	0.22
BHI	0.42	0.53	0.32	0.35

Table 3.1 shows *Arcobacter butzleri* NCTC 12481 can grow equally well when exposed to atmospheric oxygen (20%) or a reduced oxygen concentration (5%) in their growing environment. Nevertheless, in most instances, slightly higher optical density values were obtained when this strain was grown under aerobic conditions. For example when *A. butzleri* was grown in BHI broth at 37°C an optical density value of 0.53 was detected under aerobic conditions, which compares to 0.35 for microaerophilic conditions. As it was also easier to produce greater volumes of culture for experiments, it was decided to grow *A. butzleri* NCTC 12481 under aerobic conditions. Furthermore, similar optical density values were obtained at both 30°C and 37°C for all three different growth media, but generally slightly higher values were detected when *A. butzleri* was grown at 37°C.

Therefore, 37°C was used in further experiments, which was more significant to this study in relating *A. butzleri* to food poisoning as it is human body temperature. Media composition also had an effect on *A. butzleri* NCTC 12481 ability to grow. Optical density values were lower with Brucella broth and FBP supplements than with TSB and BHI broth, especially when incubated under microaerophilic conditions. Slightly higher values were detected when BHI was used, therefore, BHI was chosen for culturing *Arcobacter* spp. in future experiments. Plate counts showed that after growth in BHI broth at 37°C for 48 h, *A. butzleri* NCTC 12481 reached a cell density of 10^8 -10^9 colony forming units per ml (cfu ml^{-1}).

3.2. DETERMINATION OF *A. butzleri* NCTC 12481 TEMPERATURE GROWTH RANGE.

To determine the growth range of *A. butzleri* NCTC 12481 the RABIT technique (Section 2.6.2) was used. Impedance microbiology works by detecting changes in conductivity, which are caused by bacterial growth. The indirect method used 1% potassium hydroxide to detect the release of carbon dioxide from growing *A. butzleri* cells. When carbon dioxide reacts with the potassium hydroxide it produces potassium carbonate, which in turn lowers the measurable conductivity. Therefore, the faster the bacteria are growing and the higher the cell density the more carbon dioxide is produced. The direct method works on a similar principle, but the conductivity changes of the growth medium are measured. As the bacteria grow changes in the pH of the media occur causing the conductivity of the media to increase or decrease. Preliminary tests (Figure 3.1) showed that the indirect method produced greater conductivity changes compared to the direct method and was therefore a more sensitive technique to detect the growth of *A. butzleri* NCTC 12481. For this reason only the indirect method was used. Once the indirect method had been established the detection parameters needed to be standardised. This was achieved by using the differential plot (Figure 3.2, p124). The detection parameters were set as follows: (a) Temperature of the heating block was set to the desired temperature for each experiment. (b) The duration time of each experiment was 48 hours as the *A. butzleri* NCTC 12481 was a

particularly slow growing organism, especially under sub-optimal conditions such as high temperatures and acidic pHs. (c) The standard parameters of a temperature stabilisation period of 30 minutes followed by sampling resolution period of 6 minutes were used. Finally, from Figure 3.2 an appropriate detection criterion was determined to be -20μs/ 6 min, which was the point when the *A. butzleri* cells were detectable as shown by the rapid decrease in conductivity. Uninoculated control tubes were always included.

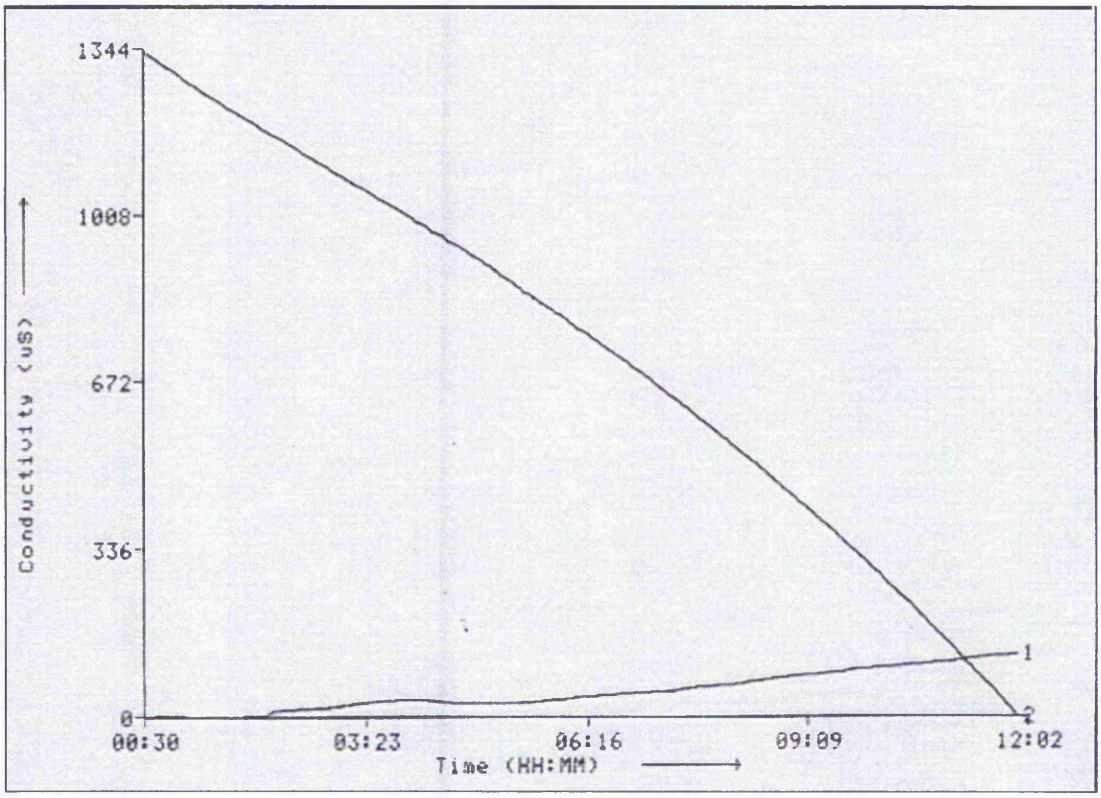


Figure 3.1: Comparison of direct and indirect impedance methods for the detection of *A. butzleri* NCTC 12481 growth.
Scanned picture from the Don Whitley Scientific RABIT machine.
1 = Direct method.
2 = Indirect method.

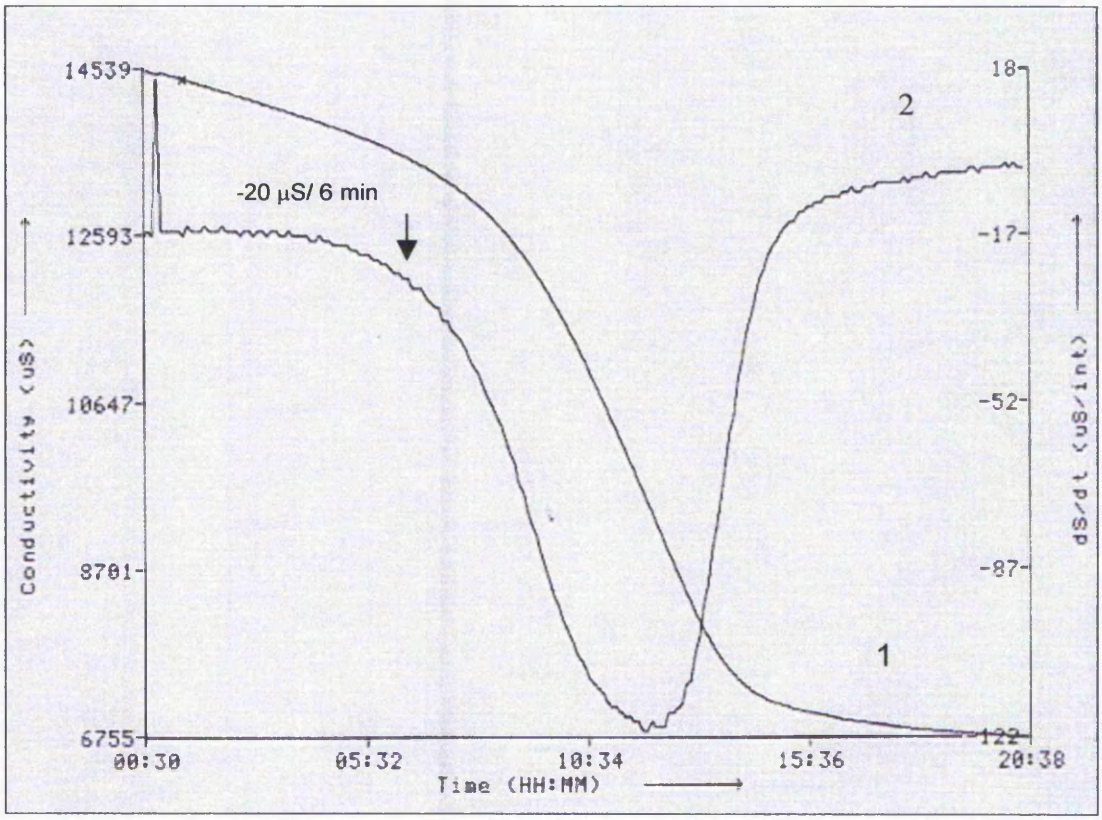


Figure 3.2: Comparison of real and differential plot to determine the detection criterion for *A. butzleri* NCTC 12481.

Scanned picture from the Don Whitley Scientific RABIT machine.

1 = Real plot.

2 = Differential plot.

It was also important to determine an optimum volume to use in the indirect conductance tubes. From the results (Figure 3.3) a 3 ml volume of BHI broth inoculated with 0.3 ml of culture produced the most accurate growth detection times. Smaller volumes (1.0 ml and 2.0 ml) produced longer detection times (3.14 h & 2.8 h respectively), which was believed to be due to there being a greater head space in the tube. Either the carbon dioxide settled in the tubes, so it took longer for a large enough volume of gas to be produced that could escape the tube and react with the potassium hydroxide causing the conductivity changes or the carbon dioxide production rate was less than that in 3 ml volumes. When a volume of 4 ml with a 0.4 ml culture inoculation was used the growth detection times were the same as those obtained for the 3 ml volume (1.24 h). However,

this volume was unsuitable for a purely physical reason that the tubes containing the culture held a maximum of 5 ml and were analysed at an angle in the temperature block. Therefore, a 3 ml volume was set as a standard condition for further experiments.

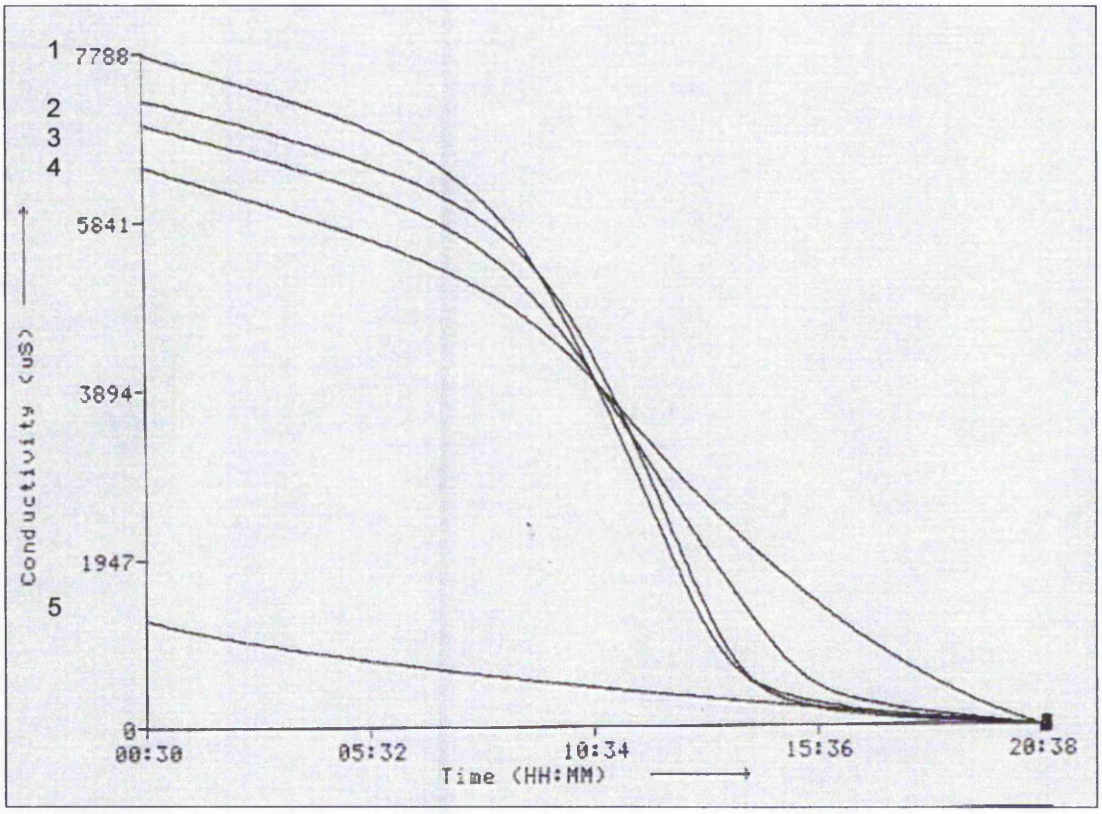


Figure 3.3: Comparison of detection rates when different volumes of BHI broth were used with a 9.1% inoculum of *A. butzleri* NCTC 12481.
Scanned picture from the Don Whitley Scientific RABIT machine.
1 = 0.3 ml of BHI broth. 4 = 0.1 ml of BHI broth.
2 = 0.4 ml of BHI broth. 5 = Uninoculated BHI control.
3 = 0.2 ml of BHI broth.
The time to detection, which is the point (-20μs/ 6 min) when a rapid decrease in conductivity occurs, can not be read directly from the real plot shown above, but is calculated directly by the impedance machine.

Once the parameters had been set it was possible to determine the growth rate of *A. butzleri* NCTC 12481 at various temperatures. This was achieved by

comparing the time to detection (TTD) of the inoculum from the RABIT technique to the corresponding viable count (cfu ml^{-1}) using the plate count method.

Table 3.2: An example of the time to detection (TTD) and viable count (cfu ml^{-1}) obtained at a growth temperature of 32°C.

Culture dilution	TTD (h)	Viable count (cfu ml^{-1})
Neat	1.4 ± 0§	1.6×10^{9}
10^{-1}	4.6 ± 0.30	1.6×10^{8}
10^{-2}	9.05 ± 1.35	1.6×10^{7}
10^{-3}	14.33 ± 0.19	1.6×10^{6}
10^{-4}	19.8 ± 0.32	1.6×10^{5}
10^{-5}	22.93 ± 0.27	1.6×10^{4}

§ = Standard error of mean where n = 3.

Table3.2 shows that the time to detection (TTD) was related to the number of *Arcobacter* cells present, in that the TTD increased with decreasing cell density. This was because the TTD is the point when sufficient carbon dioxide has dissolved into the potassium hydroxide agar to cause a detectable change in conductance. Therefore, the TTD increased with each dilution. As there were fewer cells growing in the diluted cultures, so it took longer for the levels of carbon dioxide produced to reach a detectable level. These were expected results and a typical pattern observed by all the growth temperatures used in this particular experiment. Using the TTD and viable count values a calibration graph was created. Figure 3.4 shows an example of a calibration curve created from the 32°C data.

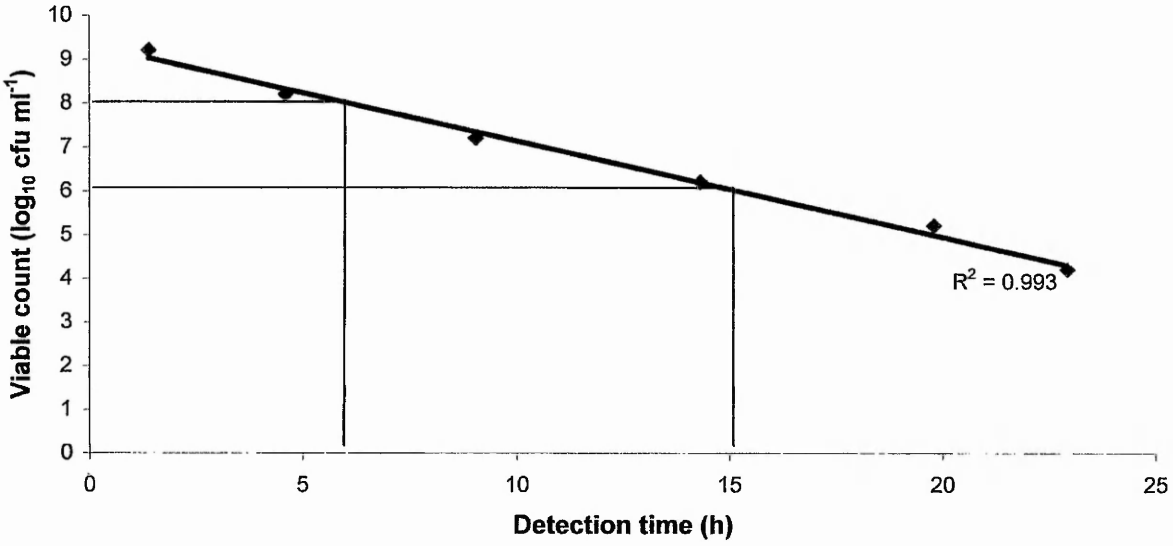


Figure 3.4: Determination of *A. butzleri* NCTC 12481 doubling time (t_d) at 32°C.

From the calibration curve a 2 log difference was determined, using this value the doubling time (h) and specific growth rate (h^{-1}) of *A. butzleri* NCTC 12481 at particular temperatures were calculated as in Section 2.6.2 (pp.80-81). These values are illustrated in Figure 3.5, with the exception of 15°C and 22°C, which were determined by turbidity measurements (see Section 2.6.2).

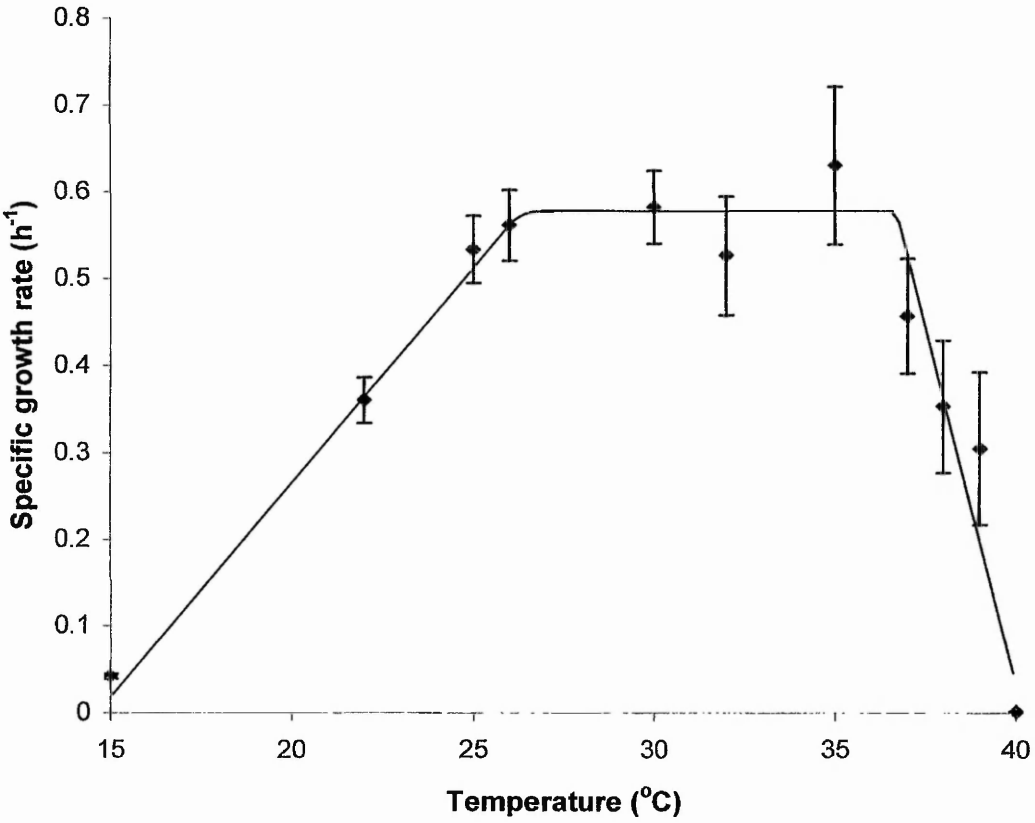


Figure 3.5: Variation in growth rate of *A. butzleri* NCTC 12481 with temperature. Error bars indicate standard error of mean where n = 3. With the exception of 15°C and 22°C, the specific growth rate was determined by the RABIT technique. See Section 2.6.2 for more details.

Figure 3.5 confirms that *A. butzleri* NCTC 12481 is a mesophilic organism, which can grow over a wide range of temperatures (15-39°C). From 25°C to 35°C a plateau was observed suggesting that these temperatures do not greatly alter the activity of the cells. The specific growth rate at 30°C was 0.57 h^{-1}. Below 22°C the growth rate was extremely slow, with a 72-98 h incubation period required instead of the usual 48 h. However, the cells were still viable, as experiments have shown that the minimum detectable growth temperature was at 15°C (μ' 0.04 h^{-1}). There was a very sharp decrease in the rate of growth above 37°C (μ' 37°C = 0.46 h^{-1} and μ' 39°C = 0.3 h^{-1}) with no detectable growth at 40°C.

Purity of the cultures used to inoculate the RABIT tubes and after growth were routinely confirmed by Gram staining, as well as culture optical density (OD 650 nm <0.6) and pH (~7.5). An example of these results are shown in Table 3.3 below.

Table 3.3: pH and optical density (650 nm) of the pooled samples from the 32°C RABIT experiment.

Original inoculum	pH	Optical density (650 nm)
Neat	7.49	0.19
10^{-1} dilution	7.54	0.18
10^{-2} dilution	7.48	0.15
10^{-3} dilution	7.53	0.20
10^{-4} dilution	7.54	0.18
10^{-5} dilution	7.47	0.14
Uninoculated control	7.54	0.02

The pH values in Table 3.3 have only altered slightly from the original uninoculated control tube. Therefore, from these results it can be stated that *A. butzleri* NCTC 12481 did not ferment the sugars in their growth media whilst exposed to different growth temperatures. The optical density values show that growth had occurred during the 48 h incubation period. Also, the optical density values were below 0.6, which was typical of NCTC 12481 growth (Table 3.1). These results were further supported by only Gram-negative rods being identified by the Gram stain.

3.3. EFFECT OF pH ON THE GROWTH OF *A. butzleri* NCTC 12481.

The impedance technique was also used to measure the effect of pH on the growth rate of *A. butzleri* NCTC 12481. As with the growth range experiment (Section 3.2) a 0.3 ml volume of preculture grown in BHI broth at 37°C was used (3 ml BHI broth).

Table 3.4: The effect of the starting pH on the optical density (650 nm) of *A. butzleri* NCTC 12481 cultures (24 h) grown at either 30°C or 37°C.

Starting pH	pH after autoclaving	pH after growth		Optical density (650nm) after growth	
		30°C	37°C	30°C	37°C
4.5	4.48	4.58	4.63	0.03	0.02
5.0	4.95	5.86	5.09	0.14	0.06
5.5	5.50	6.38	5.87	0.22	0.11
6.0	5.99	6.58	6.68	0.23	0.24
6.5	6.50	6.98	6.77	0.24	0.29
7.0	6.94	7.34	7.33	0.19	0.22
7.5	7.45	7.72	7.27	0.22	0.32
8.0	7.83	8.07	7.98	0.18	0.18
8.5	8.28	8.38	8.35	0.19	0.20
Inoculated control pH 7.4	7.35	7.75	7.77	0.27	0.25
Uninoculated control pH 7.4	7.32	7.35	7.32	0.02	0.02

The data shown above is a typical example of the effect of pH on the growth of *A. butzleri* NCTC 12481.

From Table 3.4 it can be seen that autoclaving the pH altered BHI broth did cause the starting pH to change slightly. The difference was always less than 0.1 pH units for the acidic and neutral pHs (pH 4.5-7.5). However, greater differences were detected for the more alkaline BHI broths (pH 8.0-8.5), but these were only 0.17 and 0.22 pH units respectively. Therefore, the pH of the BHI broths was approximately that of the starting pH values.

The pH value of the inoculated BHI broths increased after incubation (except the uninoculated control), with an apparently greater increase in pH after incubation at 30°C compared to 37°C. From pH 6.0 to pH 8.5, including the inoculated control

(pH 7.35), the increase in pH was less than 0.5 pH units. Whereas at pH 5.0 and 5.5 the difference in pH from the autoclaved pH was nearly one pH unit.

Table 3.4 shows that at 30°C the optical density (650 nm) value at pH 4.5 was 0.03, which suggests that *A. butzleri* NCTC 12481 were unable to grow at this pH, as a similar value was obtained for the uninoculated control. As the pH increased, so did the optical density values until it peaked at pH 6.5, after which point a gradual decline occurred. A similar pattern was observed at 37°C, however no growth was detected at either pH 4.5 or pH 5.0. These results are also shown in Figure 3.6 where growth detection time (impedance method) was compared to pH. The impedance method detects the production of carbon dioxide during microbial metabolism. Therefore, the faster the cells were growing the greater the amount of carbon dioxide produced and subsequently a detectable level of gas was reached in a shorter period of time. Generally the results showed that a longer detection time was observed at 37°C than 30°C for every pH. However, between the pH range of 6.0 and 8.0 the difference was minimal (approximately 2-3 h). Above and below these values the difference was far greater (20-30 h).

A. butzleri NCTC 12481 was unable to grow at pH 4.5. At 37°C this species was also unable to grow at pH 5.0, but growth was detected at pH 5.5. Under these conditions the detection time was extremely high (39.25 h) indicating that it was growing at a very slow rate. Growth was observed at 30°C, but a substantially lower detection time (8.43 h) was obtained. *A. butzleri* also grew slower in alkaline conditions such as pH 8.5. Over the range from pH 6.0 to 8.0 a fairly constant value of approximately 7 h detection time was recorded for 30°C and 11 h for 37°C.

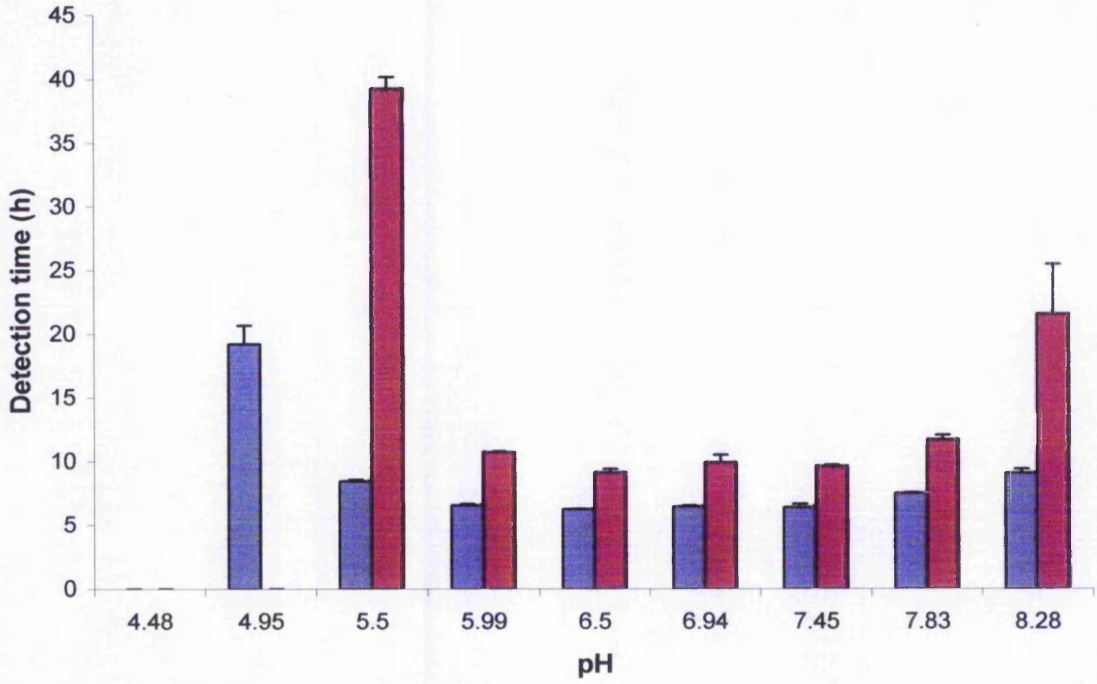


Figure 3.6: Effect of pH on the detection time (h) of *A. butzleri* NCTC 12481 at 30°C and 37°C.

Growth temperature at 30°C ( ) and 37°C ( ).

Error bars indicate standard error of mean where n = 3.

Inoculum size was 1.57×10^9 cfu m^{-1}.

The pH experiment was repeated, but a series of dilutions were used thus enabling calibration curves to be created for each pH. Therefore, it was possible to calculate the doubling times of *A. butzleri* NCTC 12481 at each pH whilst growing at either 30°C or 37°C. The doubling times were calculated as in Section 2.6.2. (pp.80-81). These results are shown in Table 3.5 over the page.

Table 3.5: Doubling times of *A. butzleri* NCTC 12481 grown at different pH values at either 30°C or 37°C.

pH of BHI broth	Doubling time (h)	
	30°C	37°C
4.49	0	0
5.01	0	0
5.46	2.31	6.25
6.01	1.54	1.74
6.45	1.05	1.07
6.93	1.1	1.09
7.44	0.80	1.09
7.84	0.83	1.46
8.35	0.98	2.71
Inoculated control (pH 7.35)	0.75	1.09

Table 3.5 shows the effect of pH on the growth rate of *A. butzleri* NCTC 12481 at 30°C and 37°C. The results confirm those seen in Figure 3.6 with *A. butzleri* growing faster at 30°C than at 37°C over the entire pH range used in this experiment. It is also confirmed that the optimum pH range for growth was between pH 6.0-8.0, with the fastest doubling times occurring at pH 7.5 (30°C = 0.8 h, 37°C = 1.09 h). The results also showed that no growth occurred at pH 4.5 or pH 5.0 at either temperature (30°C or 37°C). Furthermore, *A. butzleri* grew slowly at pH 5.5 as shown by the doubling times being 2.31 h and 6.25 h respectively at 30°C and 37°C.

3.4. GROWTH CURVE OF *A. butzleri* NCTC 12481.

The growth curve *of Arcobacter* spp. was determined by measuring the optical density (650 nm) of a culture grown at 37°C and compared it with the appropriate viable counts (Figure 3.7). A lag period for 5 h was observed, followed by a rapid increase in turbidity where the cells were in the exponential phase of growth until 18 h where the values start to plateau and the stationary phase was entered. A

similar pattern was also determined for the viable cell counts. However, the plate count started to increase after only 2 h, but after 10.5 h the viability count started to decrease.

Therefore, after 2-5 h the culture had entered into the exponential phase and after 9 h the culture was in mid-exponential phase. In addition, the results show that *Arcobacter* spp. entered the stationary phase after approximately 18 h. Therefore, 9 h and 24 h incubation periods were used to produce exponential and stationary phase cultures respectively.

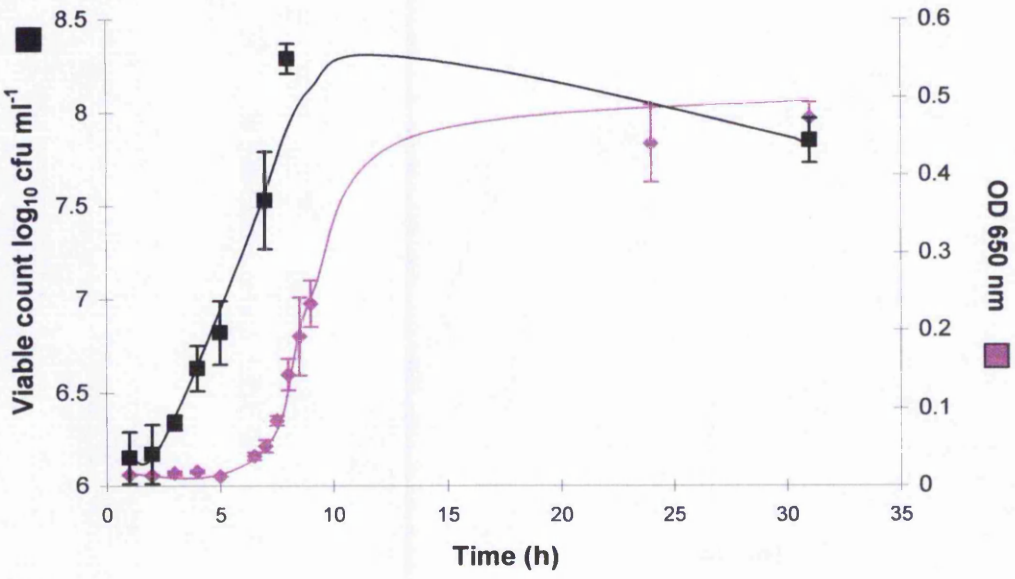


Figure 3.7: Growth curve of *A. butzleri* NCTC 12481 at 37°C.
Error bars indicate standard error of mean, where n = 3.

3.5. PRELIMINARY HEAT TREATMENT EXPERIMENTS.

Once the growth rates had been determined it was possible to study the effect of heat on *Arcobacter* spp. Blood agar and brilliant green bile agar (BGBA) were chosen as non-selective and selective media respectively as they were routinely used to isolate *Campylobacter* spp.

Table 3.6: The effect of a 3 minute exposure at various temperatures on a stationary phase *A. butzleri* NCTC 12481 culture.

Temperature (°C)	Viable count after heat treatment (cfu ml^{-1})		%[c] Survivors	%[c] Sublethal* injury
	Blood agar	BGBA		
40	$7.5x10^{8a}$	$6.0x10^{8b}$	34.72	20
45	$7.7x10^{8}$	$9.9x10^{7}$	35.46	87.1
50	$5.1x10^{7}$	$3.2x10^{4}$	2.37	99.95
55	$8.8x10^{4}$	$3.2x10^{4}$	<0.01[d]	63.69
60	$4.4x10^{5}$	$1.7x10^{5}$	0.02	62.22
65	$3.4x10^{5}$	$2.3x10^{4}$	0.02	93.83
70	$2.2x10^{4}$	$1.6x10^{2}$	<0.01[d]	99.27

[a] = Inoculum size 2.2×10^{9}. [b] = Inoculum size 1.2×10^{8}.

[c] = Definitions given in Materials and Methods. * = Structurally injured cells.

[d] = Detection limit set at 0.01.

A third of the cells present in the original sample were still viable after a 40°C heat treatment and of the survivors only 20% were sublethally injured, as seen in Table 3.6. This was probably due to the culture only being heat treated at this temperature for 3 minutes, which is a relatively short period of time. At 45°C exposure over a third of the cells remained viable. Nevertheless, this temperature has a greater effect than 40°C as 87.1% of the survivors were sublethally injured. On the other hand 50°C caused a high percentage of death with only 2.37% survivors, of which 99.95% were sublethally injured. At 55 to 65°C nearly all the cells present in the suspension were lethally injured within 3 minutes.

Therefore, 45°C was initially chosen as a treatment temperature that would produce the greatest number of sublethally injured cells, but the least amount of death. More experiments were required to determine exposure time and for this reason *A. butzleri* cultures were exposed to 45 to 55°C temperatures for 2 to 10 minutes. These results are shown below in Table 3.7 and 3.8.

Table 3.7: Effect of temperature on stationary phase *A. butzleri* NCTC 12481 for various time periods.

Temperature (°C)	Time (min)	Viable count after heat treatment (cfu ml^{-1})		%[e] Survivors	%[e] Sublethal* injury
		Blood agar	BGBA		
45	2	$1.6x10^{7}$[a]	$3.5x10^{5}$[b]	32.78	97.82
	4	$1.5x10^{7}$	$1.4x10^{4}$	30.71	99.9
	6	$1.2x10^{7}$	$2.7x10^{4}$	2.39	97.68
	8	$9.8x10^{6}$	$1.1x10^{5}$	2.04	88.72
	10	$1.3x10^{7}$	<100[b]	<0.01[d]	100
50	2	$4.3x10^{5}$	$5.8x10^{1}$	0.89	99.99
	4	$7.0x10^{4}$	<100[c]	0.15	100
	6	$4.4x10^{3}$	<100[c]	0.01	100
	8	$9.8x10^{4}$	$5.0x10^{2}$	0.2	99.49
	10	$9.9x10^{3}$	$2.5x10^{1}$	0.02	99.75
55	2	<100[c]	<100[c]	<0.01[d]	<0.01[d]
	4	<100[c]	<100[c]	<0.01[d]	<0.01[d]
	6	$5.2x10^{5}$	$6.5x10^{2}$	1.1	99.87
	8	<100[c]	<100[c]	<0.01[d]	<0.01[d]
	10	$3.3x10^{1}$	<100[c]	<0.01[d]	<0.01[d]

[a] = Inoculum size 4.8×10^{7}. [b] = Inoculum size 1.6×10^{6}.

[c] = Detection limit set at 100 cfu ml^{-1}.

[d] = Detection limit set at 0.01.

[e]= Definitions given in Materials and Methods. * = Structurally injured cells.

The results in Table 3.7 show that a treatment temperature of 45°C for between 2 and 4 minutes resulted in two thirds of the stationary phase cells being killed, with nearly all the survivors being sublethally injured. A 6 and 8 minute exposure time caused the number of survivors to decrease from approximately 30% to just over 2%. After 10 minutes at 45°C nearly all of the viable population were killed.

At 50°C an exposure time of 2 minutes caused a high percentage of cell death with only 0.89% survivors of which 99.99% were sublethally injured. This was also shown by the plate counts as no growth was detected on the selective BGBA. After 2 minutes exposure at this temperature nearly all of the cells were killed. However, of the small percentage of survivors 100% were sublethally injured. At 55°C the effect was even greater with cell death occurring after only 2 minutes. Nevertheless, a low plate count was observed after 6 and 10 minutes. This may have been an aberrant result.

From these results a temperature of 45°C for 3 minutes was chosen to heat treat stationary phase cells, which gave a high proportion of sublethally injured survivors.

Similar results were also detected for exponential phase cultures (Table 3.8), with increasing temperature and exposure time reducing the number of survivors, but increasing the percentage of sublethally injured cells that were present. However, the exponential phase culture seemed to be more resistant to heat than stationary phase cells. As a higher percentage of survivors were detected for both 45°C and 50°C. It was also observed that *A. butzleri* in the exponential phase could survive for longer exposure times than stationary phase cells. For example a 6 minute exposure time at 45°C caused a high percentage of kill of stationary phase cells with only 2.39% of survivors being detected. This compares to 36.36% of survivors being observed for exponential phase cells.

Table 3.8: Effect of heat on exponential phase *A. butzleri* NCTC 12481 exposed for various periods of time.

Temperature (°C)	Time (min)	Viable count after heat treatment (cfu ml^{-1})		%[e] Survivors	%[e] Sublethal* injury
		Blood agar	BGBA		
45	2	$7.2x10^{7}$	$5.2x10^{7}$	59.1	26.71
	4	$5.9x10^{7}$	$2.3x10^{7}$	48.93	61.15
	6	$4.4x10^{7}$	$2.8x10^{7}$	36.36	37.05
	8	$2.1x10^{7}$	$2.7x10^{7}$	17.69	23.83
	10	$4.3x10^{7}$	$1.4x10^{7}$	35.37	67.29
50	2	$1.9x10^{7}$	$3.5x10^{6}$	15.29	81.24
	4	$9.4x10^{5}$	$8.8x10^{2}$	0.78	99.91
	6	$1.2x10^{5}$	<100[c]	0.1	100
	8	$3.9x10^{4}$	$1.6x10^{1}$	0.03	99.9
	10	$1.7x10^{4}$	<100[c]	0.01	100
55	2	$2.3x10^{5}$	$3.7x10^{5}$	0.19	58.97
	4	$4.1x10^{4}$	<100[c]	0.03	100
	6	$1.9x10^{3}$	<100[c]	<0.01[d]	100
	8	$1.3x10^{2}$	$2.5x10^{1}$	<0.01[d]	80.77
	10	$5.2x10^{2}$	<100[c]	<0.01[d]	100

[a] = Inoculum size 1.2×10^{8}. [b] = Inoculum size 6.8×10^{7}.

[c] = Detection limit set at 100 cfu ml^{-1}.

[d] = Detection limit set at 0.01.

[e]= Definitions given in Methods and Materials. *= Structurally injured cells.

During the course of these experiments commercially available isolation media containing cefoperazone, amphotericin B and teicoplanin agar (CAT) for *Arcobacter* spp. and charcoal cefoperazone, deoxycholate agar (CCDA), which is more specific for *A. butzleri* became available. Therefore, these media were tested as selective media in place of BGBA which is a *Campylobacter* spp. selective medium (Figure 3.8).

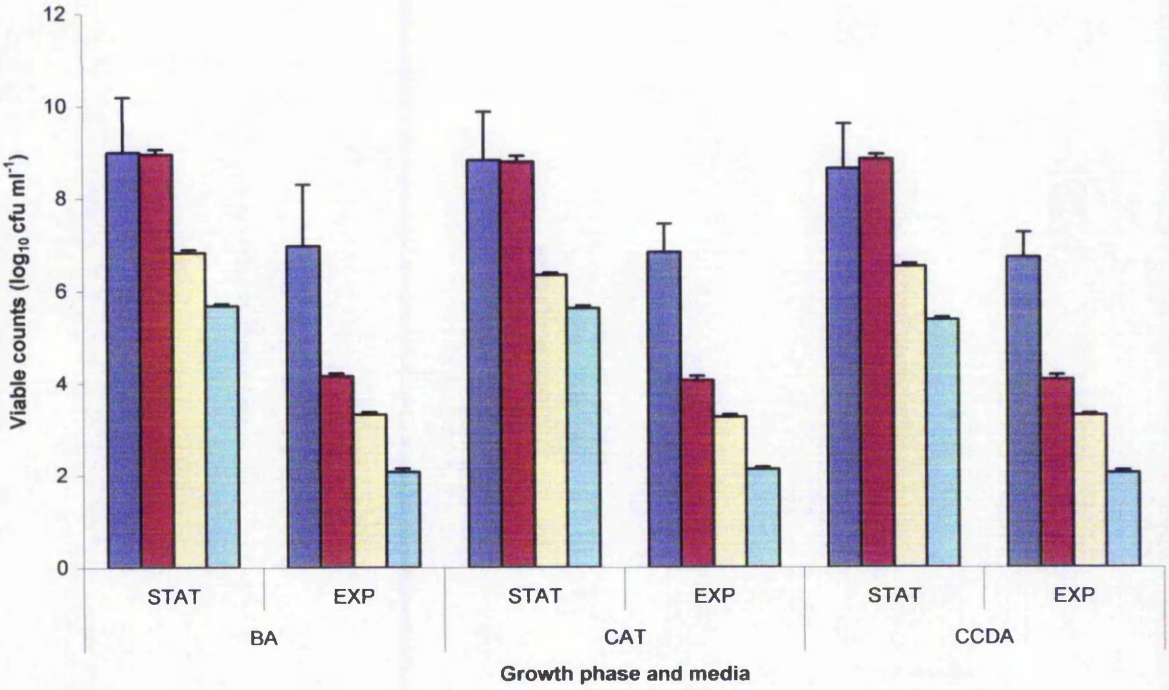


Figure 3.8: Effect of heat on *A. butzleri* NCTC 12481 with subsequent recovery on non-selective and selective media.

Temperature treatment : Untreated( ), 45°C( ), 50°C( ), 55°C( ).

Error bars indicate standard error of mean where n = 6.

STAT = Stationary phase culture. EXP = Exponential phase culture.

BA = Blood agar. CAT = Cefoperazone, amphotericin B & teicoplanin agar.

CCDA = Charcoal, cefoperazone, deoxycholate agar.

Figure 3.8 shows that as the temperature increased in 5°C increments the colony counts on the different media decreased significantly ($p<0.006$-0.03, determined by the Mann-Whitney U test 95% confidence limit). For this set of results stationary phase cells were more resistant to temperature increases. A 3 log decrease in viability was observed for stationary phase cells at 55°C, whereas a 4 log decrease was detected under the same conditions for exponential phase cells. However, these results may have been affected by the initial values of the exponential phase cells being 2 log lower than the stationary phase culture. Furthermore, the exponential phase viable count decreased gradually (1 log) with each temperature increase, whereas the stationary phase culture did not show any

signs of decrease at 45°C. These preliminary experiments were not repeated as a more detailed temperature exposure experiment was performed to determine the *D* and *z* values of *A. butzleri* NCTC 12481 (see Section 3.6).

Under these heat treatment conditions (i.e. 45°C *etc*) the colony counts on the non-selective and selective types of media were very similar for each temperature. Nevertheless, both CAT and CCDA tended to have a slightly lower colony count than the non-selective blood agar. From these results and due to both CAT and CCDA being specifically designed to selectively isolate *Arcobacter* spp. they were chosen to be used in any further experiments where a selective medium was required.

3.6. TIME EXPOSURE EXPERIMENTS TO DETERMINE *D* AND *z* VALUES.

The results in Section 3.5 showed that a temperature of 45°C to 50°C for 3 minutes would be suitable heat treatment conditions to study sublethal injury in both exponential or stationary phase cultures. As these conditions would produce a relatively high percentage of survivors, which would be sublethally injured. However, to standardise the heat treatment for future experiments with CAT and CCDA, a series of time exposure experiments at different temperatures were done and subsequently *D* and *z* values determined. Definitions of *D* and *z* values are given in the Methods and Materials section (see Section 2.9, p.86).

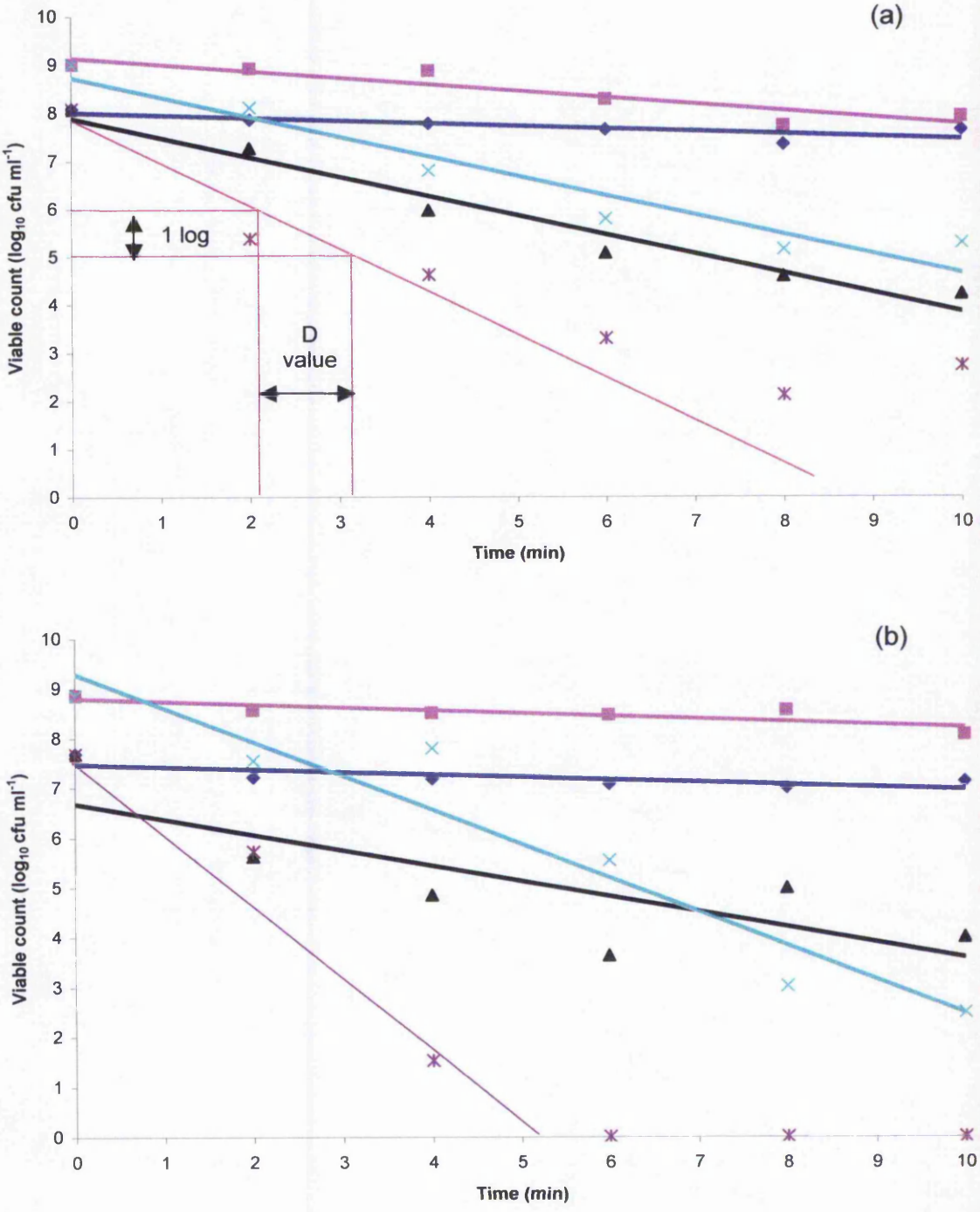


Figure 3.9: Effect of heat on *A. butzleri* NCTC 12481 in (a) exponential and (b) stationary phase of growth for various time periods. Colony counts were done on blood agar.
Exposure temperature: 45°C (◆), 47°C (■), 50°C (▲), 52°C (X), 55°C (✳).

Figure 3.9 a shows the effect increasing temperature and time exposure has on an exponential phase culture of *A. butzleri* NCTC 12481. For each temperature a similar pattern was detected, whereby as the exposure time increased the colony count decreased. As predicted, as the temperature increased the colony count decreased at a faster rate. A similar set of results was observed for the stationary phase *A. butzleri* culture (Figure 3.9 b). However, these results showed a greater variability, with increasing temperature having a greater effect on the colony counts. At 47°C and 50°C there was only a gradual decrease in viability, with less than a 1 log difference being detected at 47°C. Whereas 52°C and 55°C caused a rapid decrease after only a four minute exposure time. Furthermore, a six minute exposure time at 55°C caused a lethal affect to the *A. butzleri* cells with no colonies being detected on any of the blood agar plates. From these graphs the *D* and *z* values were calculated and the results are shown in Tables 3.9 and 3.10.

Example *D* value calculation.

From Figure 3.9a a 1 log difference was determined, using this value the following calculation was performed enabling the *D* value to be calculated:

log 5 = 2.6 min
log 6 = 3.7 min

To determine the 1 log difference the log 5 value was subtracted from the log 6 value.

3.7 – 2.6 = 1.1 min

Therefore, *A. butzleri* NCTC 12481 in the exponential phase of growth has a D_{55} value of 1.1 min when recovered on non-selective blood agar (Table 3.10).

Table 3.9: *A. butzleri* NCTC 12481 *D* values obtained using selective (BGBA, CAT and CCDA) isolation media.

Growth phase	Temperature (°C)	*D* value (min)	
		BGBA	CCDA/CAT
Exponential	45	>10	>10
	50	0.8	1.5
	55	0.43	0.6
Stationary	45	2.35	2.4
	50	0.45	1.4
	55	0.66	0.5

Statistical analysis was not performed on this set of data as not enough replicates were not performed, since the selective media was changed from BGBA (n = 1) to CCDA and CAT (n = 2) during the course of these experiment.

Table 3.10: *D* and *z* values of *A. butzleri* NCTC 12481 according to growth phase using blood agar.

Growth phase	Treatment temperature (°C)	*D* value (min)	*z* value (°C)
Exponential	45	>30.0 (± 0.1)§	8.1, r^2 = -0.911
	47	16.0 (± 2.3)	
	50	2.1(± 0.1)	
	52	2.0 (± 0.2)	
	55	1.1 (± 0.2)	
Stationary	45	>30.0 (± 0.1)	7.4, r^2 = -0.977
	47	8.0 (± 2.2)	
	50	1.7 (± 0.6)	
	52	1.5 (± 0.1)	
	55	0.4 (± 0.01)	

§ = Standard error of mean where n = 3.

Table 3.10 shows that as the temperature increased, the time required to reduce *A. butzleri* NCTC 12481 by 1 log decreased. Both exponential and stationary phase harvested cells follow a similar pattern, whereby longer exposure times (16 min and 8 min respectively) were required to kill this particular species at 47°C. However, at 50°C and above the exposure time drops considerably to two minutes or less. The results also show that at each temperature exponential phase *D* values were greater and in some cases almost double of those obtained for stationary phase cells. For example cells harvested from the exponential phase had a D_{55} 1.1 min, compared to D_{55} 0.4 min detected for stationary phase cells.

Plotting the *D* values against temperature gave a straight line relationship (data not shown). The slope of the line was used to determine the change in temperature resulting in a ten-fold increase or decrease in the *D* value. This coefficient is called the *z* value. The *z* values for exponential and stationary phase harvested cells were 8.1 and 7.4 respectively (Table 3.10). Therefore, a greater increase (7-8°C) in temperature was required to decrease the *D* value by ten-fold.

Exponential phase cells had higher *D* values on both selective (BGBA and CCDA/CAT) and non-selective (blood) media compared to stationary phase cells. This was more notable at 45°C than the higher temperatures. Nevertheless, for both growth phases a larger *D* value was obtained using blood agar (D_{50} 2.1 min) compared to CCDA/CAT (D_{50} 1.5 min) or BGBA (D_{50} 0.8 min) (Table 3.9 and 3.10). From these results it was concluded that both exponential and stationary phase cells should be heated shocked at 55°C for 3 minutes to produce a high number of survivors which are sublethally injured.

From all these preliminary heat exposure experiments the results have shown that a temperature of 45°C and 47°C for various exposure times did not greatly affect either exponential or stationary phase cultures. A 50°C temperature had a greater effect on *A. butzleri* NCTC 12481 cell viability, but it was felt that a 55°C exposure temperature stressed both exponential and stationary phase cells sufficiently to study the effect of heat on this particular species. Therefore, the heat treatment was standardised at 55°C for three minutes.

3.7. FLUORESCENT STAINING TO DETERMINE MEMBRANE INTEGRITY OF *A. butzleri* NCTC 12481.

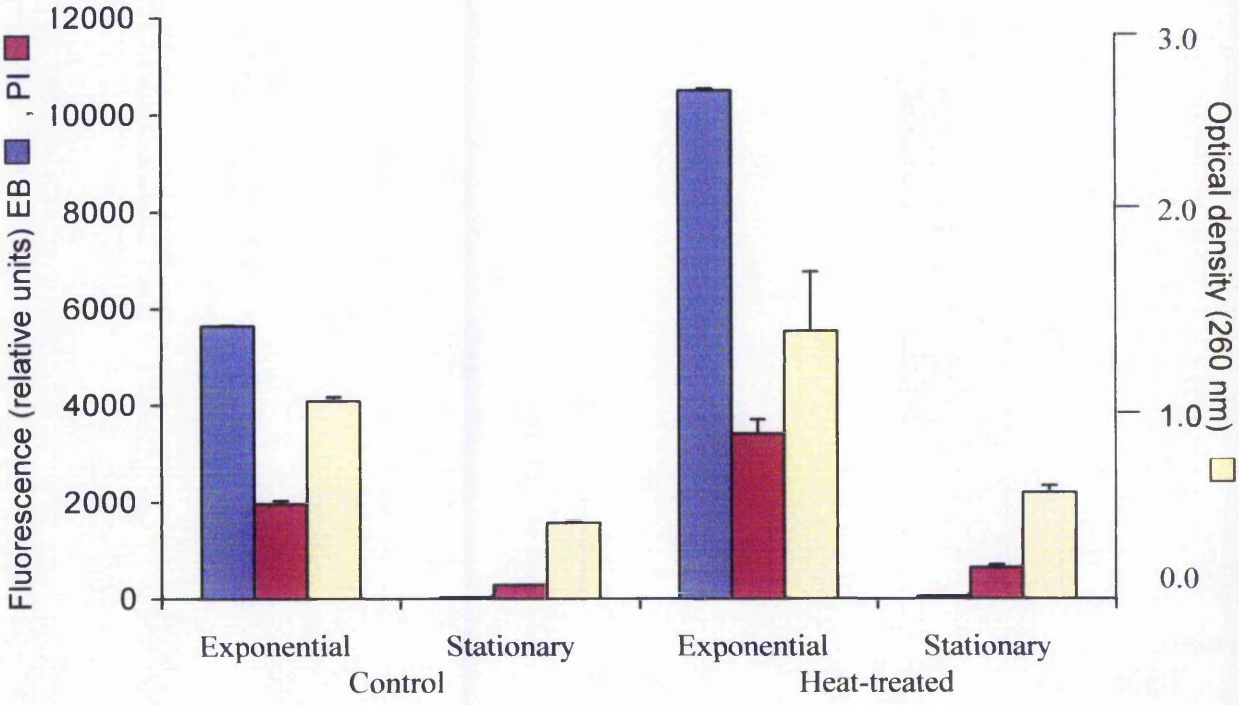


Figure 3.10: Uptake of fluorescent dyes and cytoplasmic leakage by heat-treated exponential and stationary phase *A. butzleri* NCTC 12481.
Error bars indicate standard error of mean where n = 3.
EB = Ethidium bromide. PI = Propidium iodide.

Membrane integrity was studied by the uptake of DNA-binding fluorescent dyes (ethidium bromide and propidium iodide). For presentation purposes some data is not presented here, but may be found in the Appendix (Figure 1, p.258). Figure 3.10 shows that both dyes gave higher fluorescence values when the *A. butzleri* cells were heat-treated compared with the controls. Furthermore, greater levels of fluorescence were detected for heat-treated harvested exponential phase cells compared with stationary phase cells. The integrity of the membrane was studied by measuring the leakage of 260 nm and 280 nm absorbing material from the cells. This material can only be released if the cell membrane is damaged. It was observed that cells harvested from the exponential phase released more UV-

absorbing material after heat treatment than harvested stationary phase cells. This pattern was also shown by 280 nm absorbing material, but the absorbance values were lower than those at 260 nm and for the sake of clarity these results have been omitted from Figure 3.10. Thus supporting the results obtained from the fluorescence experiments.

3.8. COLD STORAGE OF *A. butzleri* NCTC 12481.

The effect of cold storage on *A. butzleri* NCTC 12481 was studied at chilled (+4°C) and freezer (-20°C) temperatures (Figures 3.11 to 3.14).

Storage of cells from the stationary phase at +4°C caused a gradual decrease in viability as exposure time increased. The colony count on blood agar decreased from 1 x 10^9 to 8 x 10^5 cfu ml^{-1} (Figure 3.11) over a 21 day storage period. In contrast freezing (-20°C) caused a 2 log decrease in viability after only 24 h storage and thereafter the viability remained constant (0.6-1 x 10^5 cfu ml^{-1}). Differences in cell recoveries between selective (CAT and CCDA) and non-selective (blood agar) media were apparent after 3 and 7 days in storage (chilled and frozen samples, respectively). The difference between the different types of media increased with storage time, especially at -20°C. There was no apparent difference in cell recoveries between CAT and CCDA selective media. Under chilled conditions cells from the exponential phase followed a similar pattern to stationary phase cells, whereby the colony count decreased gradually. However, after 7 days in storage growth was only detected on the non-selective blood agar. Freezing had a greater effect on cells harvested from the exponential phase than the stationary phase, with a 4 log kill after only 24 h and no growth was detected on any medium after 7 days in storage (Figure 3.12).

BGBA plates were also used in this set of cold storage experiments. However, the results have not been shown in Figures 3.11 and 3.12 as no colonies were detected on any of the plates for the frozen samples for either exponential or stationary phase harvested cells. Furthermore, viable counts were detected for

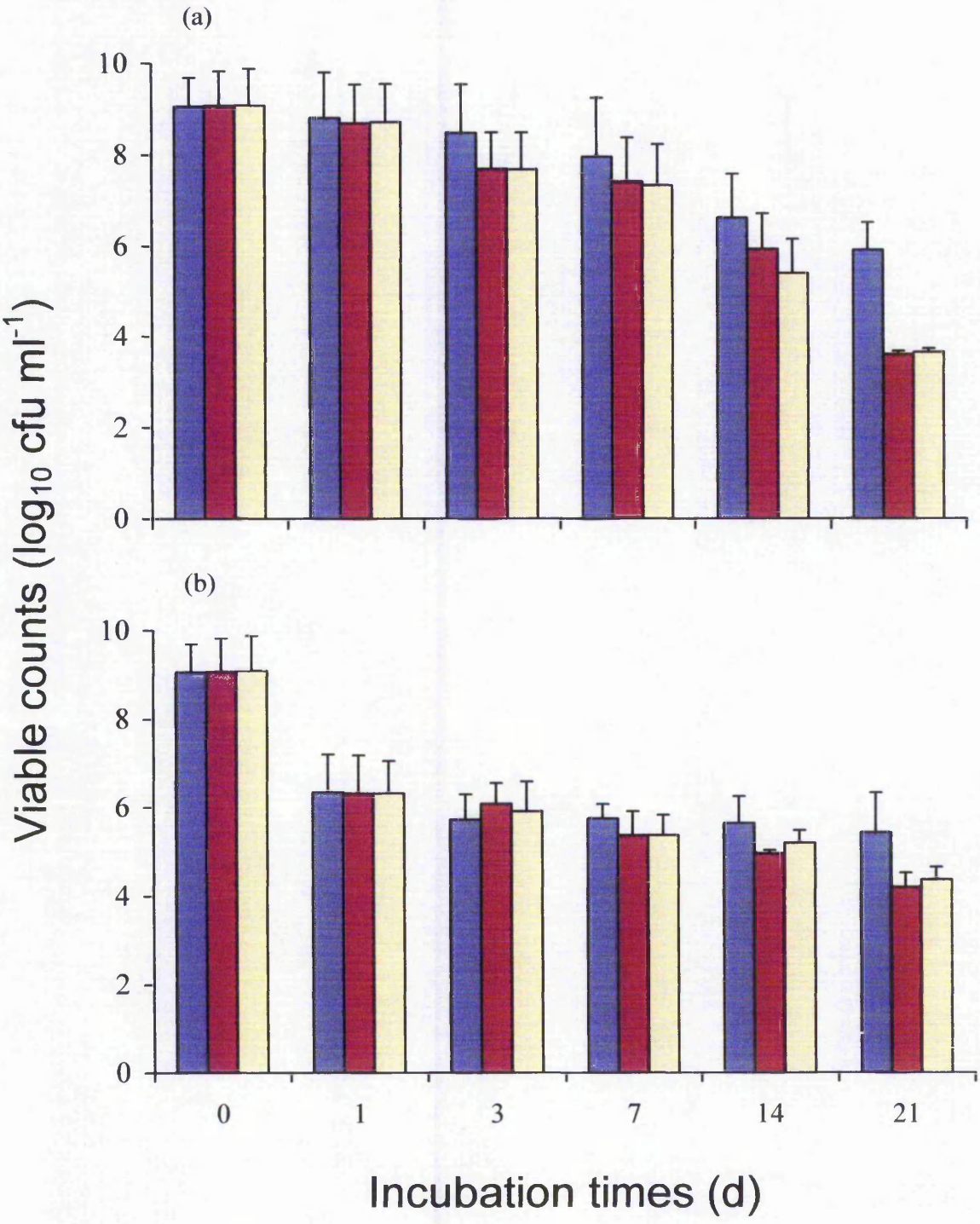


Figure 3.11: Recovery of *Arcobacter butzleri* NCTC 12481 on non-selective blood agar(■) and selective agar CAT (■) and CCDA(□) plates during storage at (a) +4°C and (b) -20°C in BHI from the stationary phase. Error bars indicate standard error of mean where n = 3.

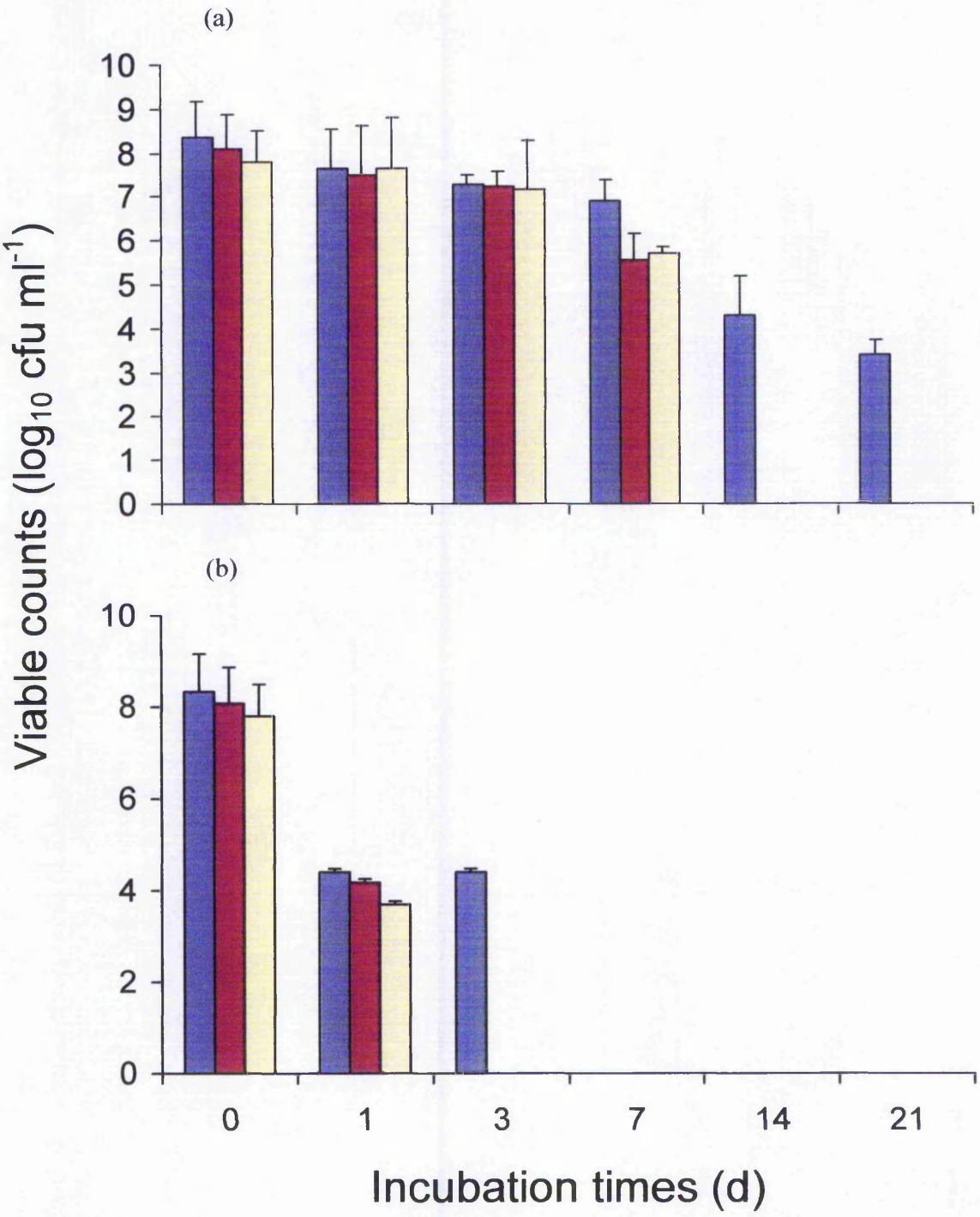


Figure 3.12: Recovery of *Arcobacter butzleri* NCTC 12481 on non-selective blood agar(■), and selective agar CAT (■) and CCDA(□) plates during storage at (a) +4°C and (b) -20°C in BHI from the exponential phase. Error bars indicate standard error of mean where n = 3.

the chilled samples, but only for cells harvested from the exponential phase after 1 and 3 days in storage (9.5 x 10^6 and 1.5 x 10^4 cfu ml^{-1} respectively).

In order to determine the affect of storage in a nutrient poor environment this experiment was repeated, with the *A. butzleri* NCTC 12481 cells being stored in phosphate buffer instead of BHI. The results showed a very similar pattern to those detected for BHI broth storage (Figures 3.13 and 3.14). However, slightly higher viability counts were observed for the cells harvested from exponential phase cultures and stored in BHI broth. The difference became more apparent after 3 days when approximately a 1 log difference occurred. Freezing in phosphate buffer also had a greater effect on both stationary and exponential phase harvested cells than BHI broth. No colonies were detected on any of the agar plates for the exponential phase cells and viable stationary phase cells were detected on both the selective and non-selective media after a 24 h storage period (2 x 10^5 cfu ml^{-1}) and only on blood agar plates after a 3 day period (1 x 10^4 cfu ml^{-1}).

3.9. HEAT AND COLD ADAPTATION OF *A. butzleri* NCTC 12481.

As well as the effect of heat (55°C) on *A. butzleri* NCTC 12481, it was important to study heat adaptation. The bacterial cells were pre-exposed to a non-lethal temperature for various time periods, followed by the usual heat treatment (55°C, 3 min). These experiments could subsequently determine if exposing *A. butzleri* NCTC 12481 to a non-lethal temperature greater than its optimum growing temperature would induce the production of heat shock proteins.

The preliminary experiments were performed at 40°C. This temperature was chosen as previous experiments (Section 3.2) had shown that *A. butzleri* was unable to grow at this temperature. The results for this initial experiment can be seen in Figure 3.15 (p.152), which shows that heat treating the cells harvested from the stationary phase at 55°C caused a 4 log decrease in cell viability on the non-selective blood agar. However, a 5 log decrease was detected on the

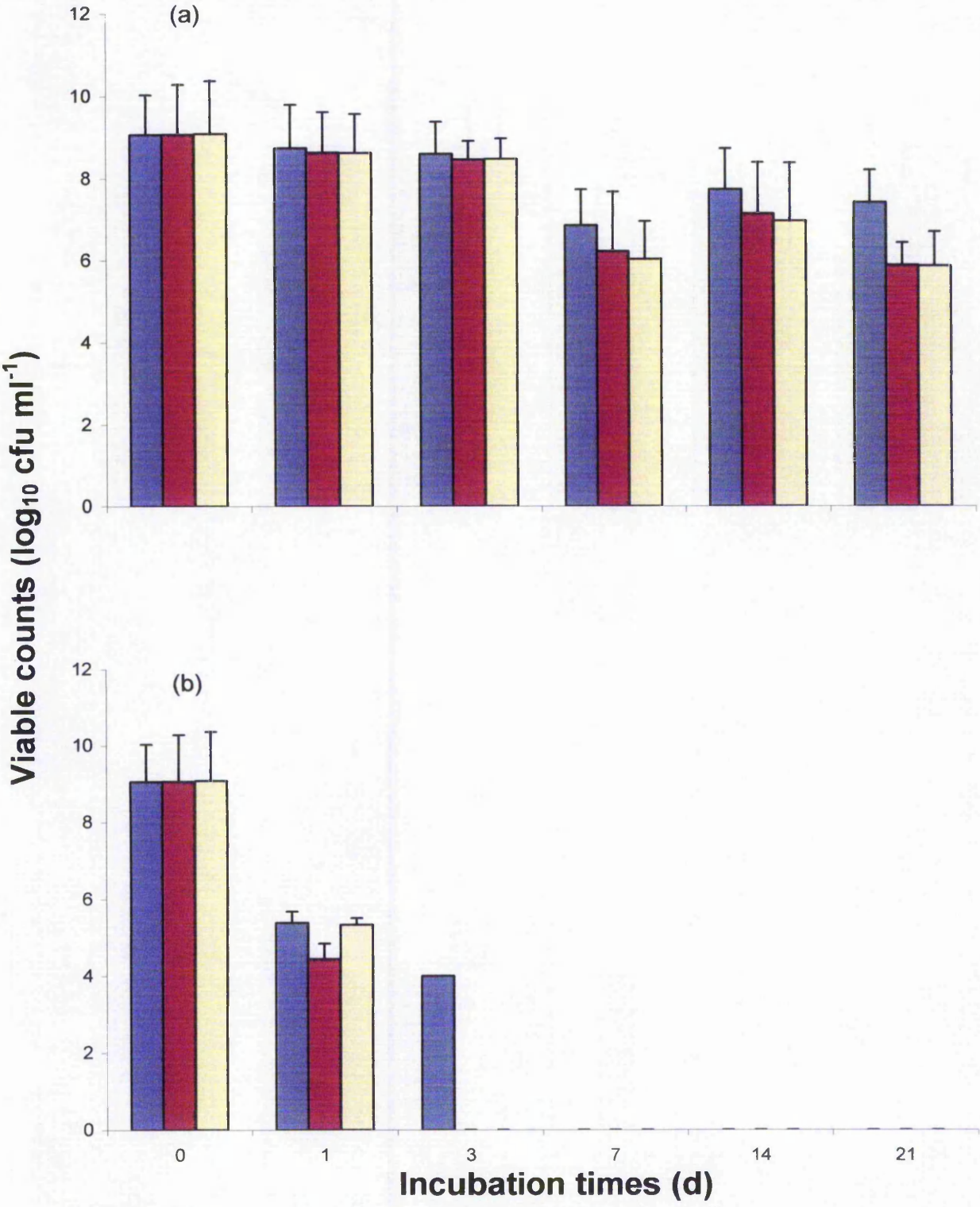


Figure 3.13: Recovery of *Arcobacter butzleri* NCTC 12481 on non-selective blood agar(■) and selective agar CAT (■) and CCDA(□) plates during storage at (a) +4°C and (b) -20°C in phosphate buffer from stationary phase. Error bars indicate standard error of mean where n = 3.

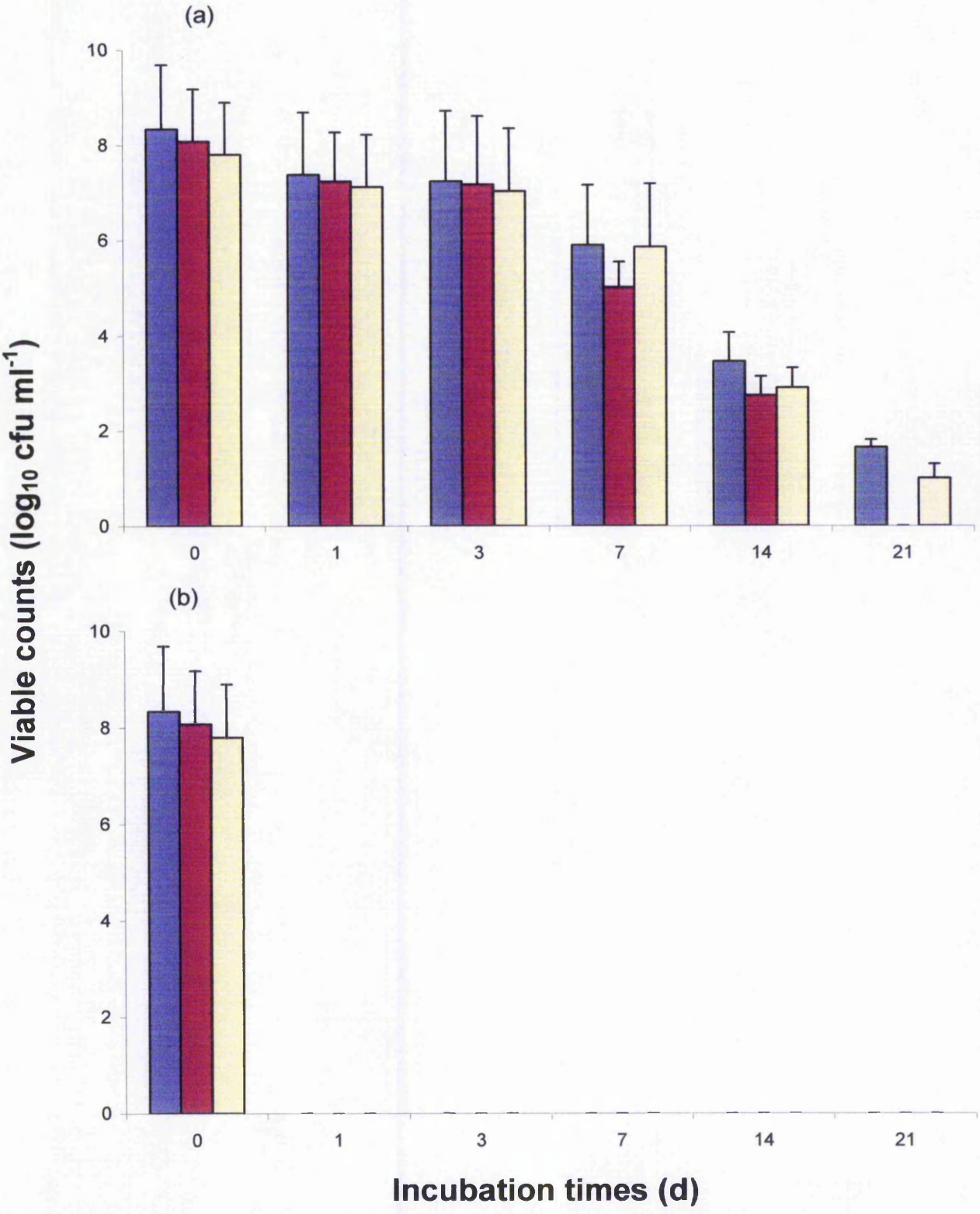


Figure 3.14: Recovery of *Arcobacter butzleri* NCTC 12481 on non-selective blood agar(■) and selective agar CAT (■) and CCDA(□) plates during storage at (a) +4°C and (b) -20°C in phosphate buffer from exponential phase. Error bars indicate standard error of mean where n = 3.

selective CAT and CCDA agar plates. When the *A. butzleri* cells were incubated at 40°C for 1 h prior to heat treating the viable counts on blood agar had only increased slightly by approximately half a log. After a 2 h exposure period at 40°C a large decrease in cell viability was observed with the colony count (blood agar) dropping from 2.3×10^8 cfu ml^{-1} in the initial population to 1.08×10^2 cfu ml^{-1}. The cell viability decreased further after a 3 h incubation with no colonies being observed on either the non-selective or selective media.

These results show that a pre-exposure of up to an hour at 40°C had a slightly beneficial effect, but after that time period it caused the *A. butzleri* stationary phase cells to become more sensitive to heat. For this reason the experiment was repeated with shorter exposure times. Unfortunately contamination problems and time constraints meant that accurate viability values could not be determined.

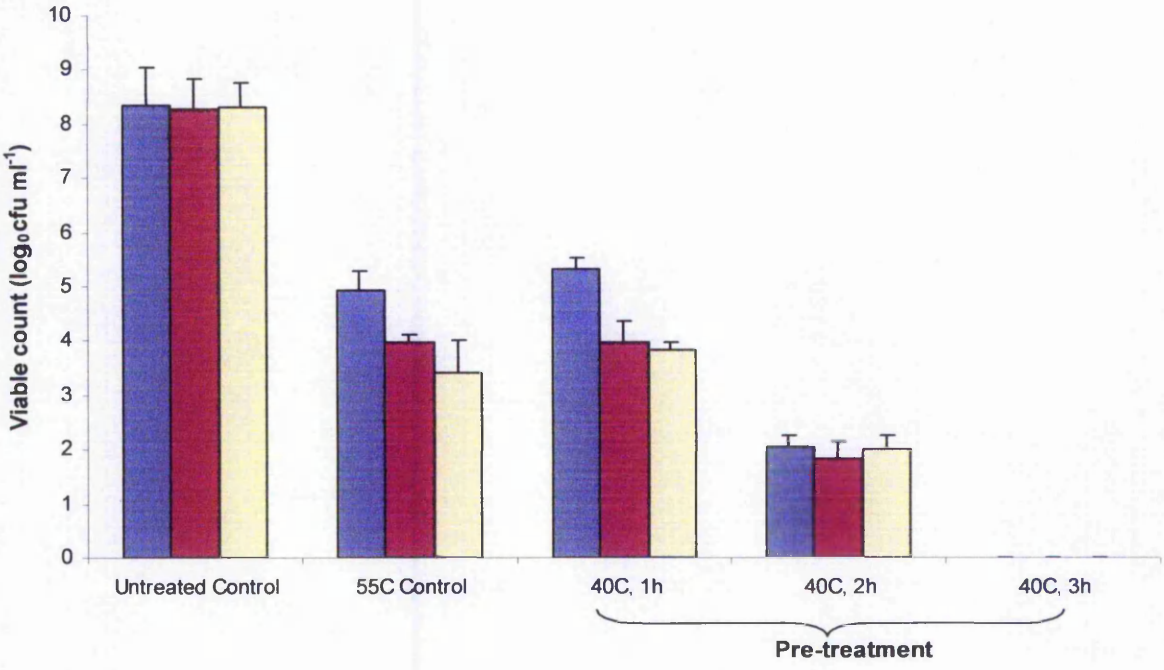


Figure 3.15: Heat adaptation of *A. butzleri* NCTC 12481 (stationary phase) by pre-exposure to 40°C followed by heat treatment (55°C, 3 min).
Media: Blood agar (■), CAT (■), CCDA (□).
Error bars indicate standard error of mean where n = 3.

These experiments were also repeated on *A. butzleri* NCTC 12481 cells harvested from the exponential phase and shown in Figure 3.16.

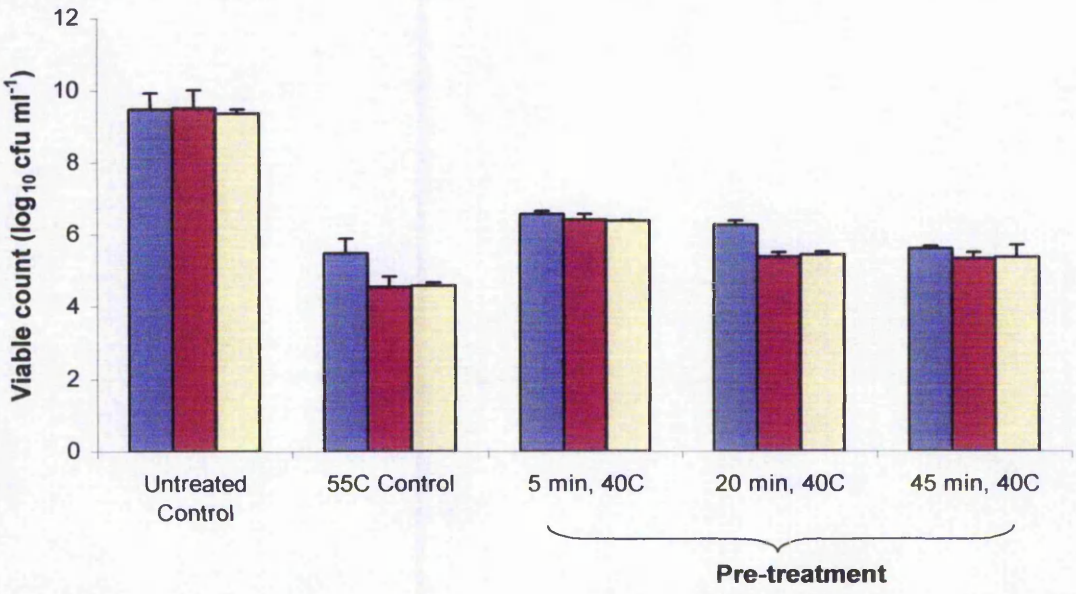


Figure 3.16: Heat adaptation of *A. butzleri* NCTC 12481 (exponential phase) by pre-exposure to 40°C followed by heat treatment (55°C, 3 min).
Media: Blood agar (■), CAT (■), CCDA (□).
Error bars indicate standard error of mean where n = 3.

Figure 3.16 shows that when the cells harvested from the exponential phase were heat treated at 55°C a 4 log decrease was detected on blood agar, whereas a 5 log decrease was observed on the selective CAT and CCDA plates. Thus suggesting that 87.9% of the population recovered on blood agar were sublethally injured by the heat treatment. However, the control experiments when the *A. butzleri* NCTC 12481 cells were only pre-exposed at 40°C for various time intervals (5, 20 and 45 min) without any further heat treatment (55°C, 3 min), the viable counts did not alter greatly from the initial untreated population of 3.0×10^9 cfu ml^{-1} on blood agar. Similar results were also detected for CAT and CCDA. For the sake of clarity this data has been omitted from Figure 3.16 (Figure 2a, p.259). After heat treating (55°C, 3 min) the 5 min and 20 min pre-exposed samples, viable counts of 3.6×10^6 cfu ml^{-1} and 1.8×10^6 cfu ml^{-1} respectively on blood agar were obtained. These counts are a 3 log decrease from the initial untreated control. Therefore, higher counts (1 log) were detected for these samples

compared to the 55°C heat-treated control (3.0 x 10^5 cfu ml^{-1}). After a 45 minute - incubation at 40°C the viability count decreased, but was still slightly greater (<0.5 log) than the 55°C heat-treated control.

To study heat adaptation further this experiment was repeated with more time periods at 40°C (5, 10, 20, 30, 45, 60, 120 and 180 min). The results from this experiment showed variation (Figure 2b, p.259). However, the general trend was similar to the previous experiment, whereby up to 1 h (with the exception of the 10 minute sample) all the viability counts were greater and in some cases 1 or 2 logs greater than the 55°C heat-treated control. For example the 45 min sample at 40°C had a cell count of 5.7 x 10^5 cfu ml^{-1} on blood agar which compared with 2.5 x 10^3 cfu ml^{-1} when the cells had only been heat-treated at 55°C for 3 minutes. Nevertheless, after extended incubation periods (2-3 h) the viability counts dropped below that of the heat-treated control.

Cold adaptation of *A. butzleri* NCTC 12481 was also studied. Figure 3.17a shows that chilling (+4°C) stationary phase cells for 24 h caused the cell viability to decrease by 1 log. A 1 log decrease was also detected for the samples that were stored at 25°C for up to 3 h prior to the chilling treatment. Therefore, storing the *A. butzleri* NCTC 12481 cells at a temperature lower than its optimum growth temperature did not induce any beneficial or harmful responses. Furthermore, very little difference was observed between enumeration on selective (CAT/CCDA) and non-selective (blood agar) media. When the harvested stationary phase cells were frozen for 24 h the viable count decreased from 1.3 x 10^9 cfu ml^{-1} on blood agar to 3.1 x 10^5 cfu ml^{-1} (Figure 3.17b). This large initial decrease was expected as it was also observed in the cold storage experiments (Section 3.8). Storing the cells at 25°C before freezing treatment had very little effect on the cell viability, although a 3 h pre-exposure period at 25°C caused the cell viability to be slightly greater than the frozen control. This affect could possibly be due to cold shock proteins being induced. Further experiments were performed whereby the pre-exposure temperature was decreased to 15°C and the *A. butzleri* cells were exposed for 30 minutes, 1, 3 and 5 hours. The results showed a similar trend for both chilling and freezing of stationary phase *A. butzleri* NCTC 12481 cells to the previous experiment (Figure 3.17, Figure 3, p. 260).

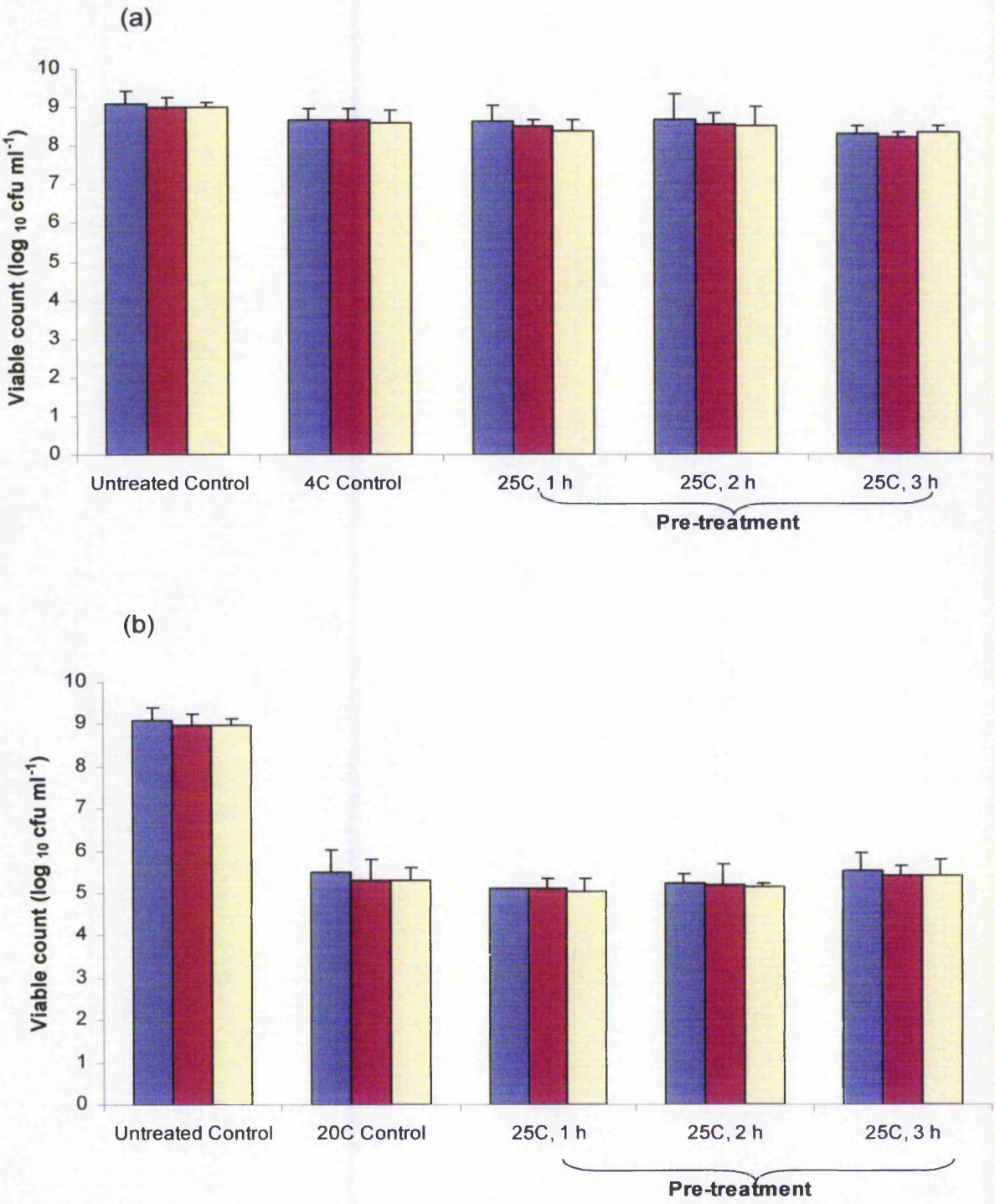


Figure 3.17: Cold adaptation (25°C) of *A. butzleri* NCTC 12481 (stationary phase) followed by (a) chilling (+4°C) or (b) freezing (-20°C) for 24 h.
Media: Blood agar (■), CAT (■), CCDA (□).
Error bars indicate standard error of mean where n = 3.

3.10. TETRAZOLIUM DYE ASSAY TO STUDY METABOLIC STATUS.

Earlier sections have shown how the plate count method was used to determine the effect of temperature treatments on the viability of *A. butzleri* NCTC 12841. However, this only provided information on the percentage of dead and sublethally injured cells that occur due to environmental stress. To further understand the mechanisms involved in sublethal injury the metabolic status of these cells needed to be studied. TTC (2,3,5-triphenyltetrazolium chloride) assay was used as an indicator of metabolic activities. The metabolic activities of both untreated and heat-treated *A. butzleri* NCTC 12481 and *E. coli* 0157:H7 toxic negative strain (comparative organism) were studied. The original method was based on that of Steward *et al.* (1991).

Untreated exponential phase *E. coli* 0157:H7 cells had a high metabolic rate as shown by a rapid red colour development (Figure 3.18). Cells harvested from the exponential phase had a metabolic rate that was almost double that of stationary phase cells. After heat treating (55°C) exponential phase cells metabolic rate was reduced by 3.5 fold, whereas stationary phase cells rate decreased by approximately 10 fold.

After studying the results it was believed that the metabolic rates observed were lower than expected, when compared with the metabolic activity of *E. coli* quoted by Steward *et al.* (1991). This decrease in metabolic activity was thought to be due to the assay being aerated when the samples were removed. As the presence of oxygen would become a competitive antagonist for reduction (Hurwitz & MacCarthy, 1986). For these reasons the assay was left in the spectrophotometer at 30°C in a constant temperature room instead of at 37°C in a water bath, thus preventing additional aeration and enabling more readings to be taken. This method produced considerably higher metabolic rates, approximately 10 fold for cells harvested from the stationary phase (rate (x 1000) = 475 abs unit min^{-1} mg^{-1}) and 3 fold the rate of the exponential phase cells (rate (x 1000) = 241 abs unit min^{-1} mg^{-1}).

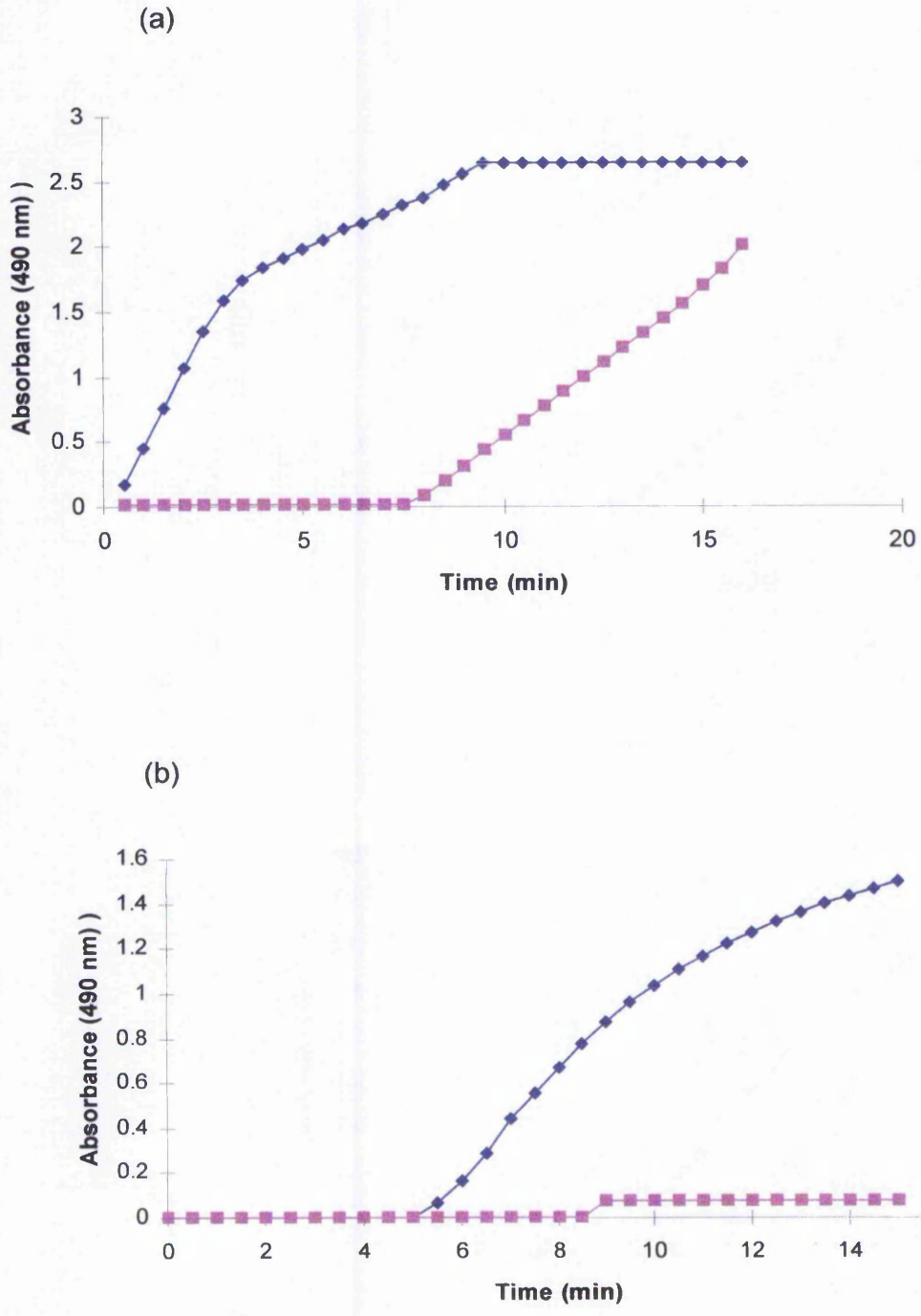


Figure 3.18: Optical density (490 nm) due to reduced tetrazolium dye by untreated (■) and heat-treated (55°C, 3 min▩) *E. coli* 0157:H7 in (a) exponential and (b) stationary phase of growth.

The metabolic activity of *A. butzleri* NCTC 12481 was also determined by using these methods (30°C and 37°C). The modified method (In the spectrophotometer at 30°C) was repeated several times with various amendments (Section 2.15), but *A. butzleri* was unable to reduce the tetrazolium salt into a red formazan. This was shown by the lack of colour development occurring after several hours, for either the untreated or heat-treated samples. These experiments were therefore not investigated further.

3.11. NITRATE AND NITRITE REDUCTION BY *A. butzleri* SPECIES.

3.11.1. PRELIMINARY SCREENING USING THE SPOT TEST.

It has been established that *A. butzleri* species are microaerophilic organisms and have the ability to tolerate an aerobic environment. Due to its ability to grow under reduced oxygen levels, the addition of nitrate or nitrite in the growth media of *A. butzleri* may be able to provide this organism with an additional source of electron acceptors in the absence of oxygen. To study the reduction of nitrate or nitrite, *A. butzleri* NCTC 12481 was screened by using the spot test, whereby the assay changed colour depending on the presence of nitrate or nitrite. Human *A. butzleri* isolates (Rigs 1714, Rigs 16799 and Rigs 15342) were also studied for comparison. The results of this preliminary screening experiment are shown over the page in Table 3.11.

Table 3.11: Determination of nitrate and nitrite reduction by four strains of *A. butzleri* after 24 h incubation.

Broth	*A. butzleri* strain			
	NCTC 12481	Rigs 1714	Rigs 15342	Rigs 16799
0.1 mM nitrate	No nitrate/nitrite detected[a]	No nitrate/nitrite detected[a]	Nitrite detected[b]	Nitrite detected[b]
1 mM nitrate	No nitrate/nitrite detected[a]	No nitrate/nitrite detected[a]	Nitrite detected[b]	Nitrite detected[b]
5 mM nitrate	Nitrite detected[b]	Nitrite detected[b]	Nitrite detected[b]	Nitrite detected[b]

[a] = Broth remained brown even upon the addition of zinc dust, therefore the nitrate had been bacterially reduced further than nitrite to ammonium ions or dinitrogen gas.

[b] = Broth turned pink due to the presence of nitrite.

The preliminary spot tests (Table 3.11) show that when low levels of nitrate (0.1 and 1 mM) were present in the BHI broth *A. butzleri* NCTC 12481 and Rigs 1714 were able to reduce the nitrate beyond nitrite to ammonium ions or dinitrogen gas. This was shown by the broth remaining a brown colour even after the addition of zinc. However, when a greater concentration was present (5 mM) the broth turned pink after the addition of 1% sulphanilamide and 0.02% naphthylene diamine. This was due to the presence of nitrite in the broth. Therefore, at the highest concentration not all the nitrate was reduced past nitrite and hence nitrite accumulated. For both *A. butzleri* Rigs 15342 and Rigs 16799 only a pink colouration was detected in all three broth types. This suggests that these strains were only able to reduce nitrate to nitrite.

These preliminary results showed that all four *Arcobacter* strains were able to reduce nitrate. Therefore, to determine if the presence of nitrate or nitrite increased *Arcobacter* species growth rate, the experiment was repeated and included plate counts and the RABIT method (Section 2.6.2).

3.11.2. IMPEDANCE MICROBIOLOGY OF *ARCOBACTER* SPECIES.

Table 3.12: Determination of nitrate and nitrite reduction and optical density (650 nm) of four strains of *A. butzleri.*

A. butzleri strain	Tests Performed	Broth		
		BHI control	BHI + 5 mM nitrate	BHI + 1 mM nitrite
NCTC12481	Spot test	Not appropriate	Nitrite detected[b]	No nitrate/nitrite detected[a]
	OD (650 nm)	0.160	0.271	0.216
Rigs 1714	Spot test	Not appropriate	Nitrite detected[b]	No nitrate/nitrite detected[a]*
	OD (650 nm)	0.121	0.094	0.126
Rigs 15342	Spot test	Not appropriate	Nitrite detected[b]**	Nitrite detected[b]
	OD (650 nm)	0.225	0.223	0.330
Rigs 16799	Spot test	Not appropriate	Nitrite detected[b]**	Nitrite detected[b]
	OD (650 nm)	0.262	0.090	0.104

[a] = Broth remained brown even upon the addition of zinc dust, therefore the nitrate had been bacterially reduced further than nitrite to ammonium ions or dinitrogen gas.

[b] = Broth turned pink due to the presence of nitrite.

* = Broth was brown, but had a slightly pink tone.

** = Deeper shade of pink was detected.

OD (650 nm) = Average of three readings.

Table 3.12 shows that the BHI control stayed brown even after the addition of zinc dust for all four *Arcobacter* strains. This was an expected result as this broth type did not contain any nitrate or nitrite. All the strains of *A. butzleri* grown in the 5 mM nitrate broth reduced the nitrate to nitrite shown by the pink colouration in the spot test. For the BHI broth containing 1 mM nitrite *A. butzleri* NCTC 12481 and Rigs 1714 reduced the nitrite to ammonium ions. However, a small amount of nitrite

was present in the broth inoculated with Rigs 1714 as a slight pink tone was observed. The other two strains were unable to reduce the nitrite present as a pink colour was detected. The optical density of each sample was taken to determine if the culture was pure after growth. Table 3.12 has shown that all the optical density values were below 0.6 which was typical of *Arcobacter* cultures and purity was confirmed by the Gram stain.

Using impedance technology, as per Section 2.6.2, the detection time of four *A. butzleri* strains were determined. These values were plotted against the viable counts on blood agar. An example set of results is shown in Figure 3.19.

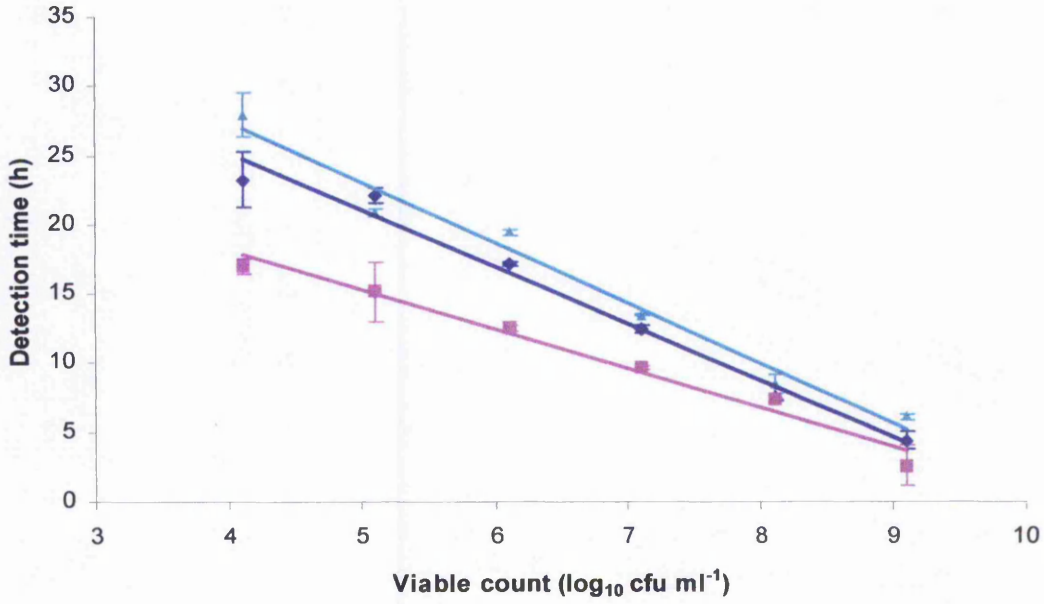


Figure 3.19: The effect of nitrate and nitrite on the growth of *A. butzleri* NCTC 12481.

Growth media: BHI broth (◆), 5 mM Nitrate broth (■), 1 mM Nitrite broth (▲)

Error bars indicate standard error of mean, where n = 3.

Figure 3.19 shows that, as expected the time to detection decreased as the viable count increased. The results also show that *A. butzleri* NCTC 12481 grew and multiplied at a faster rate when nitrate (t_d = 0.85 h) was present in the BHI broth, whereas 1 mM nitrite (t_d = 1.37 h) produced the lowest detection times, so growth occurred at a slower rate. Similar graphs were produced for the other 3 strains of *A. butzleri*, therefore this data has not been shown (Figure 4, pp.261-262). These graphs were used to determine the doubling times of each *A. butzleri* strain grown in different broths. The doubling times were calculated as in Section 2.6.2 (p.80) and shown in the table below (Table 3.13)

Table 3.13: Doubling times of four *A. butzleri* strains grown in the presence and absence of nitrate or nitrite.

A. butzleri cultures	Doubling time (h)		
	BHI control Broth	5 mM Nitrate broth	1 mM Nitrite broth
NCTC 12481	1.23	0.85	1.37
Rigs 1714	2.29	0.74	1.56
Rigs 15342	0.65	0.64	0.64
Rigs 16799	1.15	0.85	1.72

For three *A. butzleri* strains (NCTC 12481, Rigs 1714 and Rigs 16799) the fastest doubling time was detected when the cultures were grown in the presence of 5 mM nitrate in the BHI broth (Table 3.13). Therefore, the presence of nitrate increased the growth rate for the majority of *A. butzleri* strains. Furthermore, the rate of growth of the four strains in the nitrate BHI broth was similar with only 0.09 h difference between *A. butzleri* strain Rigs 15342, and NCTC 12481 (0.64 h and 0.85 h respectively). With the exception of Rigs 1714, the *A. butzleri* strains were able to grow and multiply at a slightly faster rate in the BHI broth control compared to the nitrite-BHI broth. Finally, *A. butzleri* Rigs 15342 was the only strain, where the doubling times did not vary greatly between the three different types of growth media. Consequently, the presence of nitrate or nitrite does not increase Rigs 15342 growth rate.

3.12. CYTOTOXICITY SCREENING OF *ARCOBACTER* STRAINS.

The physiological aspect of this study has provided some valuable information on *A. butzleri* NCTC 12481, especially on its survival ability. However, literature has shown that very little progress has been made into the pathogenicity of this particular organism, which is an important characteristic of any potential human pathogen. Therefore, this study investigated the possible virulence mechanisms of *Arcobacter* spp, concentrating on the cytotoxicity.

3.12.1. PRELIMINARY CYTOTOXICITY SCREENING WITH *A. butzleri* NCTC 12481.

Preliminary experiments were used to determine if cell extracts of *A. butzleri* NCTC 12481 could exert cytotoxicity towards mammalian cells (CHO, ECV and N2a) *in vitro* when the cells had been heat-treated (45, 50, 55 and 60°C, for 3 min) or left untreated. Cytotoxicity was assessed by a dye reduction assay, in which the MTT dye is reduced from a yellow to a blue colour in viable mammalian cells. Therefore, the lighter the blue colour the fewer viable cells that were present and hence the more cytotoxic the sample was. See Materials and Methods Section 2.18.5 for more details. For presentation purposes certain supporting data can be found in the Appendix (Figures 5 & 6, pp.263-264).

The MTT assay gave approximately 100% values for viability with the 2.5% sample concentration (Figure 3.20a). Therefore, the same number of N2a cells had been killed by the phosphate control as the cell-free extract. However, when the N2a mammalian cell lines were exposed to a 10% concentration of the cell-free extract the cell viability decreased greatly producing values between 80% for the untreated sample to 50% for the 60°C heat-treated sample. Also, as the temperature of the heat treatment increased the cell viability decreased gradually. For the untreated and 50°C heat-treated sample a 10% concentration was required to produce a significant decrease in N2a cell viability. On the other hand, significant levels of cytotoxicity were detected with the lower applied concentrations of cell-free extracts from 55°C (5% and 10% concentration) and 60°C (2.5%, 5% and 10% concentration) heat-treated cells.

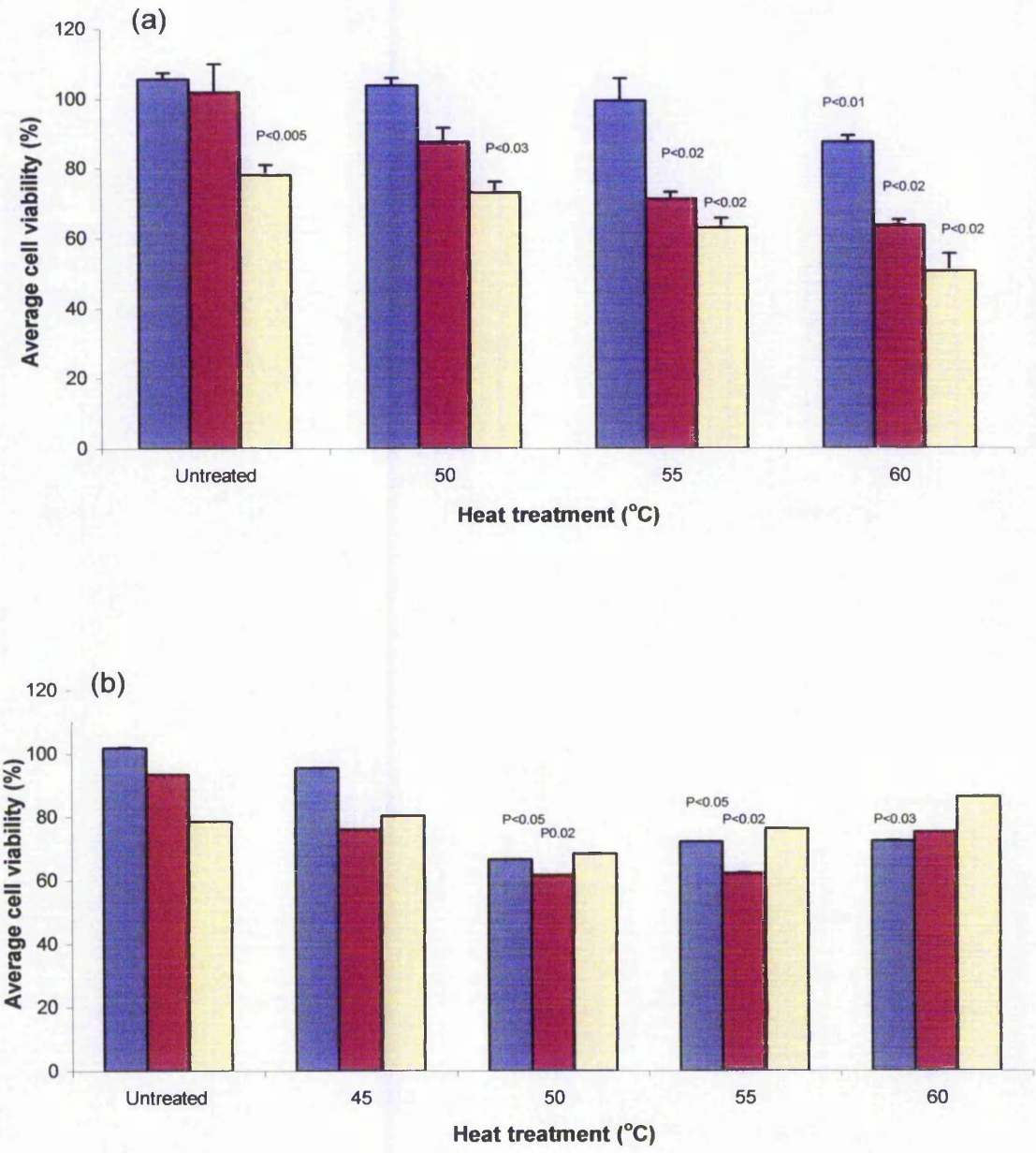


Figure 3.20: The effect of cell-free extracts from (a) exponential and (b) stationary phase *A. butzleri* NCTC 12481 on N2a cell lines.

Concentration of cell-free extract applied to mammalian cell lines:
2.5% ( ), 5%( ) and 10%( ).
Error bars indicate standard error of mean where n = 9.
For Figure 3.20 (b) error bars were too small to be presented.
p = The statistical significance compared with controls determined using Mann-Whitney U test for non-parametric distributions using confidence limit of 95%. Unless otherwise specified there was no significant difference from controls.

Upon comparison of the untreated and heat-treated cell-free extracts significant differences in cytotoxicity were only detected between the untreated samples and the samples, which had been heat-treated at 55°C (5% and 10% concentration) and 60°C (2.5%, 5% and 10% concentration).

These experiments were also repeated with CHO and ECV mammalian cell lines (Figure 6, p.264). The ECV results follows an almost identical trend to those observed by the N2a cell lines. This trend was also observed for CHO cell lines, but the viability of the CHO cells for the three different concentrations (2.5, 5 and 10%) were considerably higher than those detected for N2a cell lines. The lowest CHO viability values were detected for the 55°C and 60°C heat-treated samples applied at a 10% concentration, which produced 75.9% and 77.5% values respectively. Therefore, CHO cells were less sensitive to *Arcobacter* cytotoxin(s) than N2a cells.

Cell-free extracts harvested from stationary phase cultures (Figure 3.20b) showed a similar pattern to the results obtained for the exponential phase (Figure 3.20a). However, the cell viability of the N2a cells was greater for the stationary phase cell-free extract compared to the exponential phase values. For example after the application of cell-free extract from heat-treated (55°C) exponential phase cells the viability of the N2a cells was 62.9%, compared to 76.2% for stationary phase (10% applied concentration). Furthermore, when the N2a cells were exposed to a 10% concentration of the cell-free extract the viability was greater than the 5% concentration, and in some cases, for example at 50°C and 55°C heated samples were greater than the 2.5% concentration. This particular set of results was believed to be inaccurate as more N2a cells were likely to be killed by a higher concentration of cell-free extract, especially when it caused nearly a 40% kill (50°C) after a 5% concentration was applied to the cells. These preliminary experiments were not repeated as they provided an overview of *Arcobacter* cytotoxin(s) production. This information was used as part of an in-depth study into cytotoxin(s) from both exponential and stationary phase cells (see Section 3.12.2). The results obtained from the cell-free extracts harvested from stationary phase cells also showed that significantly different cytotoxicity levels were only

detected between the untreated samples and the 50°C, 55°C samples (2.5% and 5% concentration only) and 60°C sample (2.5% concentration).

The cell-free extracts harvested from a stationary phase culture were applied to both CHO and ECV cell lines (Figure 5, p.263). For all three concentrations (2.5, 5 and 10%) the cell viability of both ECV and CHO was approximately 100%. Therefore, the cell-free extracts harvested from the stationary phase culture did not show any cytotoxic effect on either CHO or ECV cells.

From these preliminary results only a gradual decrease in cell viability of all three mammalian cell lines was detected when the temperature of the heat treatment of the bacterial cell suspension was increased in 5°C increments. Therefore, it was decided that for further experiments only one heat treatment was required to determine the ability of *A. butzleri* strains to exert a toxic affect on mammalian cell lines. For this reason a 55°C heat treatment was chosen as it showed relatively high toxic affect on both N2a and ECV cell lines. Furthermore, this temperature had been used as a standard heat treatment in previous experiments (Section 2.10).

3.12.2. DETERMINATION OF TOXIN LOCATION.

From these preliminary cytotoxicity experiments it was shown that *A. butzleri* NCTC 12481 extracts had a toxic affect on mammalian cell lines. However, the location and identity of the toxin needed to be determined. For this reason the spent media, cell-free extract and cell sonicate were tested for cytotoxicity. To determine if the cytotoxin(s) was released into the growth medium during incubation or if environmental stresses such as heat or sonication caused the toxin to be induced or released after cell lysis. Although little cytotoxicity was detected from the cell-free extracts harvested from stationary phase cells in the preliminary experiments, it was necessary to study the effect growth phase had on the cytotoxicity of this species in more depth. Therefore, cells harvested from both stationary and exponential phase were used. The cytotoxicity results from the samples harvested from the stationary phase are shown below (Table 3.14).

Table 3.14: Determination of cytotoxin location from *A. butzleri* NCTC 12481 harvested from the stationary phase.

Sample	Applied concentration	CHO		ECV		N2a	
		Not heat treated	Heat treated†	Not heat treated	Heat treated	Not heat treated	Heat treated
Spent media	2.5%‡	101.0 ± 3.0§	102.4 ± 1.6	138.2 ± 11.3 p<0.04*	115.8 ± 10.2	97.0 ± 6.7	105.9 ± 2.6
	5%	113.3 ± 3.3 p<0.03*¶	112.3 ± 0.3	107.0 ± 6.7	94.2 ± 1.0	89.7 ± 5.7	91.5 ± 2.9
	10%	111.5 ± 4.5	123.8 ± 8.1 p<0.05*	117.7 ± 5.4 p<0.04*	116.1 ± 2.2 p<0.008*	94.7 ± 6.1	98.1 ± 3.7
Cell-free extract	2.5%	113.0 ± 4.0	110.7 ± 8.1	106.5 ± 4.4	109.3 ± 2.7	82.2 ± 9.3	73.5 ± 12.2
	5%	120.7 ± 3.6 p<0.01*	111.5 ± 0.26	114.2 ± 5.9 p<0.04*	100.5 ± 1.1#	76.1 ± 9.2 p<0.02	60.0 ± 6.3 p<0.002
	10%	105.1 ± 1.2	110.8 ± 3.4 p<0.04*	115.2 ± 7.7	99.7 ± 0.9	73.0 ± 3.7 p<0.002	55.1 ± 4.3# p<0.002
Cell sonicate	2.5%	93.9 ± 1.6	91.7 ± 4.4	88.4 ± 0.4 p<0.04	88.3 ± 2.0 p<0.03	95.3 ± 5.2	90.1 ± 6.3
	5%	109.1 ± 3.2 p<0.04*	106.3 ± 1.9	93.4 ± 1.8	95.6 ± 2.4	73.8 ± 5.7	89.9 ± 3.9 p<0.002
	10%	116.6 ± 2.4 p<0.002*	105.0 ± 1.2#	97.5 ± 5.7	106.6 ± 8.8	72.5 ± 1.9 p<0.002	87.5 ± 4.7 p<0.03

† = Heat-treated cells (55°C, 3 min). See Materials and Method for details.
‡ = Volume of material added to mammalian cell line (%).
§ = Standard error of mean where n = 9.
* = Statistically significant toxicity levels, except cell viability shows an increase compared with controls.
¶ = The statistical significance compared with controls determined using Mann-Whitney U test for non-parametric distributions using confidence limit of 95%. Unless otherwise specified there was no significant difference from controls.
# = The statistical significance between not heat-treated and heat-treated samples using Mann-Whitney U test with confidence limit of 95%.

Table 3.14 shows that the BHI spent media did not exert any cytotoxic effects which would cause the cell viability of any of the mammalian cell lines to decrease even when increasing concentrations of sample were applied. This was shown by values of approximately 100% viability being detected, whereby the levels of mammalian cells being killed off by the BHI spent media were equivalent to the BHI control. Nevertheless, for both the heat-treated and untreated BHI spent media applied to CHO and ECV cell lines, significant levels of cytotoxicity were detected. This cytotoxicity was shown by an increase in cell viability (>100%), whereby components of the BHI spent media cause the mammalian cell lines to increase proliferation and/or metabolic activity. Due to time constraints this type of toxicity was not studied further, with toxic effects causing a decrease in cell viability being favoured. Small levels of toxicity were detected for the cell sonicate samples. However, this was mainly detected on N2a cell lines and only when a high concentration was applied. For example untreated and heat-treated samples respectively produced a 27.5% and 12.5% decrease in N2a cell viability when applied at a 10% concentration. The highest levels of cytotoxicity were detected for the cell-free extracted samples, but again toxicity was mainly found on the N2a cell line, as a 55.1% cytotoxicity level was observed when a 10% concentration of heat-treated cell-free extract was applied. The toxicity of the cell-free extract harvested from the stationary phase also increased with increasing applied volumes. This experiment was also repeated using Rigs 1714, Rigs 15342 and Rigs 16799 however the data has not been shown here, as no significant toxicity was detected for any of the sample for any of the cell lines or applied concentration (2.5%, 5% and 10%, Tables 1-3, pp.265-267).

From these results it can be stated that the toxicity produced by stationary phase *A. butzleri* NCTC 12481 cells was detected mainly in the cell-free extract and the level of toxin(s) present was increased after a 3 minute heat treatment at 55°C. However, a small amount of toxicity was induced by cell lysis after sonication treatment. The levels of toxicity produced from these samples that were harvested from stationary phase cells tended to be lower and less reproducible compared with parallel studies with exponential phase cells (Table 3.15). This suggests that the level of cytotoxicity decreased as the *A. butzleri* cells aged.

Table 3.15 MTT toxicity testing of *A. butzleri* strains cell sonicate and cell-free extract harvested from the exponential phase.

Cell line & volume of material added (%)	*A. butzleri* strain											
	NCTC 12481				1714		15342		16799			
	CFE¥		CS†	CFE	CS	CFE	CS	CFE	CS	CFE	CS	
	Heat treated	Not heat treated										
CHO 2.5‡	117.2 ± 3.8§ p<0.004*¶	110.6 ± 4.1 p<0.05*	105.9 ± 3.0	93.0 ± 2.4	111.4 ± 3.5 p<0.04*	124.9 ± 6.6 p<0.03*	89.9 ± 2.4	141.0 ± 8.9 p<0.002*	106.0 ± 3.3			
5	110.7 ± 2.4 p<0.0004*	112.5 ± 2.4 p<0.0003*	107.3 ± 5.5	99.2 ± 3.3	106.4 ± 1.3	122.2 ± 8.3 p<0.02*	111.4 ± 5.4	109.9 ± 9.4	99.8 ± 4.4			
10	89.0 ± 5.8#	100.4 ± 1.8	98.6 ± 5.9	99.4 ± 2.6	97.7 ± 0.7	118.6 ± 11.2	111.2 ± 2.3 p<0.02*	113.0 ± 4.0	109.2 ± 12.2			
ECV 2.5	106.3 ± 2.7	106.2 ± 7.4	104.8 ± 7.9	100.9 ± 0.5	91.7 ± 3.5	97,2 ± 7.0	112.3 ± 2.3	102.3 ± 1.8	103.6 ± 1.8			
5	95.4 ± 8.8	108.2 ± 10.9	105.9 ± 10.7	86.6 ± 4.4	97.8 ± 3.4	98.3 ± 4.1	92.2 ± 2.9	83.3 ± 5.1	105.7 ± 3.1			
10	76.5 ± 7.4 p<0.01	85.9 ± 9.5	109.8 ± 9.9	69.4 ± 6.3 p<0.03	95.9 ± 3.1	89.1 ± 8.4	92.9 ± 3.6	58.4 ± 7.9 p<0.03	98.9 ± 3.4			
N2a 2.5	90.18 ± 8.9	89.5 ± 8.3	95.1 ± 3.2	96.1 ± 1.0	100.5 ± 0.2	115.2 ± 0.8 p<0.004	125.1 ± 13.2	94.0 ± 10.2	107.6 ± 0.5			
5	59.7 ± 5.6# p<0.001	82.7 ± 9.2	92.8 ± 4.2	83.3 ± 3.8 p<0.002	102.5 ± 5.9	104.2 ± 1.1	113.4 ± 0.7	72.0 ± 10. P<0.03	98.6 ± 1.6			
10	47.8 ± 6.0# p<0.0001	69.9 ± 4.6 p<0.0001	78.3 ± 1.4 p<0.002	55.0 ± 3.9 p<0.002	103.2 ± 5.6	108.5 ± 5.2	101.3 ± 4.4	62.3 ± 7.5 p<0.01	105.5 ± 5.5			

Footnotes for Table 3.15.
¥CFE = Cell-free extract from heat-treated cells (55°C, 3 min) unless otherwise indicated. See Materials and Methods for details.
†CS = Cell sonicate, see Materials and Method for details.
‡ = Volume of material added to mammalian cell line (%).
§ = Standard error of mean where n = 9.
* = Statistically significant toxicity levels, except cell viability shows an increase compared with controls.
¶ = The statistical significance compared with controls determined using Mann-Whitney U test for non-parametric distributions using confidence limit of 95%. Unless otherwise specified there was no significant difference from controls.
# = The statistical significance between not heat-treated and heat-treated samples using Mann-Whitney U test with confidence limit of 95%.

The MTT cytotoxicity results in Table 3.15 showed that cytotoxic activity towards N2a and to a lesser extent ECV was detected in cell-free extracts from bacterial cells harvested in the exponential phase of growth. This toxicity was greater following heat treatment of bacterial cells (55°C, 3 min). For the sake of presentation only comparative data for strain NCTC 12481 are presented in Table 3.15 (Table 4 & 5 pp.268-269).

As Table 3.15 shows, no cytotoxicity causing the cell viability to decrease was detected with CHO cells when either cell-free extract or cell sonicate from any of the four *A. butzleri* strains was applied. However, an increase in MTT activity due to an increase in cell proliferation and/or metabolic activity was detected with the cell- free extracts from *Arcobacter* NCTC 12481, Rigs 15342 and Rigs 16799 and the cell sonicate from *Arcobacter* Rigs 1714 and Rigs 15342.

Heat-treated cell-free extracts of *A. butzleri* NCTC 12481 from the exponential phase of growth produced comparable cytotoxic activity (on cell weight basis) to the other strains, whereas *A. butzleri* Rigs 15342 produced the lowest cytotoxic activity (Table 3.15). Prior heat treatment (55°C, 3 min) increased the cytotoxicity activity of extracts from all *A. butzleri* strains. For example a value of 47.8% MTT activity was detected when the heat-treated cell-free extract (10% concentration) of *A. butzleri* NCTC 12481 was applied to N2a cell line, which compares to a value of 69.9% for the non heat-treated cell-free extract. No cytotoxicity was detected in the BHI spent media for any of the *A. butzleri* strains.

3.12.3. THE EFFECT OF GROWTH CONDITIONS ON *A. butzleri* NCTC 12481 CYTOTOXICITY.

Since *A. butzleri* NCTC 12481 was the type strain for the *Arcobacter* genus and was originally isolated from a human case of gastroenteritis, it was selected for further detailed investigation. As well as trying to determine the cytotoxin(s) location it was also important to study if growth conditions had any effect on the toxicity levels produced by this particular organism. For this reason, *A. butzleri* NCTC 12481 was grown at 30°C instead of 37°C or under microaerophilic conditions unlike previous broth cultures, which had been grown under aerobic conditions (Figure 3.21 a & b).

Samples from exponential phase *A. butzleri* NCTC 12481 grown at 30°C produced comparable cytotoxicity on CHO, ECV and N2a cell lines (10% concentration only) to the cultures grown at 37°C. For example, a cytotoxicity value of 49% was detected for the cell-free extract from cultures grown at 37°C (10% concentration on N2a cells), which compares to a value of 47.8% for the 30°C sample. Significant toxicity levels were detected on both N2a and ECV cell lines for cell-free extracts and to a lesser extent cell sonicate samples. Using the Mann-Whitney U test (95% confidence levels) all three cell lines showed that the cell-free extracts produced greater cytotoxicity levels than cell sonicate. However, the N2a cell lines (2.5% and 5% concentration) showed that growing the *Arcobacter* cells at 37°C caused the cytotoxicity levels to significantly increase compared to the 30°C sample (Figure 3.21a). Therefore, growing the cells under more stressful temperatures (37°C) increased the cytotoxin(s) production. This effect was not observed for the 10% sample as both cytotoxicity values were extremely high for the cell-free extract harvested from the exponential phase cells (30°C = 49%. 37°C = 47.8%).

A similar pattern of results was obtained for the untreated samples, however lower levels of cytotoxicity were determined. For example a value of 74.9% was observed when a 10% concentration of untreated cell-free extract was applied to ECV cells, whereas a value of 56.7% was detected for the heat-treated sample. This experiment was also repeated with cells harvested from the stationary phase and a similar set of results to those shown in Table 3.14 was obtained.

Nevertheless, like previous experiments the levels of toxicity, detected by the stationary phase extracts were generally lower compared to those produced by exponential phase cultures. Due to the similarities in the untreated and stationary phase results with those shown in Figure 3.21a it was deemed unnecessary to present this data here (Figures 7 & 8, pp.270-271).

When the *A. butzleri* NCTC 12481 cultures were grown under microaerophilic conditions (static in the microaerophilic cabinet) instead of shaking in aerobic conditions cytotoxicity was again mainly detected on N2a and to a lesser extent ECV cell lines (Figure 3.21b, Figure 9, p.272). Furthermore, the greatest toxicity levels were detected for the cells harvested from the exponential phase, which had been heat- treated (55°C, 3min) before being applied to the mammalian cell lines. Comparing these results to those when *A. butzleri* NCTC was grown at 37°C under aerobic conditions (Table 3.14 & 3.15, Figure 3.20a & b) the levels of cytotoxicity did not alter greatly, but the samples were slightly more cytotoxic under the more stressful conditions of aerobic growth. This effect was more evident for the untreated (2.5%-10% concentration) and heat-treated (2.5% & 5% concentration only) cell- free extracts harvested from stationary phase cells with significantly higher cytotoxicity values being detected when the cells were grown aerobically. Therefore, changing the growth temperature and oxygen availability only slightly altered the cytotoxic activity of *A. butzleri* NCTC 12481.

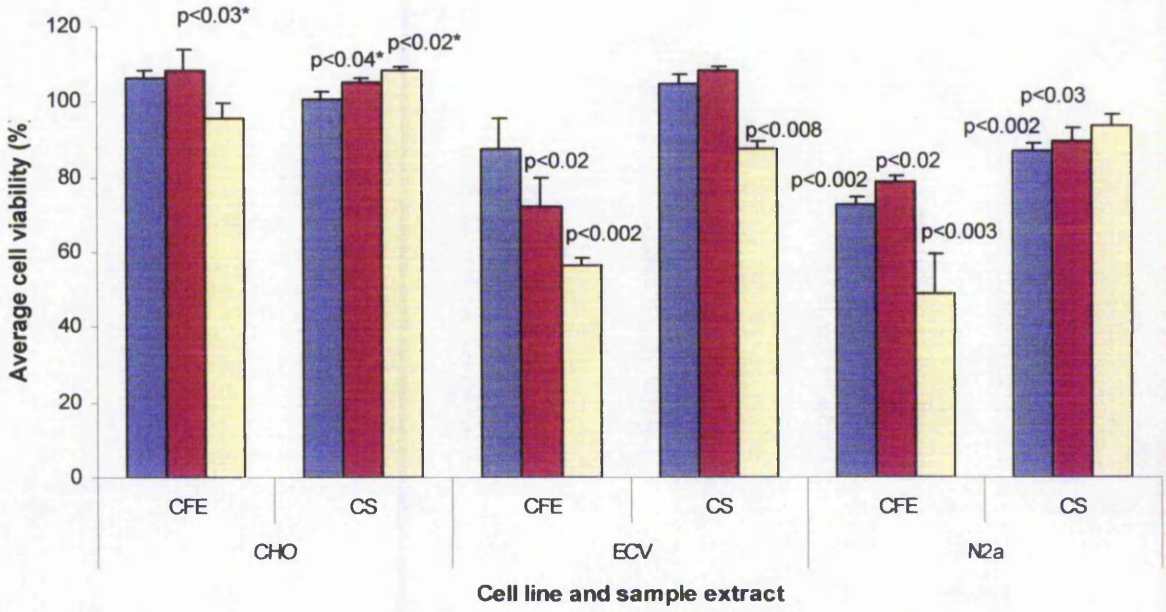


Figure 3.21a: Cytotoxicity of heat-treated *A. butzleri* NCTC 12481 grown at 30°C under aerobic conditions (exponential phase).

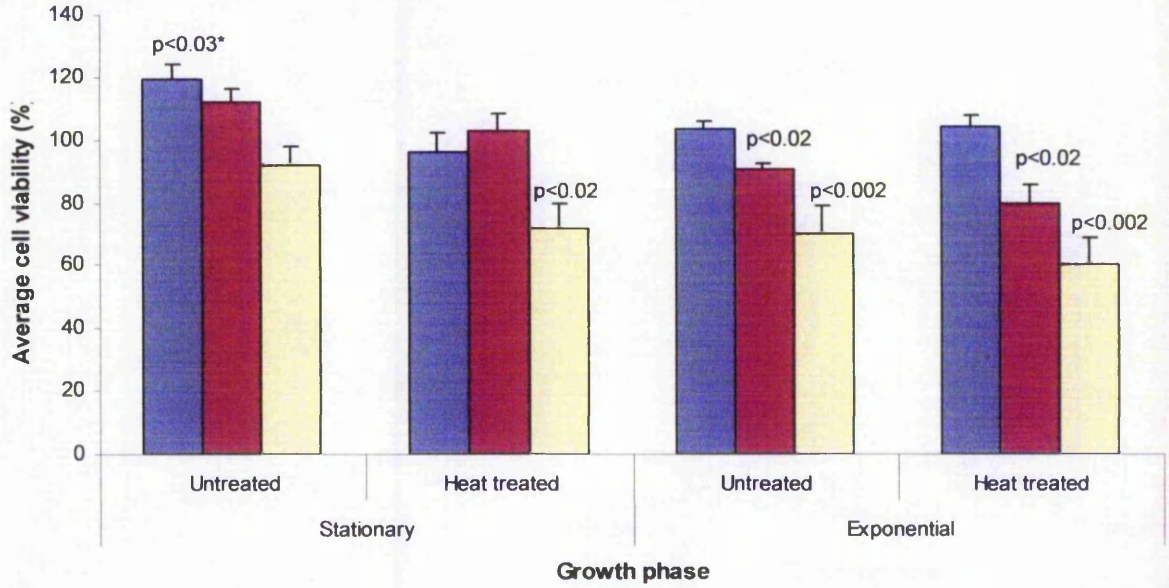


Figure 3.21b: Cytotoxicity of *A. butzleri* NCTC 12481 grown at 37°C under microaerophilic conditions towards N2a cell line.
Concentration of cell-free extract (CFE) or cell sonicate (CS) applied to mammalian cell lines: 2.5% (▨), 5%(■) and 10%(□).
Error bars indicate standard error of mean where n = 9.
* = Statistically significant toxicity levels, except cell viability shows an increase compared with controls.
p = The statistical significance compared with controls determined using Mann-Whitney U test for non-parametric distributions using confidence limit of 95%. Unless otherwise specified there was no significant difference from controls.

3.12.4. DETERMINATION OF TOXIN TYPE.

It was important to determine whether the type of cytotoxin(s) produced by *A. butzleri* NCTC 12481 was heat-stable and/or susceptible to proteolysis. Cell-free extracts from heat-treated (55°C, 3 min) cells were boiled for 10 minutes or exposed to either trypsin or pronase (for more details see Section 2.17.2 in Materials and Method). N2a cell line cytotoxicity results are shown below in Figure 3.22.

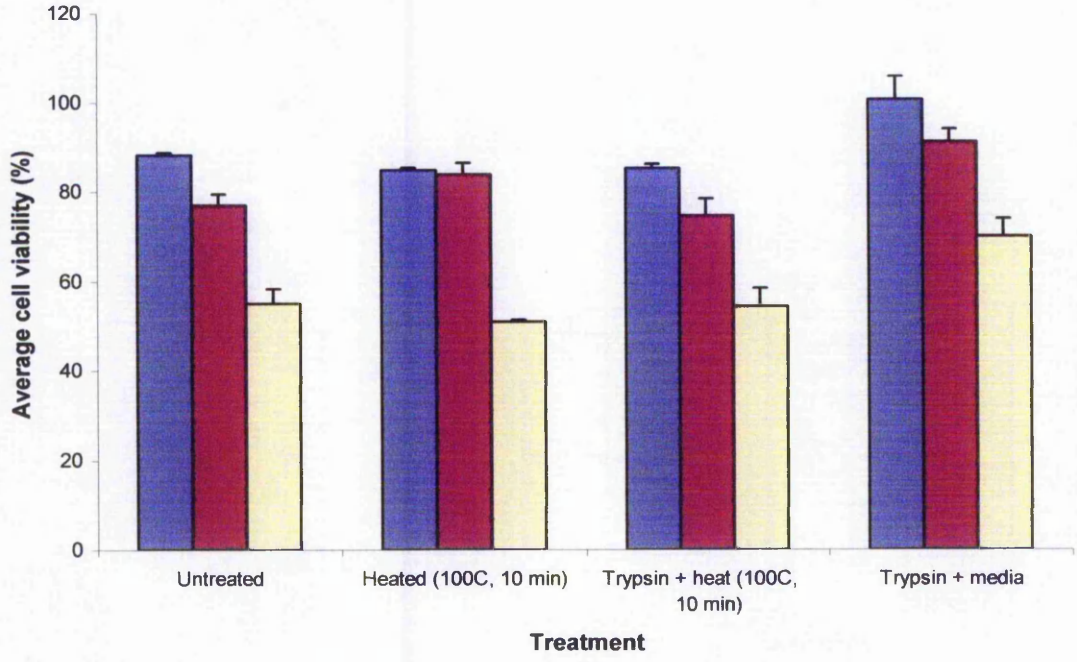


Figure 3.22: Heat and proteolytic stability of cytotoxin(s) from *A. butzleri* NCTC 12481 towards N2a cells (exponential phase).

Untreated control = Heat-treated (55°C, 3 min) cell-free extract (CFE).
Heated = Heat-treated (55°C, 3 min) CFE followed by boiling (100°C) for 10 minutes.
Trypsin + heat = Heat-treated (55°C, 3 min) CFE followed by incubation (30 min) with 0.1% Trypsin which was deactivated by boiling (100°C) for 10 minutes.
Trypsin + media = Heat-treated (55°C, 3 min) CFE followed by incubation (30 min) with 0.1% Trypsin which was neutralised by mixing with DMEM media containing 10% foetal calf serum.
Concentration of cell-free extract applied to mammalian cell lines:
2.5% (▩), 5%(■) and 10%(□).
Error bars indicate standard error of mean where n = 9.

Figure 3.22 shows that all the samples were cytotoxic to a certain extent with increasing sample concentration causing the viability of the N2a cell line to decrease. The toxicity level did not alter greatly between the untreated control and the heated (100°C, 10 min) and the trypsin treated sample which was deactivated by boiling for 10 minutes. However, the trypsin treated sample, which was neutralised with DMEM media containing 10% foetal calf serum showed less cytotoxicity compared to the other samples and a significant difference was observed between this sample and the untreated control. Since the trypsin/heat-treated sample had no effect on the cytotoxin(s) produced by *Arcobacter* NCTC 12481, this effect is probably due to the cytotoxin(s) being diluted out slightly when the trypsin was neutralised by the DMEM. Therefore, these results suggest that either the cytotoxin(s) was a heat and trypsin stable protein or an LPS molecule. The experiment was repeated with pronase at a 0.25% concentration. High levels of toxicity were obtained with pronase exposed samples (Figure 11c, p.275). A similar pattern of toxicity results was detected for ECV cells, but again no toxicity was detected for CHO cells and so no comparisons between the untreated control and heat and/or protease treated samples could be made (Figures 10 & 11, pp. 273-275).

To further determine if the cytotoxin(s) was a heat stable protein or an LPS molecule analysis was performed using a series of protein and LPS gels (see Figures 3.23 & 3.24).

The results obtained from the exponential phase samples showed that proteins of varying sizes were present in the samples and a well-banded profile was detected (Figure 3.23). Heat-treating (55°C, 3 min) the samples produced a higher concentration of proteins, which was shown by the intensity of the bands increasing. Nevertheless, the gel does show that the more treatments (i.e. heat or proteolytic enzymes) used on the samples the less proteins that are detectable. This was especially evident in the trypsin and boiled samples. These results did not correspond with the cytotoxicity results (Table 3.15) since if the cytotoxin was a protein then as the protein content decreased with treatment, so would the cytotoxicity levels of the *A. butzleri* samples. This decrease in protein content, but

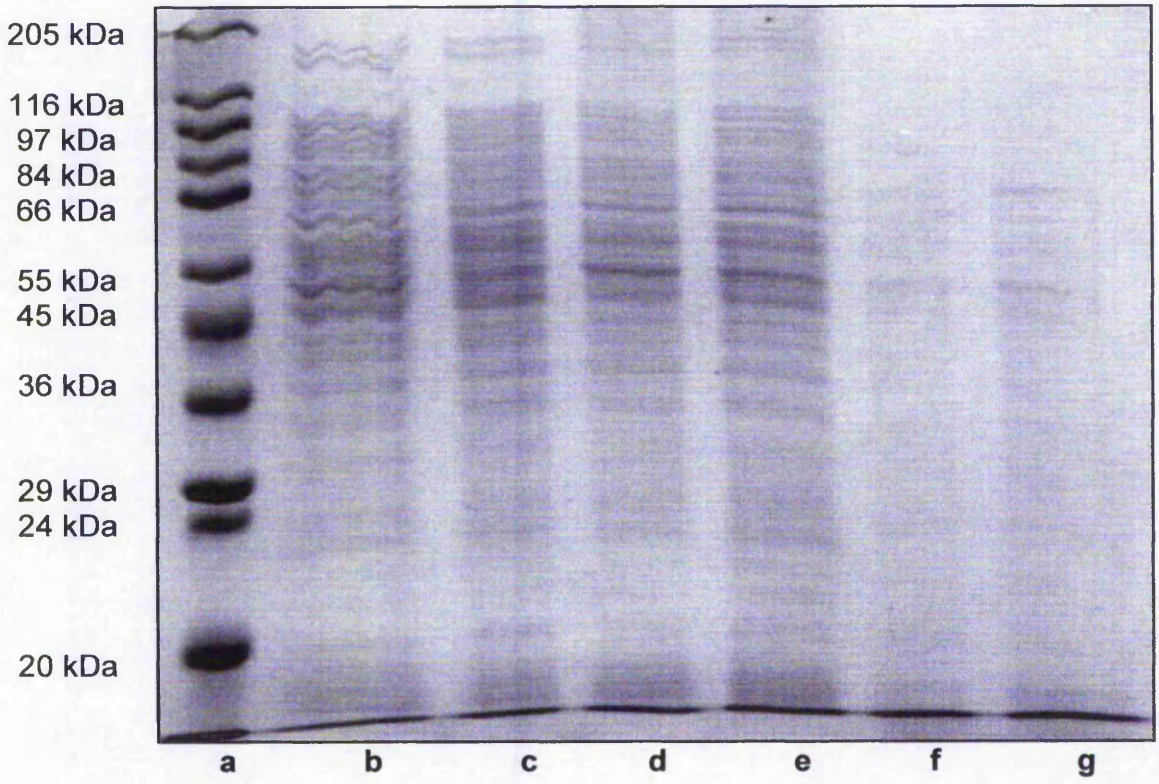


Figure 3.23: Detection of proteins in cell-free extracts harvested from exponential phase *A. butzleri* NCTC 12481 cells, which have been exposed to heat treatment (100°C, 10 min) and/or proteolytic enzymes.

Arcobacter heat-treated and non heat-treated cell free extracts prepared from exponentially growing *A. butzleri* NCTC 12481 cells prior to separation by polyacrylamide gel electrophoresis as described in Material and Methods. Shown is a Coomassie blue stained gel of protein from (a) protein molecular marker (6.5-205 kDa, the 6.5 and 14 kDa bands do not appear on a 10% resolving gel), (b) cell-free extract from non heat-treated exponentially growing cells (control), (c) cell-free extract from heat-treated exponentially growing cells (control) (d) cell-free extract from non heat-treated exponentially growing cells, which were boiled for 10 minutes. (e) cell-free extract from heat-treated exponentially growing cells, which were boiled for 10 minutes, (f) cell-free extract from non heat-treated exponentially growing cells, which was Trypsin treated for 30 minutes followed by being boiled for 10 minutes, (g) cell-free extract from non heat-treated exponentially growing cells, which was Trypsin treated for 30 minutes followed by being boiled for 10 minutes.

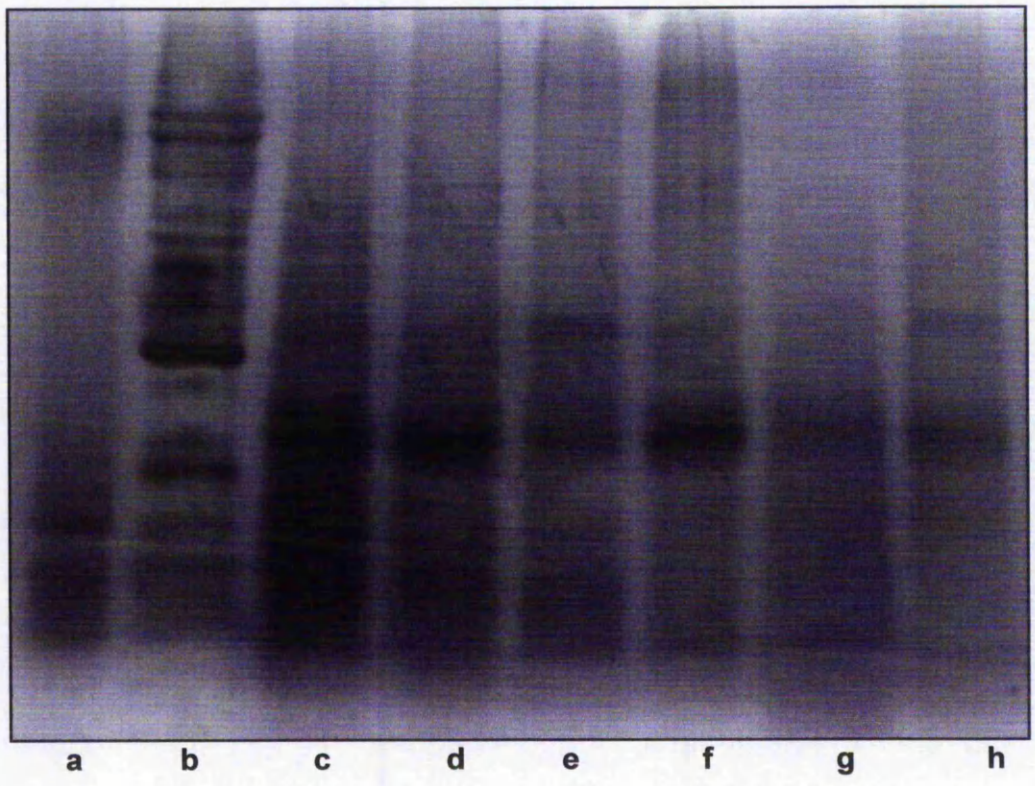


Figure 3.24: Detection of lipopolysaccharide in cell-free extracts harvested from exponential phase *A. butzleri* NCTC 12481 cells, which have been exposed to heat treatment (100°C, 10 min) and/or proteolytic enzymes.

Arcobacter heat-treated and non heat-treated cell free extracts prepared from exponentially growing *A. butzleri* NCTC 12481 cells prior to separation by polyacrylamide gel electrophoresis as described in Material and Methods. Shown is a silver stained gel of lipopolysaccharide from (a) *Salmonella* Typhimurium (marker) (b) silver stain molecular marker (6.5-180 kDa) used only as a positive control to check that the staining procedure was working correctly, (c) cell-free extract from non heat-treated exponentially growing cells (control), (d) cell-free extract from heat-treated exponentially growing cells (control) (e) cell-free extract from non heat-treated exponentially growing cells, which were boiled for 10 minutes. (f) cell-free extract from heat-treated exponentially growing cells, which were boiled for 10 minutes, (g) cell-free extract from non heat-treated exponentially growing cells, which was Trypsin treated for 30 minutes followed by being boiled for 10 minutes, (h) cell-free extract from non heat-treated exponentially growing cells, which was Trypsin treated for 30 minutes followed by being boiled for 10 minutes.

not toxicity was also observed for the cell-free extract harvested from stationary phase cells.

A series of electrophoresis gels of LPS were performed in parallel with the protein analysis. The gel (Figure 3.24) shows that cell-free extract harvested from exponential and stationary phase cells produced ladder-like profiles proving that LPS was present in these samples. However, most of the bands were near the bottom of the gel suggesting the presence of low molecular LPS. Bands with greater intensity were detected for the exponential phase sample which was heat-treated. This result corresponds to the toxicity results shown in Table 3.15. It was also important to prove that there was no residual protein in these samples, which could effect the migration of the LPS and the staining of the gel. For this reason a duplicate gel was performed, but stained with Coomassie blue stain. The gel showed that there was no protein present in the LPS samples (not presented).

Subsequently, the LPS profiles of the cell-free extract harvested from both exponential and stationary phase cells were studied in more detail (Figure 3.25).

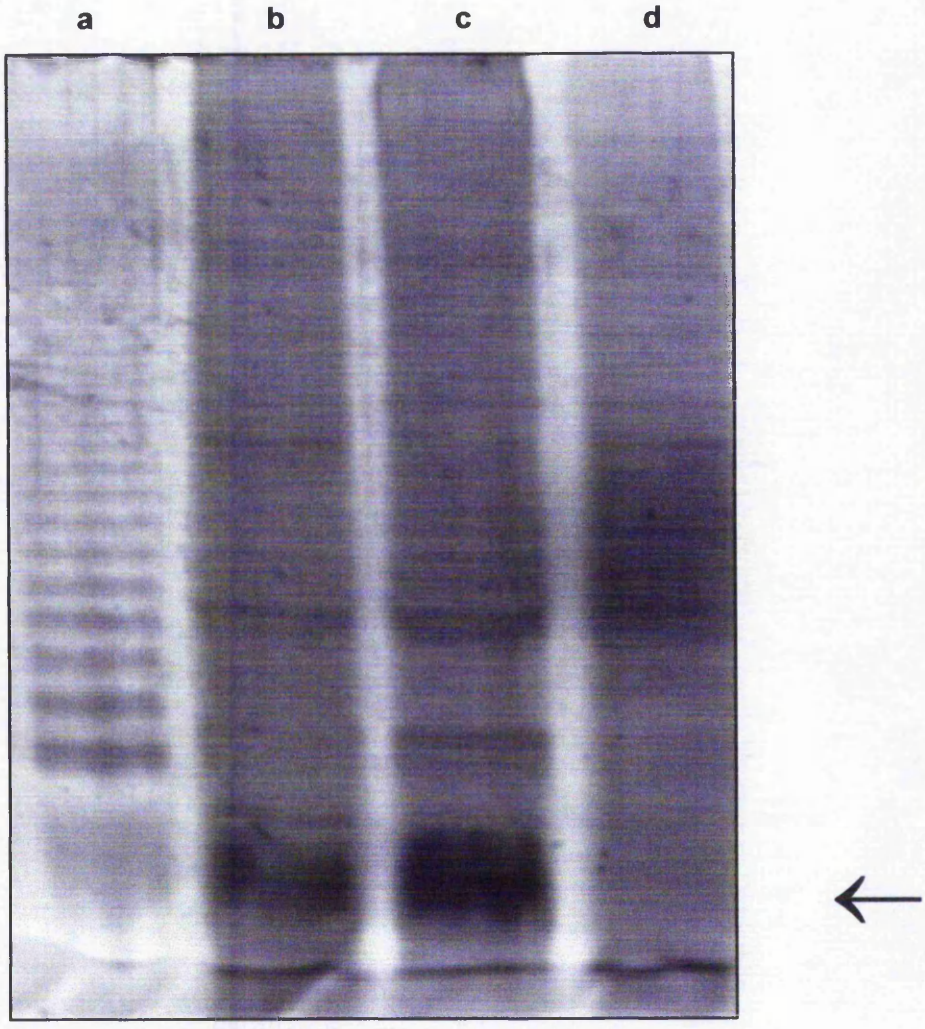


Figure 3.25: Detection of lipopolysaccharide fractions from *A. butzleri* NCTC 12481.

Arcobacter heat-treated and non heat-treated cell extracts prepared from exponentially growing (b, c) and stationary phase (d) cells prior to separation by polyacrylamide gel electrophoresis as described in Materials and Methods. Shown is a silver stained gel of lipopolysaccharide from (a) *Salmonella* Typhimurium (marker) (b) cell-free extract from non heat-treated exponentially growing cells, (c) cell-free extract from heat-treated exponentially growing cells and (d) cell-free extract from heat-treated stationary phase cells. The arrow indicates low molecular weight lipopolysaccharide band that was more pronounced in exponentially growing cells especially after heat treatment.

Figure 3.25 shows that the exponential and stationary phase samples produced ladder-like LPS profiles with more bands being concentrated at the bottom of the gel. However, a band was more pronounced in the exponential phase LPS profile. This band was darker in the heat-treated cell-free extract than in the untreated sample. These results correspond with the cytotoxicity results shown in Table 3.15. A parallel Coomassie blue stained gel showed only the molecular markers and positive protein control (not presented). Therefore, no protein was present in the LPS fractions.

The cell-free extracts from the other three *A. butzleri* strains (Rigs 1714, Rigs 16799 and Rigs 15342) were also run on LPS gels. The results showed that Rigs 1714 and Rigs 16799 showed similar ladder-like profiles to *A. butzleri* NCTC 12481 (Figure 12 a & b, p.276). The LPS band intensity was greater for the samples harvested from the exponential phase and when the samples were heat-treated (55°C, 3min) prior to use. Nevertheless, for Rigs 15342 a different set of results was obtained (Figure 3.26). LPS banding profiles were only detected for the heat-treated samples and the bands had a greater intensity in the stationary phase sample. This suggests that Rigs 15342 may have a different growth pattern compared to the other three *A. butzleri* strains, which will have a direct affect on the type of LPS and proteins it produces and consequently, the level of cytotoxicity. This may explain the decreased toxicity observed for this particular strain compared with NCTC 12481 (Table 3.15).

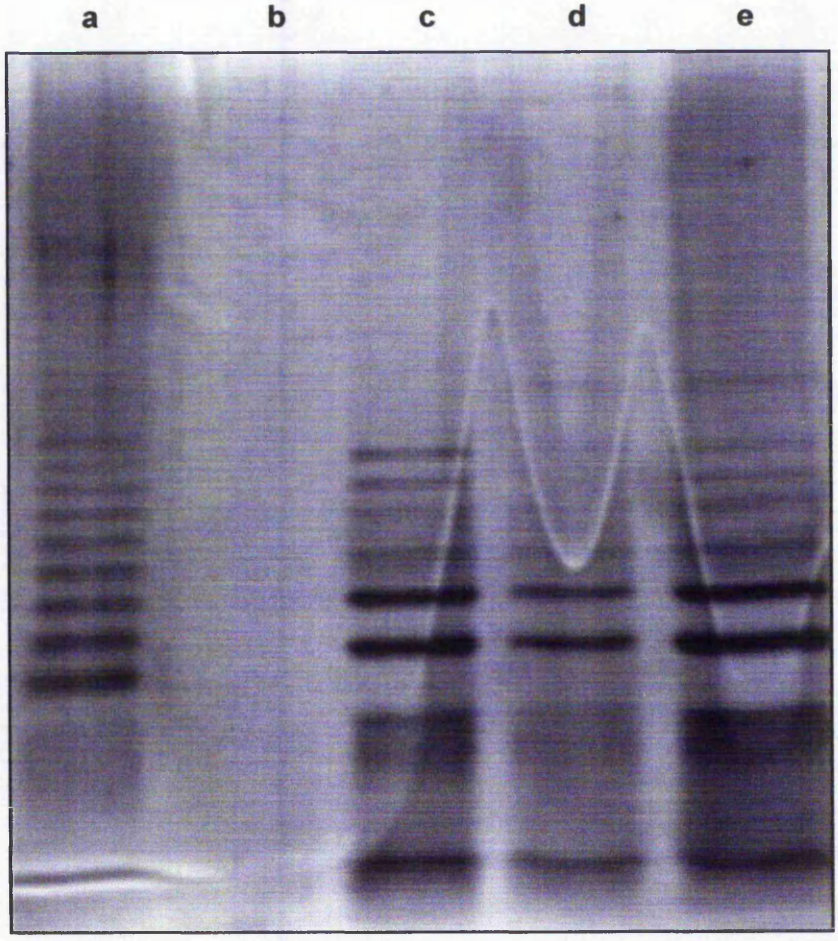


Figure 3.26: Detection of lipopolysaccharide fractions from *A. butzleri* Rigs 15342.

Arcobacter heat-treated and non heat-treated cell-free extracts prepared from exponentially growing (b, c) and stationary phase (d, e) cells prior to separation by polyacrylamide gel electrophoresis as described in Materials and Methods. Shown is a silver stained gel of lipopolysaccharide from (a) *Salmonella* Typhimurium (marker) (b) cell-free extract from non heat-treated exponentially growing cells, (c) cell-free extract from heat-treated exponentially growing cells, (d) cell-free extract from non heat-treated stationary phase cells and (e) cell-free extract from heat-treated stationary phase cells.

3.12.5. CYTOTOXICITY OF LPS EXTRACTED FROM *A. butzleri* NCTC 12481.

To provide additional evidence that the cytotoxic factor produced by *A. butzleri* NCTC 12481 was LPS, the LPS was extracted from exponential phase cells and untreated and heat-treated (55°C, 3 min) cell-free extract. The three mammalian cell lines (CHO, ECV and N2a) previously used were exposed to the extracted LPS.

Lipopolysaccharide extracted from whole *A. butzleri* NCTC 12481 cells (exponential phase) and heat-treated cell-free extracts were also found to be cytotoxic in the MTT assay (Figures 3.27 a & b). The N2a cells were the most sensitive cell line to the cytotoxin(s) produced by *A. butzleri* NCTC 12481. In addition, the LPS from whole cells had a greater cytotoxicity on the cell lines than the LPS extracted from the cell-free extracts. For example, LPS from whole cells caused a 66.63% decrease in N2a cell viability when a 10% concentration was applied, whereas a 35.33% kill was detected for the heat-treated cell-free extract sample. No cytotoxicity was detected for the untreated cell-free extract sample with any of the cell lines tested (Figure 13, p.277). No protein was detectable in the LPS preparations using SDS-PAGE and Coomassie blue staining procedure (not presented). Comparison of LPS profiles from whole cell and from untreated and heat-treated cell-free extracts showed ladder-like profiles, which had increased levels of low molecular weight LPS (see Figure 3.28). Furthermore, LPS extracted from whole cells showed the greatest intensity of bands suggesting that more LPS was obtained in these preparations. In contrast the untreated cell-free extract showed the lowest LPS band intensity. When compared to the MTT cytotoxicity assay (Table 3.15) the results showed that the whole-cell LPS sample produced the greatest levels of cytotoxicity.

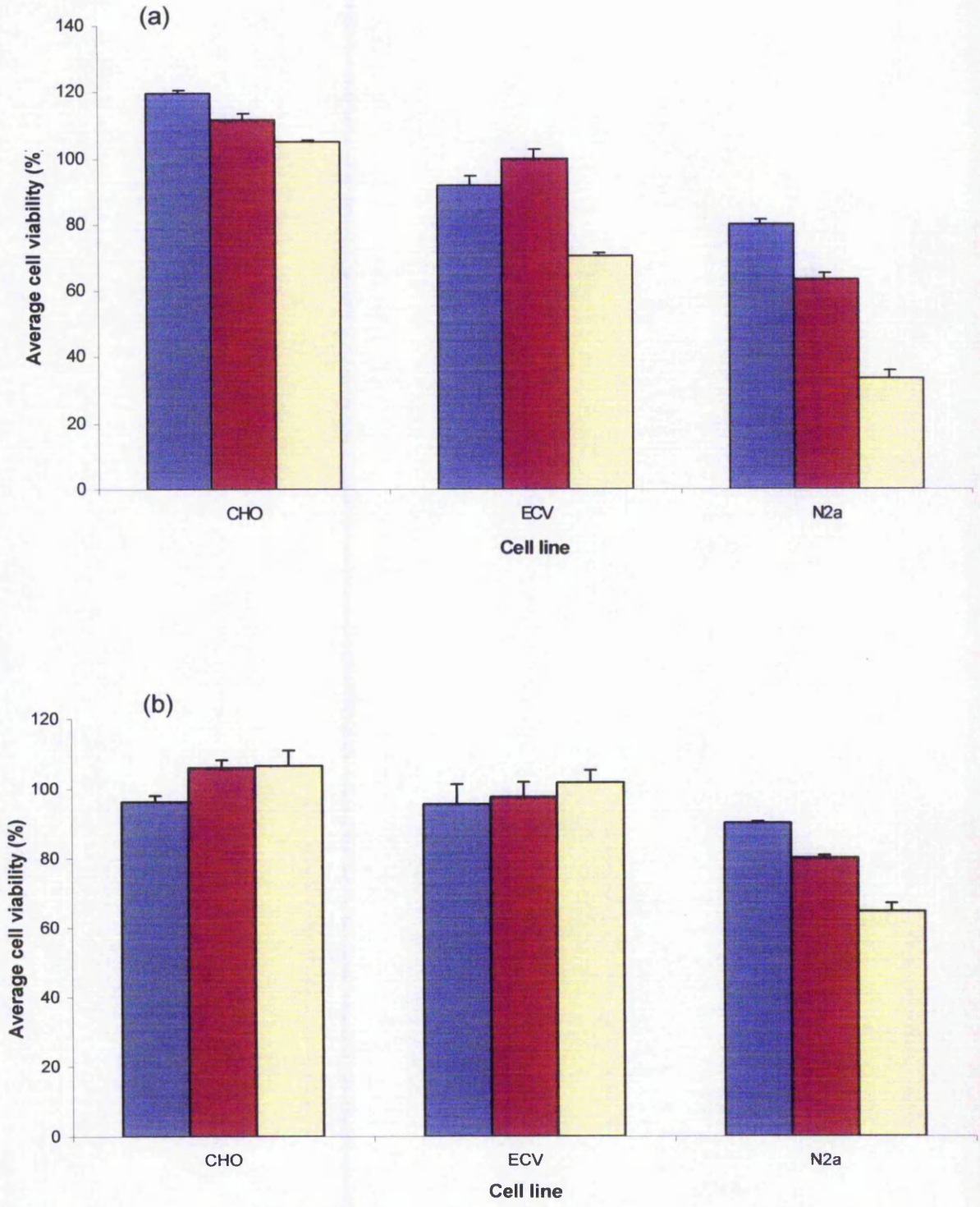


Figure 3.27: Cytotoxicity of LPS extracted from (a) whole cells and (b) heat-treated cell-free extracts of *A. butzleri* NCTC 12481 in the exponential phase. Concentration of LPS extract applied to mammalian cell lines: 2.5% (▩), 5%(■) and 10%(□). Error bars indicate standard error of mean where n = 9.

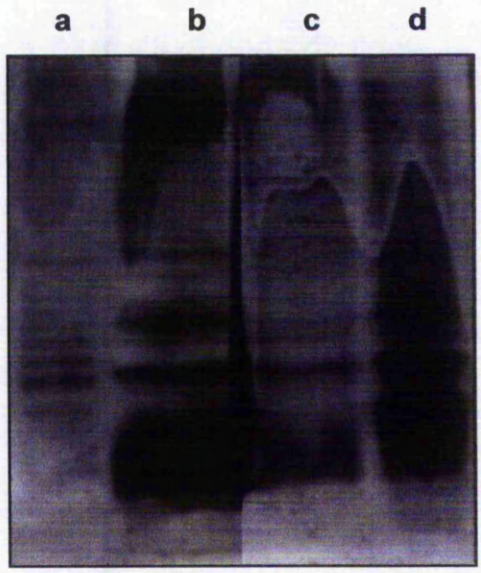


Figure 3.28: Detection of lipopolysaccharide extracted from exponential phase *A. butzleri* NCTC 12481 cells and heat-treated and non heat-treated cell-free extracts.

Shown is a silver stained gel of lipopolysaccharide extracted from (a) *Salmonella* Typhimurium (marker), (b) whole *A. butzleri* NCTC 12481 cells in the exponential phase of growth, (c) cell-free extract from non heat-treated exponential phase cells and (d) cell-free extract from heat-treated exponential phase cells. See Figure 2.2 (insert) for more details.

3.12.6. EFFECT OF CYTOTOXIN ON THE MORPHOLOGY OF CELL LINES.

Many toxins exert a direct effect on the morphology of cell lines. Therefore, it was important to study the affect the LPS toxin from *A. butzleri* NCTC 12481 had on the morphology of the three cell lines (CHO, ECV and N2a) previously used in cytotoxicity experiments. This was achieved by staining the mammalian cell lines with Coomassie blue stain after exposure to untreated and heat-treated cell-free extract for a 24 h and 48 h period. The cell-free extracts were applied at 2.5% and 10% concentrations to give sublethal and lethal levels of cytotoxicity. A MTT assay was also performed to monitor the level of cytotoxicity.

Table 3.16: Morphological changes induced by the presence of cell-free extract from heat-treated *A. butzleri* NCTC 12481 (exponential phase) after forty-eight hours.

Cell-free extract (%)	CHO morphologies			ECV morphologies		
	Round†	Flat	Elongated	Round	Flat	Process
2.5%	21.4 ± 1.1*	66.0 ± 1.1	12.6 ± 1.1	19.6 ± 1.5	46.6 ± 0.9	33.8 ± 0.7
2.5% Control	15.3 ± 1.1	69.4 ± 1.0	15.2 ± 0.2	17.7 ± 0.2	49.6 ± 1.6	32.7 ± 1.8
10%	15.1 ± 1.1	73.5 ± 2.2	11.6 ± 1.3*	15.2 ± 0.9	55.3 ± 2.3*	29.5 ± 1.4*
10% Control	12.1 ± 1.1	67.4 ± 2.1	20.7 ± 1.0	20.4 ± 0.5	44.5 ± 2.6	35.1 ± 2.2

† = For a more detailed description on cell line morphologies see Materials and Methods Section 2.18.7.

Numbers in parenthesis are standard errors of mean (n = 3).

Asterisk indicates a significant difference between the sample and the control (95% confidence level).

Cell-free extracts from heat-treated *A. butzleri* NCTC 12481 induced increased numbers of rounded (10% applied volume) and decreased numbers of elongated cells (10% applied volume) in the CHO cell line (Table 3.16 & Figure 3.29 a, b). However, in ECV cells there was an increase in the proportion of flat cells and a decrease in the number of elongated cells with processes (10% applied volume; Table 3.16 & Figure 3.29 c, d). In the neuronal cell line N2a there was a considerable (over 50%) decrease in the outgrowth of neurites (Figures 3.29 e, f & 3.30). These observations were also noticed for the cell-free extracts from non heat-treated cells, however the differences between the cell types were not so great. Furthermore, this experiment was repeated with cell-free extracts from stationary phase cells, but no significant morphological differences between the samples and the PBS controls were detected (Tables 6-12, pp.278-280).

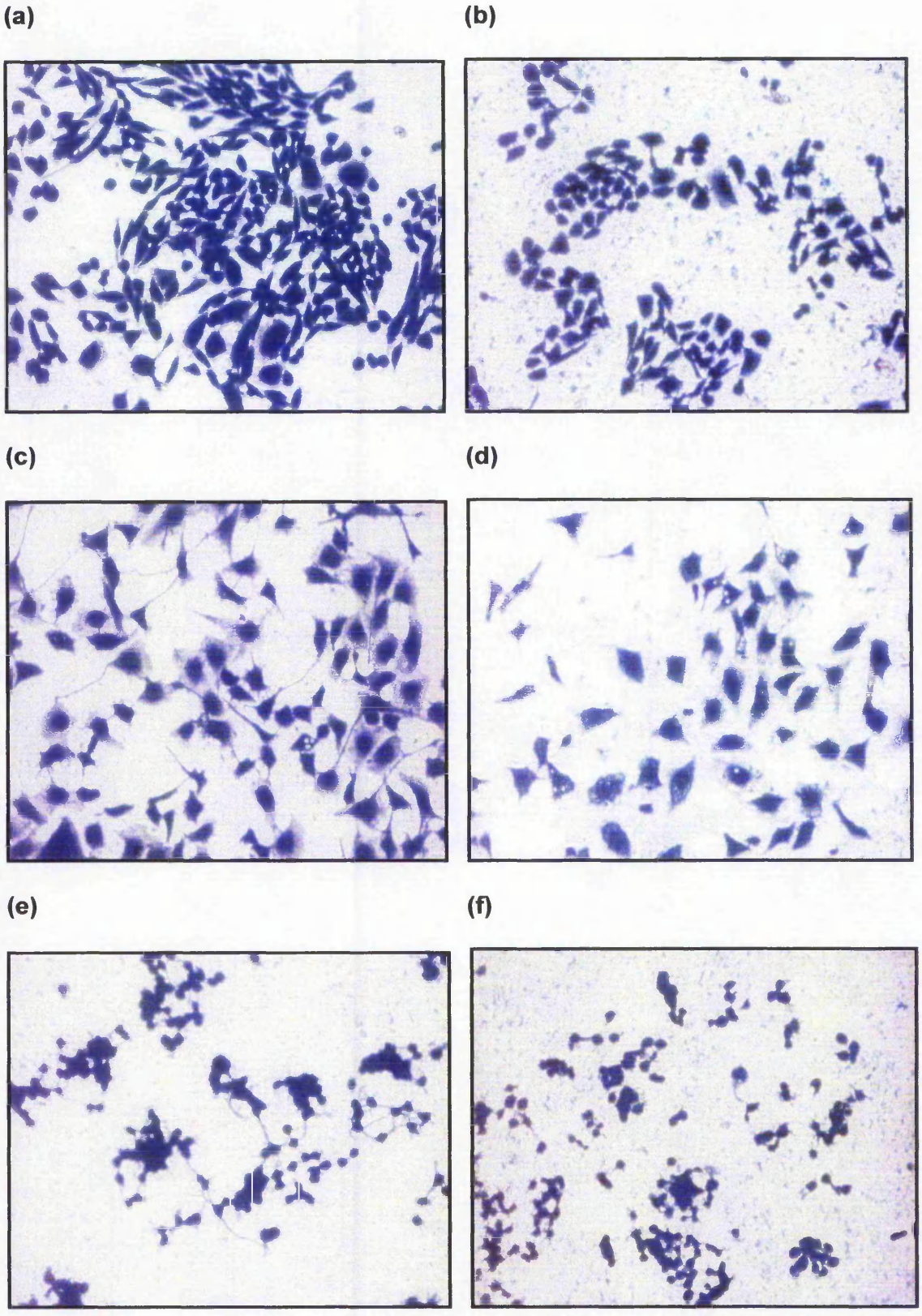


Figure 3.29: The effect of cell-free extract harvested from heat-treated exponential phase *A. butzleri* NCTC 12481 cells on the morphology of various mammalian cell lines.

Footnotes for Figure 3.29.

The pictures show Coomassie blue stained CHO (a, b), ECV (c, d) and N2a (e, f) cells. Filter sterilised 0.1 M potassium phosphate buffer was applied to the mammalian cell lines (a, c, e) at a 10% concentration. These act as controls, for comparative purposes. Heat-treated cell-free extracts from exponentially growing *A. butzleri* NCTC 12481, were applied at a 10% concentration to the mammalian cell lines (b, d, f). The cell lines were exposed for a 48 h period.

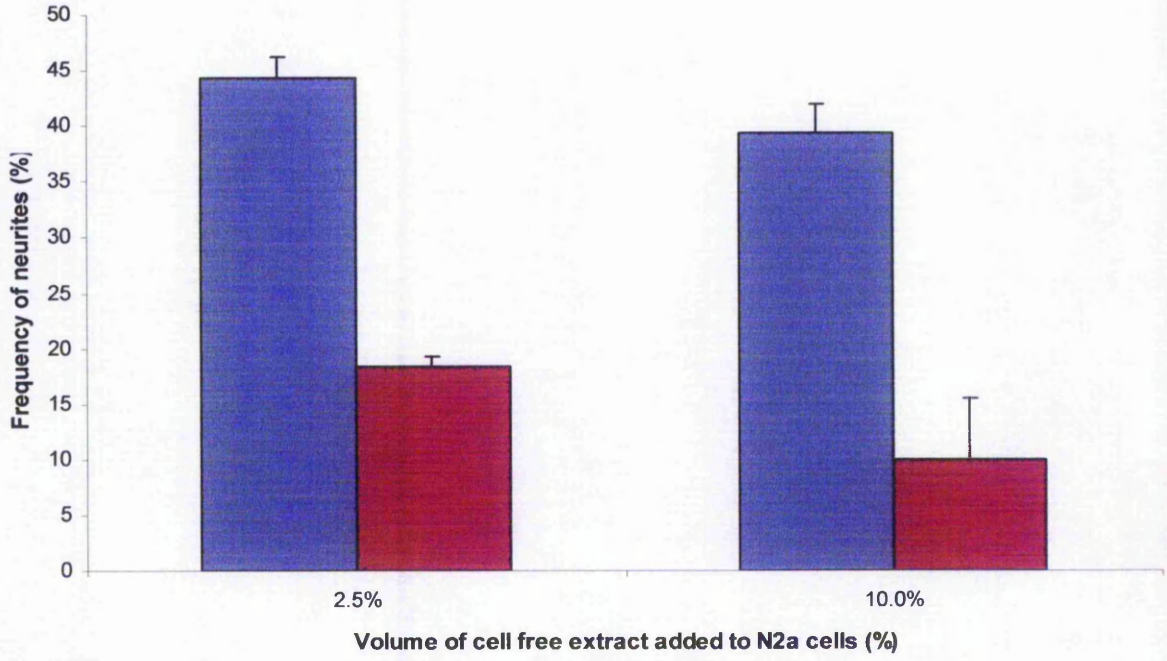


Figure 3.30: Inhibition of neurite outgrowth by cell-free extract of *A. butzleri* NCTC 12481 in exponential phase.

PBS treated control N2a cells ( ), Bacterial extract treated N2a cells ( ).

Error bars indicate standard error of mean (n = 3).

The cytotoxicity of the cell-free extracts, are shown in Table 3.17. No toxicity was detected on CHO or ECV cell lines for either the untreated or heat-treated cell-free extracts. This was an expected result for the CHO cell lines, but not for the ECV cell line as these samples normally caused some cell death (see Table 3.15). Therefore, this may have had an effect on the morphology study. Nevertheless, the N2a cell line was the most sensitive to the toxin present in the cell-free extract especially the heat-treated sample.

Table 3.17: MTT toxicity testing of *A. butzleri* NCTC 12481 cell-free extracts used in the morphology study (exponential phase, 48 h).

Cell line	Sample concentration (%)	CFE from untreated *A. butzleri* cells	CFE from heat-treated *A. butzleri* cells
CHO	2.5%*	109.1 ± 1.7§	119.1 ± 2.8
	10%	123.1 ± 3.8	103.1 ± 5.2
ECV	2.5%	102.4 ± 2.2	100.5 ± 4.8
	10%	102.6 ± 9.2	107.4 ± 4.9
N2a	2.5%	86.5 ± 5.7	44.5 ± 3.2
	10%	67.6 ± 7.5	30.1 ± 3.0

* = Volume of material added to mammalian cell line (%).

CFE = Cell-free extract, see Materials and Methods for detail.

§ = Standard error of mean where n = 3.

3.13. DETECTION OF CD14 ON MAMMALIAN CELL LINES (CHO, ECV and N2a).

The cytotoxicity and morphological results show that *A. butzleri* strains were able to exert a cytotoxic affect on mammalian cells by causing various morphological changes and cell death after exposure. These studies have also shown that the cytotoxin was likely to be lipopolysaccharide in the outer membrane. In order to identify potential receptors for the LPS toxin, Western blots of mammalian cell extracts were probed with antibodies against the known LPS receptor CD14.

The blot (Figure 3.31) shows that a polypeptide band(s) of similar molecular weight to CD14 (as indicated by the arrow) was detected in N2a and ECV and to a lesser extent in CHO cells. The control blood sample also showed an identical band. Nevertheless, to prove the presence of LPS receptor CD14 on the surface of the three mammalian cell lines an ELISA technique probing with antibodies to CD14 was used (Figures 3.32 & 3.33).

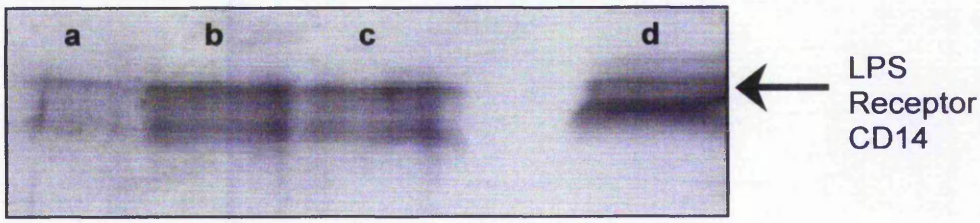


Figure 3.31: Probing Western blots of mammalian cell extracts with antibodies to CD14.

Mammalian cell extracts and human blood were separated by SDS-PAGE and transferred onto nitrocellulose as described in Materials and Methods (Section 2.21.1). Blots were then probed with polyclonal antibody to the recognised LPS receptor CD14 followed by secondary antibody conjugated to alkaline phosphatase. Shown are probed blots of equivalent protein amounts of (a) CHO, (b) ECV and (c) N2a cell extracts. Human blood (d) was used as a positive control for white cell-associated CD14.

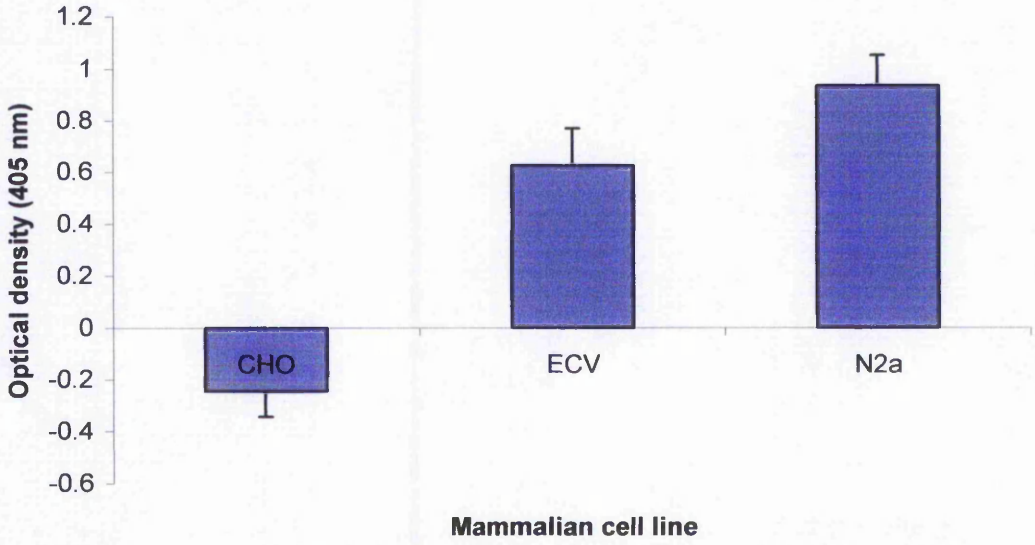


Figure 3.32: Mammalian cell lines probed with rabbit polyclonal IgG primary antibody (1:500 dilution) against CD14 and conjugated with alkaline phosphatase. Error bar indicate standard error of mean where n = 3.

Figure 3.32 supports the Western blot (Figure 3.31) results whereby CD14 was detected in high levels for N2a and ECV cell lines (0.93 and 0.62 arbitrary units respectively). However, a negative result was observed for the CHO cell line as the background colouration in the control samples (used to measure non-specific binding of the antibodies) was greater than the CHO samples. This suggests that no CD14 receptors were detected on the surface of CHO cell lines. To clarify the results they were repeated except a horseradish peroxidase conjugate was used in place of the alkaline phosphatase conjugate. It is believed that this conjugate is more sensitive to the rabbit polyclonal IgG primary antibody used therefore more accurate results would be obtained.

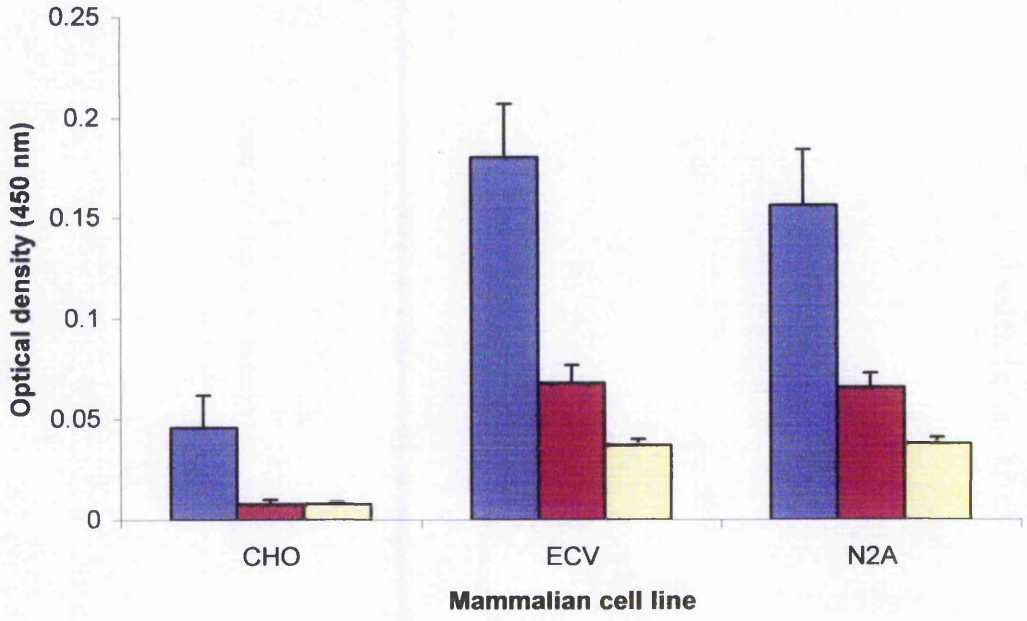


Figure 3.33: Mammalian cell lines probed by rabbit polyclonal IgG primary antibody against CD14 and conjugated with horseradish peroxidase.
Primary antibody dilutions: 1:200(■), 1:500 (■), 1:1000(□)
Error bars indicate standard error of mean where n = 3.

CD14 receptors were detected on the surface of three different types of mammalian cell lines, supporting the previous results (Figure 3.33). However, the level of receptor present varied depending on the cell line type. High levels were

detected for N2a and ECV lines (0.156 and 0.18 arbitrary units respectively, 1:200 dilution), whereas low levels of the receptor were detected for CHO cells (0.046, 1:200 dilution). This pattern was also detected with all three dilutions (1:200, 1:500 and 1:1000) used in this experiment.

From these experiments it can be stated that LPS receptor CD14 was detected in all three cell line types, but the levels at which they were detected varied. Subsequently, flow cytometry was used in an attempt to determine the levels of CD14 receptors present on the surface of N2a, ECV and CHO cell lines (Table 3.18).

Table 3.18: Total fluorescence values detected from the presence of CD14 receptors on different mammalian cell lines.

Cell line	Fluorescence (%)†	
	1:50 Dilution§	1:100 Dilution§
CHO	4.2	0.14
ECV	13.6	3.4
N2a	22.9	11
Blood monocytes*	63	57.9

*= Blood monocytes were used as a CD14 positive control.

† = Percentage of fluorescence detected from the total population of cells. The values have been corrected by subtracting the isotype control value, which shows fluorescent background caused by non-specific binding.

§ = Dilution of primary antibody (rabbit polyclonal IgG primary antibody).

CD14 receptors were detected on the surface of the three mammalian cell lines. Again the level of receptor present varied in accordance with cell type. The highest level of fluorescence detected for the mammalian cells lines was once more for the N2a cells (22.9% and 11% for 1:50 and 1:100 dilutions respectively), which did provide further support to the previous experiments (Table 3.18). Even though this data was consistent with the Western blot and ELISA technique results, only low levels of fluorescence were detected. For example 63% (1:50 dilution) and 57.9% (1:100 dilution) fluorescence values were detected for the

blood monocyte control. This was an ideal positive control as monocytes from fresh blood should bind to the antibodies and produce a high level of fluorescence. Various antibodies, isotypes and controls were used, but these factors did not make any difference as no significant levels of fluorescence were detected for any of the samples, even the positive blood monocyte control. After various discussions with companies that supply antibodies, the antibodies, which had already been tried were the only ones suitable for this type of experiment. Consequently, it was decided that this line of investigation was the best achievable.

CHAPTER FOUR

DISCUSSION

4.0: DISCUSSION.

4.1. DETERMINATION OF THE OPTIMAL GROWTH CONDITIONS.

Before any experimental work could be carried out on *A. butzleri* NCTC 12481 it was important to become familiar with this organism. Preliminary experiments were conducted to determine the optimum growth conditions to produce broth and plate cultures, which are suitable for investigative experimental work. Firstly, three types of broth (TSB, BHI and Brucella plus FBP supplement), which are commonly used for culturing *Campylobacter* spp. have been and quoted in the literature on *Arcobacter* spp. (Fernandez *et al.*, 1995; Dickson *et al.*, 1996) were tested for their suitability in growing *A. butzleri* NCTC 12481. Growth in the presence of atmospheric oxygen (20%) or a reduced oxygen concentration (5%) and at different temperatures (30°C and 37°C) was investigated. The results (Section 3.1) show that *A. butzleri* NCTC 12481 grew equally well when oxygen was present in high or a reduced concentration. This is due to *Arcobacter* spp. being a microaerophile. However, unlike *Campylobacter* spp. which are strict microaerophiles, *Arcobacter* spp. are aerotolerant to atmospheric oxygen after primary isolation in microaerophilic conditions (Neill *et al.*, 1978). Due to these results aerobic conditions were used as standard growth conditions as it was easier to produce the large volumes of culture required for experimentation. Furthermore, similar optical density values were detected for both 30°C and 37°C growth temperatures in all three growth media. Due to these similarities 37°C was chosen as the optimum growth temperature as it reflects human body temperature and therefore is more significant to this study in relating *Arcobacter* spp. to food poisoning in humans.

The results in Table 3.1 show that a difference in growth yield was detected for the different growth media. This method, however, did not consider growth rates of the organism. *A. butzleri* NCTC 12481 was able to grow in all three media. However, slightly higher optical density values were detected for BHI broth, which is rich in peptones that are necessary for growth of *Arcobacter* species. This

suggested that BHI broth provided *A. butzleri* NCTC 12481, which is a particularly fastidious species, with all the relevant nutrients to support growth. Furthermore, plate counts on blood agar supported the use of this medium as it produced cultures with a colony count between 10^8-10^9 cfu ml^{-1} which is a satisfactory cell mass for experiments. For these reasons BHI broth was chosen for further experiments.

4.2. DETERMINATION OF *A. butzleri* NCTC 12481 TEMPERATURE GROWTH RANGE.

As well as looking at the optimum growth conditions for *A. butzleri* NCTC 12481, it was important to determine the temperature range at which this particular organism can grow. This was achieved using the indirect impedance technique and the results are shown in Figure 3.5. The results from this study show that *A. butzleri* NCTC 12481 was a mesophile as it grows well between 25°C and 35°C, with a specific growth rate of 0.57 h^{-1} at 30°C in BHI broth. Above and below this range growth could still occur, but at a slower rate. For example a very sharp decrease in growth rate occurred above 37°C, with no detectable growth at 40°C. This result is supported by a previous study by Vandamme *et al.* (1991), whereby *Arcobacter* spp. (n = 9) were unable to grow at 42°C. Furthermore, *A. butzleri* NCTC 12481 was unable to grow at the upper temperature limits of *Campylobacter* spp. which has a maximum detectable growth temperature of 47°C (Doyle & Roman, 1981). Thus suggesting that this species is more sensitive to high temperatures and does not possess any thermotolerant properties. Therefore, *Arcobacter* should not be a particular problem in the food industry as cooking temperatures are usually higher than 60°C. Nevertheless, this does suggest that food maintained at room temperature (25°C) and below could allow *A. butzleri* NCTC 12481 to grow.

Arcobacter spp. are able to survive and grow at lower temperatures. Experimental data has shown that *A. butzleri* NCTC 12481 had a minimum detectable growth temperature of 15°C. This result is supported by many groups such as

Vandamme *et al.* (1991), Burnens *et al.* (1992) and On and Holmes (1995). However, Neill *et al.* (1985) observed that *A. cryaerophilus* was able to grow at 5°C, but this ability was not detected for *A. butzleri* NCTC 12481 in this particular study. Growth at low temperatures was an expected result as Dhamabutra *et al.* (1992) stated that the major reservoirs of *Arcobacter* spp. were water sources. Their ability to survive at low temperatures would be important in the food industry as +4°C (fridge) and -20°C (freezer) temperatures are commonly used as food storage temperatures, so this is an important physical aspect of this organism and will be discussed in more detail in Section 4.8.

Following the termination of the impedance experiment the pH of the BHI growth media from a triplicate set of tubes was taken. Consequently, *A. butzleri* NCTC 12481 ability to ferment its growth media was investigated. The pH of the growth medium (Table 3.3) altered only slightly compared to the pH of the uninoculated control, so this particular strain does not have the ability to ferment carbohydrates present in the BHI broth. This finding is consistent with those of Vandamme *et al.* (1992a) who observed that *Arcobacter* species are chemoorganotrophs, which are unable to ferment or oxidise carbohydrates. They also observed that *Arcobacter* species obtain their energy from amino acids and intermediates of the tricarboxylic acid cycle.

4.3. EFFECT OF pH ON THE GROWTH OF *A. butzleri* NCTC 12481.

The impedance technique was used to study the effect pH has on the growth of *A. butzleri* NCTC 12481 at two growth temperatures (30°C and 37°C). Table 3.4 shows that after incubation the pH of the BHI broths increased, except the uninoculated control. This effect was generally greater when the culture was incubated at 30°C compared to 37°C, suggesting that *A. butzleri* NCTC 12481 produced alkaline waste products whilst growing. Vandamme *et al.* (1992a) supported this result by showing that *Arcobacter* spp. are methyl red and Voges Proskauer negative, which means that neither acids or acetoin, which are common bi-products of fermentation reactions, are produced by this genus. Furthermore,

before amino acids can be used as a source of carbon for the synthesis of cell constituents, the amino group must be removed by the process of deamination. This results in excess nitrogen in the form of ammonium ions being excreted into the medium, thus producing a more alkaline environment.

The results (Figure 3.6) show that 30°C was the optimum growth temperature for *A. butzleri* grown in pH-altered BHI broth, as pH had a greater effect on the cells, resulting in slower growth when incubated at 37°C. This is due to this temperature causing some stress to the cells, probably at the cell membrane. If the membranes were damaged they would no longer serve as a protective barrier against the penetration of detergents and other substances e.g. salt and antibiotics. Also, the pH-altered broth may damage certain cellular components, especially enzymes, thus affecting the viability of the cells. This effect was observed especially under acidic conditions such as pH 4.5 and 5.0 where no growth occurred when incubated at 37°C and was supported by the optical density values (Table 3.4), which were similar to the uninoculated control. In addition, growth was detected at pH 5.5, but the detection time (39.25 h) was extremely high as the cells were not under ideal growth conditions, so it took longer for the cell density to increase and produce sufficient carbon dioxide to cause a detectable change in conductance. Therefore, *A. butzleri* NCTC 12481 were growing under these conditions, but at a very slow rate (t_d = 6.25 h). No growth was detected at pH 4.5 when incubated at 30°C, but growth was detected at both pH 5.0 and 5.5, so acidic pH had a lesser affect on the viability at 30°C compared to 37°C. This effect has also been described for *C. jejuni* by Doyle & Roman (1981), who stated that death rate increases as pH decreases and temperature increases. This suggests that a combination of elevated temperatures and an acidic environment would effectively kill *A. butzleri* NCTC 12481. This is useful information for the food industry, which use organic acid sprays to decontaminate meat carcasses, especially pork products, which are heavily contaminated by *Arcobacter* species.

It was also observed that *A. butzleri* NCTC 12481 grows slower in alkaline conditions such as pH 8.5, but the effect was not as great as in acidic conditions,

suggesting that *Arcobacter* spp. favour more neutral to alkaline conditions for growth.

Between the pH range of 6.0 and 8.0 the difference between detection times at 30°C and 37°C was minimal (2-3 h), though growth was slower at 37°C (Table 3.5). Above and below these values the difference was far greater (20-30 h) with the doubling times increasing to 2.31 h and 6.25 h respectively at 30°C and 37°C (pH 5.5). These results are further supported by the optical density values which peak over this pH range, suggesting that pH 6.0 to 8.0 is the optimum pH range for *A. butzleri* NCTC 12481. Neill *et al.* (1985) also observed that *Arcobacter* strains could grow between pH 5.5-9.5, with pH 6.8-8.0 being the preferred pH range for growth. Furthermore, the pH dependence of *A. butzleri* NCTC 12481 observed in this work is comparable with the reported growth range (pH 4.9-9.0) for *C. jejuni* grown at 30°C (Anonymous, 1996).

4.4. GROWTH CURVE OF *A. butzleri* NCTC 12481.

The growth curve of *Arcobacter* spp. was determined to establish how this organism grows in a liquid media and at what time periods the different phases of growth occurred. The growth curves were achieved by measuring the optical density of the culture at 37°C and comparing them to appropriate viable counts. Figure 3.7 shows that after 5 h a rapid increase in turbidity occurred. After 18 h the growth rate decreased as the media has depleted nutrients, but increased waste products which have a toxic effect in high concentrations. This causes the bacteria to stop multiplying as the synthesis of cellular components slows down. Therefore, the number of cells remains constant as the number that are dividing equals the cells that are dying. This pattern was mirrored by the viable counts, but occurred at a faster rate. The difference occurs because the viable counts are more sensitive to changes in growth, while a significant amount of growth must occur to change the turbidity of the culture for it to be detected by the spectrophotometer. The results also show that after 9 h the cultures were in mid-exponential phase, whereas a 24 h period produces a stationary phase culture.

These results are comparable to the data obtained for *C. jejuni* NCTC 11351 whereby the cultures were grown in brucella broth with FBP supplement for 6 h and 24 h to produce exponential and stationary phase cultures respectively (Kelly *et al.*, 2001). Therefore, the growth curve data provided valuable information on the behaviour of *A. butzleri* NCTC 12481 *in vitro* and determined growth rates which were then used for further experiments enabling the different growth phases of *A. butzleri* NCTC 12481 to be studied.

Using the above growth curve method, the doubling time of *A. butzleri* NCTC 12481 at 37°C was determined and compared with the values obtained from the impedance experiments (see Section 4.2). The growth curve method showed that *A. butzleri* had a doubling time of 1.7 h, which is very similar to the value determined by the impedance method (1.5 h). These results were also comparable to the doubling times observed by Dickson *et al.* (1996), with *A. butzleri* in BHI broth having a value of 1.5 h, although a faster rate (0.869 h) was detected in a biphase culture. Furthermore, these values were considerably faster than *C. jejuni* NCTC 11168 which has a similar growth pattern to *A. butzleri* NCTC 12481, but at a slower rate with a doubling time of 4 h. This data was provided by Dr K. Holmes (Ph.D. Thesis, 2001).

4.5. PRELIMINARY HEAT TREATMENT EXPERIMENTS ON *A. butzleri* NCTC 12481.

Blood agar and BGBA are recommended as non-selective and selective media respectively for *Campylobacter* spp. (Palumbo, 1984; Ridsdale *et al.*, 1998; Wesley & Baetz, 1999). For this reason, these media were used to study the effect of heat on *A. butzleri* NCTC 12481. Firstly, it was important to determine the temperature that produced the highest percentage of survivors that were sublethally injured, but the least amount of death. These experiments showed that a difference of approximately 1 log (cfu ml^{-1}) was detected for the untreated control on blood agar and BGBA, thus demonstrating the existence of sublethally injured cells in the initial inoculum (Table 3.6, Inoculum size [a] & [b]). This effect was also

observed for *C. jejuni* cells in a heat and aeration survival study (Kelly *et al.*, 2001).

The results also show that a temperature of 45°C produced the best results (Table 3.6) with over a third of the cells remaining viable, of which 87.1% were sublethally injured. This was shown by the low plate counts on BGBA as sublethally injured organisms no longer form colonies on this selective media, which contains bile, a selective inhibitory agent that has no effect on uninjured *A. butzleri*, but causes secondary injury to already damaged cells which may even result in cell death. The lowest temperature (40°C) did not produce a significant level of sublethally injured cells, shown by the high plate counts on both selective and non-selective plates (Blood agar = $7.5x10^8$, BGBA = $6.0x10^8$), whereas above 50°C a large percentage of the population were lethally injured. *Arcobacter* strains only grow up to 39°C, above this temperature they may survive for a short period of time, which may be lengthened if they possess protection mechanisms such as heat shock proteins. Gill and Harris (1982) also observed that *C. jejuni* was rapidly inactivated when heated to 50°C or above. Hence the reason why these temperatures are used to wash chicken carcasses in poultry plants.

This initial experiment determined a temperature range (45-55°C) which produced sublethally injured *A. butzleri* cells. Nevertheless, further experiments were required to determine the optimum exposure time. From these experiments it was shown that increasing temperature and exposure time reduced the number of survivors, but increased the percentage of sublethally injured cells. The results show that a period of 3 minutes at 45°C produced a high percentage of sublethally injured cells harvested from both stationary and exponential phases of growth (Table 3.7 and 3.8). Greater exposure times caused the number of survivors to decrease to about 2%, which would be too low to study the effect of heat injury. This was expected because a longer exposure time will produce a greater amount of kill. These results also support the further use of 45°C as the optimal temperature as 50°C and 55°C caused too much damage to the cells, which was irreversible, resulting in a high percentage of cell death. This was most evident for exposure times greater than 2 minutes.

In this study cells harvested from the exponential phase seemed to be more resistant to temperature than those from the stationary phase, as higher percentages of survivors were detected for exponential phase cells at both 45°C and 50°C. Exponential phase cells could also survive for longer exposure times than stationary phase cells. This was an unexpected result, with *A. butzleri* not demonstrating the normal phenotypic stationary phase response which results in enhanced stress resistance. Exponential phase cells are actively growing and multiplying, which requires a constant replication of all cellular material, such as proteins which are greatly affected by temperature. However, because exponential phase cells are still growing they may be able to replace denatured proteins, whereas stationary phase cells are in an environment with depleted nutrients and increased waste products causing the synthesis of cellular components to slow down and stop. Consequently, fewer active systems are present to be affected by temperature or other environmental factors. Therefore, entry into the stationary phase is accompanied by profound structural and physiological changes that often result in increased resistance to heat shock, oxidative, osmotic and acid stresses (Kolter *et al.*, 1993; Jorgensen *et al.*, 1999; Trainor *et al.*, 1999). For example, under starved conditions or upon entry into the stationary phase, *E. coli* induce the expression of 30 or more genes which are regulated by the stationary phase sigma factor RpoS and are essential for survival under unfavourable conditions (Kelly *et al.*, 2001).

This phenomenon of exponential phase cells being more resistant to mild heat stress (50°C) than stationary phase cells has also been observed in *C. jejuni* NCTC 11351 (Kelly *et al.*, 2001). The authors believed that *Campylobacter* spp. do not have regulated resistance mechanisms in the stationary phase like other Gram-negative bacteria. Analysis of the *C. jejuni* 11168 genome sequence indicated the absence of the *rpoS* genes (Parkhill *et al.*, 2000). These findings are supported by Kelly *et al.* (2001) who were also unable to isolate the *rpoS* genes in the genome of *C. jejuni* NCTC 11351 using hybridization based assays and a plasmid library. Therefore, the absence of an RpoS homologue is entirely consistent with the failure of *C. jejuni* NCTC 11351 to induce stress resistance in the stationary phase. Due to these findings it is possible that *A. butzleri* NCTC 12481 also lacks the *rpoS* genes. In addition, Kelly *et al.* (2001) suggested that

upon entry into the stationary phase both the inner and outer membrane structures or functions are disrupted. Therefore, these cells are already sublethally injured prior to heat treatment.

These results are also supported by the *D* values for *A. butzleri* NCTC 12481 (Tables 3.9 and 3.10) obtained for both exponential and stationary phase cells. As cells harvested from the exponential phase had larger *D* values on both selective (BGBA) and non-selective (blood agar) media compared to stationary phase cells. This observation was more significant at 45-47°C (D_{47} on blood agar, exponential phase = 16.0 min, stationary phase = 8.0 min) than at the higher temperatures, thus proving that stationary phase cells are more sensitive to heat as it required less heat exposure time than exponential phase cells to reduce the number of survivors by a factor of 10. However, for both growth phases, a larger *D* value was detected for growth on blood agar (D_{50} = 2.1 min) compared to BGBA (D_{50} = 0.8 min). This is due to only uninjured cells being able to form colonies on BGBA, whereas both uninjured and sublethally injured cells can grow on blood agar. Consequently, less damage has to occur to the cells to prevent them from growing on BGBA, hence less time was required to reduce the number of bacteria.

During the course of these experiments commercially available isolation media, CAT and CCDA, which are specific for *Arcobacter* spp. became available. Therefore, these media were tested as selective media in comparison with BGBA, which is a *Campylobacter* spp. selective media. The results (Figure 3.8) show that as the temperature increased in 5°C increments (45-55°C) the colony counts on the different media decreased, but under these conditions colony counts on the different media were very similar for each temperature. However, CAT and CCDA had slightly lower counts compared to the non-selective blood agar (Figure 3.8). Poor growth was detected on BGBA plates even on the untreated control plates. From these results and previous ones it was decided that BGBA was inappropriate as a selective isolation medium for *Arcobacter* species. It was decided that in previous experiments a greater percentage of sublethal injury and cell death might have been obtained for BGBA compared to CAT/CCDA. This is probably due to *A. butzleri* NCTC 12481 being unable to tolerate the growing environment provided by BGBA, which was usually used for *Campylobacter* spp. Dickson *et al.*

(1996) supported this theory by reporting that *Arcobacter* spp. grows poorly on *Campylobacter* isolation media. The difference in inhibition of sublethally injured cells by BGBA and CAT/CCDA is due to the severity of the injury and the concentrations of the selective agent (Mackey, 2000). Kelly *et al.* (2001) observed that 1% NaCl was more inhibitory to injured *C. jejuni* cells than 0.1% sodium deoxycholate (DOC), because loss of resistance to NaCl preceded loss of resistance to DOC during stationary phase and because injured cells in the inoculum were sensitive to NaCl, but not DOC. This inhibitory effect was also observed for *A. butzleri* with injured cells showing a greater resistance to 0.1% deoxycholate in CCDA media compared to 0.5% NaCl in the BGBA media. However, the large inhibitory effect of BGBA on *A. butzleri* NCTC 12481 may not entirely be due to the NaCl concentration, but a combined action of NaCl and 2% bile, causing lethal injury to already delicate cells. Due to these facts CAT and CCDA were used in further experiments, as they were the most appropriate selective plating media to detect the number of sublethally injured cells.

The results in Section 3.5 showed that a temperature of 45°C to 50°C for 3 minutes would be suitable heat treatment conditions to study sublethal injury in both exponential or stationary phase cultures. As these conditions would produce a relatively high percentage of survivors, which would be sublethally injured. However, to standardise the heat treatment for future experiments with CAT and CCDA, a series of time exposure experiments at different temperatures was done and subsequently *D* and *z* values were determined.

4.6. TIME EXPOSURE EXPERIMENTS TO DETERMINE *D* AND *z* VALUES.

D and *z* values of *A. butzleri* NCTC 12481 were determined by using timed exposure experiments at different temperatures (45, 47, 50, 52 and 55°C). It was observed (Table 3.9 and 3.10) that the viable count decreased rapidly with raised temperatures. At 47°C the *D* value for cells harvested from the exponential and stationary phase was 16 min and 8 min respectively. However at 50°C and above

the *D* value dropped dramatically to 2 minutes or less. A similar pattern was detected for cells harvested from both the exponential and stationary phase of growth. The *D* values of cells harvested from the stationary phase over the range of temperatures tested were lower than corresponding values for exponential phase cells, indicating that the cells from the stationary phase were more heat-sensitive than those from the exponential phase. This is believed to be due to exponential phase cells being in an actively growing state, so they may be able to produce a heat shock response to protect their proteins thus reducing viability loss.

As no literature was available on *D* values for *Arcobacter* spp. these values were compared to those of *C. jejuni*, which is the closest related organism to this genus. At 51°C in 1% peptone water *C. jejuni* had a *D* value between 4.9-7.02 min (Blackenship & Craven, 1982), whereas *A. butzleri* NCTC 12481 had a *D* value of 1.7 min for stationary phase cells and 2.1 min for exponential phase cells. Both of which are considerably lower than *C. jejuni* values. This is probably due to *C. jejuni* having a maximum growth temperature of 45-47°C, compared to the maximum detectable growth temperature of 39°C for *A. butzleri* NCTC 12481. Therefore, it would take less heat exposure time to reduce the number of *A. butzleri* NCTC 12481 by a factor of 10.

The *D* values for *A. butzleri* NCTC 12481 at 55°C were 0.4 min for cells harvested from the stationary phase and 1.1 min for exponential phase cells. These values are comparable with those published for *C. jejuni* (D_{55} 0.6-2.1 min; Anonymous, 1996). The values were quite low, however this was expected as both species are mesophilic organisms which means that they are unable to survive at high temperatures such as 55°C and above for long periods of times.

The *z* values for exponential and stationary phase cells were 8.1°C and 7.4°C, respectively. Very little difference was detected between the two values, however this was expected as unlike the *D* values the *z* value remains fairly constant for a given organism over a range of test conditions. It is plausible that sublethally injured cells were generated during heat treatment, which were only recoverable

on the non-selective blood agar. A comparable *z* value for *C. jejuni* of 5°C was determined by Park *et al.* (1991).

4.7. FLUORESCENT STAINING TO DETERMINE MEMBRANE INTEGRITY OF *A. butzleri* NCTC 12481.

As well as studying the effect of heat on cell viability, it was important to determine the sites of heat treatment damage, such as cellular membranes. This meant that the integrity of the cell membranes of *A. butzleri* NCTC 12481 in different growth phases needed to be studied. Assessment of dye permeation into cells was a convenient approach for measuring the impairment of the cell envelope. Puchkov and Melkozernov (1995, as quoted by Robinson *et al.*, 1997) described a technique using ethidium bromide to indicate the disruption of the bacterial permeability barrier after a freeze-thaw treatment. The authors observed a good correlation between viability and ethidium bromide uptake since this dye can only fluoresce when it enters into the cell and intercalates with nucleic material such as DNA. Propidium iodide is another fluorescent stain for nucleic acid that has been widely used in microscopy and flow cytometry to quantify the percentage of injured cells in microbiological populations (Robinson *et al.*, 1997). Therefore, both ethidium bromide and propidium iodide were used to assess the membrane integrity of *A. butzleri* NCTC 12481.

Higher fluorescent values for both DNA-binding dyes were detected for cells harvested from the exponential phase compared to stationary phase cells (Figure 3.10). These results suggest that stationary phase cells were more heat resistant compared to exponential phase, which is contradictory to the heat treatment (Section 4.5) and time exposure (Section 4.6) experiments. However, it should be noted that the cell membrane integrity methods did not assess any subsequent repair of cell membranes or resuscitation of damaged cells, as would occur during incubation on blood agar plates used in the determination of *D* values. Heat treatment increased the fluorescence values for both growth phases. This was expected, as dyes such as ethidium bromide are generally impermeable to intact

cell membranes. Therefore, as the temperature increased so did the permeabilization of the cell envelope, which allowed the dyes to intercalate with nucleic material which in turn emits fluorescence. Some fluorescence was detected for the untreated samples. Then again, it is possible that some background fluorescence may have occurred if all the excess stain had not been washed away, or that sublethal damage occurred when the cells were harvested. This cellular permeabilization would also explain the decreased viability on the selective agar (BGBA, CCDA & CAT) in the heat treatment experiments (Sections 4.5 & 4.6). Since increased sensitivity to bile salts, sodium chloride or antibiotics in Gram-negative organisms is often taken to indicate loss of integrity or impairment of homeostatic functions associated with the outer and cytoplasmic membranes respectively (Mackey, 2000).

These results are supported by the 260nm/280nm leakage experiment, whereby more UV-absorbing material was released when the *A. butzleri* NCTC 12481 cells were heat-treated. This indicates that heat treatment caused damage to the cell membranes specifically the cytoplasmic membrane of *Arcobacter* spp., as the leakage of 260 nm absorbing material, amino acids and inorganic acids is indicative of thermal injury to the cytoplasmic membrane (Hitchener & Egan, 1977). Furthermore, the peptidoglygan layer appears to be comparatively heat stable and there are few reports of direct damage to it as a result of sublethal heating. This leakage phenomenon has also been reported for *C. jejuni* and other microorganisms (Palumbo, 1984).

4.8. EXPERIMENTS ON COLD STORAGE OF *A. butzleri* NCTC 12481.

As well as studying the effect of heat it was important to look at the effect cold has on *Arcobacter* spp, which have an increased tolerance to low temperatures. Both fridge (+4°C) and freezer (-20°C) temperatures were used to study the viability of *A. butzleri* NCTC 12481, under nutrient rich and poor conditions, Thus simulating

Arcobacter's survival abilities under cold and starved conditions, which are commonly experienced in the natural environment and processing plants.

The data from the cold storage experiments indicate a gradual decrease in viability following storage at +4°C as exposure time increased over a 21 day period for both growth phases. However, chilling conditions had a greater affect on exponential phase cells with colonies only being detected on the non-selective blood agar after storage for 7 days compared to >21 days for stationary phase cells (Figures 3.11a & 3.12a). Freezing on the other hand caused a 2-log decrease in viability for stationary phase cells after only 24 h storage, after which time the viability remained constant. This large decrease in viability is probably due to the production of ice crystals which damage the internal components and membranes of the cells. Also, the production of ice in the suspending medium resulted in dissolved solutes becoming more concentrated, and exert more of an osmotic stress on the cells. The high concentrations of salt/solute are toxic and will affect the viability of the cells. The viability will also be affected by the thawing process, which causes additional damage to already weak cells. This pattern was observed by Christopher *et al.* (1982) for *C. jejuni* in frozen skim milk during frozen storage at -15°C to -70°C. Initially a relatively fast death rate was followed by a much slower rate. For *C. jejuni* it was observed that the initial decrease in the viable count was often more than 10-fold, but in some cases as much as 10^3 to 10^5. This initial decline usually occurred within one day of freezing (Barrell, 1984). More recently Fearnley and co-workers (1998) supported this finding, reporting that freeze-thaw injured *C. jejuni* cells were still able to colonize chickens.

A similar pattern was detected for cells harvested from the exponential phase. However, freezing had a greater effect on these cells with a 4-log kill occurring after a 24 h period and no growth being detected on any media type after a 7 day storage. These results suggest that exponential phase cells are more susceptible to low temperatures compared to stationary phase cells. This is contradictory to the heat-treated results, but is probably due to exponential phase cells actively growing when they were initially exposed to this temperature, whereas stationary phase cells are nearing the death phase. Therefore, their active cellular mechanisms will not be greatly affected by these temperatures. Lorca and Valdez

(1998) proposed that sensitivity of *Lactobacillus acidophilus* cells to cold shock increased during cell division, but it should be noted that it is a Gram-positive organism, so may have different survival mechanism to Gram-negative bacteria such as *A. butzleri*. These results also show that freezing had a greater effect on cell viability compared to chilling. These findings are supported by Gill and Harris (1984), Hood *et al.* (1988) and more recently Buswell *et al.* (1998) who showed that *C. jejuni* survived better in chilled foods than in foods held at ambient temperatures or frozen conditions. Also, it was observed that at retail outlets *Campylobacter* spp. were present in higher numbers on chilled-fresh chicken than frozen ones. This phenomenon may be due to cellular functions such as oxygen consumption, catalase activity, ATP generation, chemotaxis and protein synthesis still functioning at 4°C, which may assist in survival (Hazeleger *et al.*, 1995; 1998).

Differences in cell recoveries between selective (CAT and CCDA) and non-selective (blood agar) media were apparent after 3 and 7 days (chilled and frozen samples, respectively) and increased with storage time, especially at -20°C. This difference was probably due to the increased number of sublethally injured cells, possibly due to the antibiotics in CAT and CCDA causing secondary (lethal) injury to the already delicate cells. On the other hand blood agar does not contain any selective ingredients and furthermore it is rich in nutrients, so it would be able to sustain growth of both uninjured and sublethally injured cell. There was no apparent difference in cell recoveries between CAT and CCDA. Although *Arcobacter* spp. are resistant to the level of cefoperazone present in CCDA (32 μg/ml compared with 8 μg/ml in CAT), growth is generally better on CAT suggesting that there may be a synergistic inhibitory effect of deoxycholate and cefoperazone present in CCDA (Corry & Atabay, 1997).

The above experiment was repeated, but the *A. butzleri* NCTC 12481 cells were stored in nutrient poor phosphate buffer instead of BHI broth. The cells that were chilled (+4°C) and stored in phosphate buffer showed similar results to those obtained for BHI broth. However, slightly higher viable counts were detected for cells harvested from the exponential phase and stored in BHI. The difference became more apparent with storage time, with a 1 log (cfu ml^{-1}) difference being observed after a 3 day period (Figure 3.14). This result was expected as these

cells are actively growing, so are more likely to be affected by the low temperature. Therefore, the nutrient-rich environment would be able to maintain a high percentage of viability by providing a suitable environment for damaged cells to repair. In addition, these nutrients may enable the cells to produce a cold shock response or act as a cryopreservative. Freezing, on the other hand, had a great effect on cell viability of both growth phases stored in phosphate buffer. No growth was detected for exponential phase cells on either media, whereas no growth occurred after a 24 h period on selective (CAT and CCDA) media and after 3 days of storage on the non-selective blood agar for cells harvested from the stationary phase. These results show that after 24 h storage the *A. butzleri* NCTC 12481 cells become sublethally injured and these cells are killed off after a further 48 h incubation period. It is probable that nutrient-poor phosphate buffer was unable to provide the damaged cells with any protection or the necessary requirements for repair. This infers that the organism can repair at 4°C. Nevertheless, for some bacteria the presence of a starved environment induces a stress response which provides additional tolerance to other harsh environments such as cold, heat and pH *etc* (Lazaro *et al.*, 1999). For example Cappelier *et al.* (2000) reported an increase in heat resistance in *C. jejuni* strain 79 as cells entered starvation in a surface water microcosm.

The ability of *Arcobacter* spp. to remain viable under chilled or frozen conditions was an expected result as *Arcobacter* have been routinely refrigerated in semi-solid media for extended periods of time without loss of viability (Wesley personal observation, as quoted by Collins *et al.*, 1996a). Furthermore, decreasing temperature appears to have an effect in reducing growth, but *A. butzleri* is still able to survive both in culture and in chicken at low temperatures (Phillips & Long, 2001; Phillips & Duggan, 2002). This survival ability also provides some explanation for the transmission of *Arcobacter* spp., as the organism would be able to exist in water and on meat products for prolonged periods of time. Consequently, the ability to survive refrigeration and freezing is of obvious relevance to food safety and public health.

4.9. HEAT AND COLD ADAPTATION OF *A. butzleri* NCTC 12481.

A variety of environmental stimuli including temperature, osmolarity, pH and exposure to atmospheric oxygen are known to trigger the synthesis of a set of highly conserved proteins named stress proteins (Thies *et al.*, 1999 a, b). *Campylobacter* spp. are known to possess such proteins, for example the GroESL bicistronic operon was cloned and sequenced in *C. jejuni* and was found to show a high level of homology to other bacterial GroES and GroEL proteins (Thies *et al.*, 1999a; Murayama *et al.*, 2003). In many bacteria GroEL and GroES proteins work together to prevent permanent injury after heat treatment by binding and stabilising proteins at intermediate stages of folding, assembly, translocation across membranes and degradation. Therefore, it is possible that these proteins have the same function in *C. jejuni* as a marked increase in production of GroEL and GroES occurred after a temperature upshift (Thies *et al.* 1999 a). The *dnaK* gene has also been sequenced in *C. jejuni* which encodes a protein with a high degree of homology with other bacterial 70 kDa heat shock proteins (Thies *et al.*, 1999 b). This protein along with DnaJ and GrpE act as negative modulators to form a three part chaperone system that regulates a heat shock response. Due to these facts it was of interest to investigate the possibility that *Arcobacter* spp. may have stress proteins. This was achieved by pre-exposing *A. butzleri* NCTC 12481 to non-lethal temperatures for various periods of time followed by the usual heat treatment (55°C, 3 min).

The preliminary experiments showed that pre-exposing stationary phase cells for 1 h at 40°C caused a slight increase (approximately half a log) in cell viability compared to the heat-treated control (Figure 3.15). However, longer pre-exposure times (2-3 h) caused a greater loss in cell viability. This suggests that the *A. butzleri* cells become more sensitive to temperature over time, possibly due to the presence of large numbers of sublethally injured cells as 40°C is slightly above this organisms growth range (15-39°C). Then again, it should be noted that this temperature was chosen as it was determined by the impedance technique, as this is an extremely accurate way of measuring growth at particular temperatures (± 0.001°C), compared to the plate count methods which show accuracy to

approximately ±1°C. Therefore, a slightly higher pre-exposure temperature may have been more appropriate for this particular experiment and hence the lack of a noticeable survival mechanism in stationary phase *A. butzleri* cells. It is also plausible that pre-exposing the cells at 40°C for less than an hour may have induced a survival mechanism such as heat shock proteins, however the long term expression of HSPs (i.e. 2-3 h) can be lethal and hence the decrease in cell viability. Nevertheless, as previously stated in Section 4.5 it is possible that *A. butzleri* cells in the stationary phase of growth are unable to induce any heat shock mechanisms due to the lack of sigma factor RpoS, which regulates the expression of essential genes for survival under unfavourable conditions (Kelly *et al.*, 2001). Consequently, further experiments were required, whereby the *Arcobacter* cells were pre-exposed to higher temperatures (i.e. 45-50°C) or exposure time decreased to less than 1 hour. Work was started in this area, but aberrant plate count results, which contradicted the previous data were obtained, so this data was not presented. Unfortunately time constraints prevented the experiments from being repeated, as it was felt that the time would be better spent looking at the effect of heat adaptation on exponential phase cells as they were more likely to induce survival mechanisms.

These experiments were also performed on exponential phase *A. butzleri* NCTC 12481 cells (Figure 3.16). After heat treating (55°C, 3 min) the 5 and 20 minute 40°C pre-exposed samples, the viable counts were approximately 1 log (cfu ml^{-1}) higher on blood agar than the 55°C heat treated control. The 45 minute pre-exposed (40°C) sample also had a viable count slightly greater (<0.5 log, cfu ml^{-1}) than the heat-treated control. However, like the stationary phase cells this survival effect was only observed up to one hour incubation at 40°C. These results suggest that exponential phase cells have an enhanced survival capacity compared to stationary phase cells, which again supports the previous heat treatment experiments (Section 4.5 & 4.6).

This data also suggests that exponential phase cells were able to adapt to a non-lethal temperature before being exposed to a higher temperature. Therefore, it is likely that *A. butzleri* cells have some sort of heat survival mechanism that is induced by adaptation at 40°C. It is possible that this mechanism includes heat

shock proteins since *GroELS* and *dnaK* genes have been sequenced in *C. jejuni*. Alternatively, mild heat treatment can lead to adaptation of the cell membrane by increasing the saturation and the length of the fatty acids in order to maintain optimal fluidity of the membrane and activity of intrinsic proteins (Abee & Wouters, 1999). However, this increased resistance to heat may be due to the cells being heat adapted in 0.1 M potassium phosphate buffer, which is a nutrient poor environment. Therefore, the cells are under starved conditions which have been reported to increase the heat shock resistance in *C. jejuni* cells (Cappelier *et al.*, 2000). The ability of starved bacteria cells to survive in hostile conditions is thought to be due to the synthesis of starvation proteins such as Cst proteins, which increase cell nutrient assimilation and Pex proteins which deal with the resistance mechanisms. Furthermore, Martin (1990, as quoted by Cappelier *et al.*, 2000) showed that the increase of heat resistance in starved cells is more efficient than the increase of resistance in heat shock pre-adapted cells. Cross protection against heat, osmotic or oxidative shock has been previously reported for various starved bacteria (Jenkins et al., 1988; 1990; Nystrom et al., 1992 as quoted by Cappelier *et al.*, 2000). This increased resistance to temperature is extremely important in the food processing industry as food preservation by elevated temperatures is a common process in use today. For this reason a more in-depth study on *Arcobacter* spp. survival capabilities is required. As various groups (Collins *et al.*, 1996b; Rice *et al.*, 1999; Otth *et al.*, 2001; Houf *et al.*, 2002a; 2003) have observed that *Arcobacter* have a good resilience to environmental factors such as irradiation, desiccation and low temperatures (see Section 1.10.1) enabling them to survive in food processing plants, thus presenting a risk to public safety. Unfortunately, the time limitations of this project meant that further investigations such as increased pre-exposure temperatures were not possible, but work is still continuing at the Nottingham Trent University.

As well as looking at the effect of heat adaptation, cold adaptation was studied as *Arcobacter* species have been shown to be tolerant to cold temperatures. *A. butzleri* is able to grow slowly at 15°C (Section 3.8) and is able to survive in ground water for up to 16 days (Rice *et al.*, 1999). Therefore, the ability of *A. butzleri* NCTC 12481 to adapt to both refrigerated (+4°C) and freezer (-20°C) temperatures were investigated, which is an important aspect of food microbiology

due to the increased popularity of convenience foods. As seen previously in Section 3.2, the stationary phase viability counts of the chilled control had decreased by approximately 1 log (cfu ml^{-1}) compared to the untreated control; whereas the frozen control viability decreased by 4 log (cfu ml^{-1}) orders on blood agar after only 24 h. This showed that *Arcobacter* spp. survived better under chilled conditions compared with frozen conditions.

Figure 3.17 a and b showed that no beneficial or harmful effect was induced when stationary phase *A. butzleri* cells were pre-exposed at 25°C for 1 to 3 h before chilling or freezing. This may be due to *A. butzleri* being able to grow slowly at 25°C, so cold adaptation may not be induced. Further experiments were performed, but a lower pre-exposure temperature (15°C) was used. However, a similar trend was observed, which could be explained by 15°C being the minimum detectable growth temperature for *A. butzleri*. Therefore, these temperatures were unable to induce cold adaptation. Consequently, *A. butzleri* NCTC 12481 is probably capable of synthesising cold acclimation proteins (CAPs) during continuous growth at cold temperatures. This enables them to tolerate low temperatures by producing antifreeze substances or maintaining some metabolic function, but provides no protection outside their growth range. Alternatively, transitions in the structure of a key enzyme(s) or regulatory compound(s) may play an important role in *A. butzleri* survival. Also, *A. butzleri* are probably unable to induce cold shock proteins (CSPs) and hence, no additional protection was detected at chilling or freezer temperatures. Even though the information on cold shock responses is limited, these suggestions are possible as CPSs have not been detected in *H. pylori* (Tomb *et al.*, 1997) and *C. jejuni* (Hazeleger *et al.*, 1998). However, to prove this theory it would be necessary to expose *A. butzleri* to temperatures below 15°C prior to storage at +4°C or -20°C. The presence of CAPs or CSPs could then be determined by 2-D gel electrophoresis.

This adaptation study could have been taken further with the combination of heat and cold shock being investigated, because exposure to a number of harsh conditions are normally experienced in the natural environment or processing plant. Especially as incidental data from the *D* value experiments had shown that *A. butzleri* NCTC 12481 had a greater sensitivity to cold shock after being heat-

treated at various temperatures. This was shown by a decrease in viable counts when the heat-treated samples were placed on ice prior to enumeration, compared to storage at room temperature.

4.10. TETRAZOLIUM DYE ASSAY TO STUDY METABOLIC STATUS.

As well as studying the percentage of dead and sublethally damaged cells that occur due to heat treatment it was necessary to investigate the effect heat has on metabolic activity. This was achieved by using 2,3,5-triphenyltetrazolium chloride (TTC), a colourless water soluble salt that is reduced to a red water insoluble triphenylformazan that can be detected by a spectrophotometer. TTC is a redox dye which can be reduced by electrons from an active biological system, such as the respiratory chain.

A. butzleri NCTC 12481 did not reduce the tetrazolium salt, probably due to *Arcobacter* using proteins and amino acids as their source of carbon and energy. This was previously seen in Sections 4.2 and 4.3 whereby *A. butzleri* was unable to ferment the carbohydrates present in the BHI broth, instead the media became more alkaline due to the deamination of amino acids. This causes *Arcobacter* to have a low metabolic activity, which in turn is related to this organism lacking a complete electron transport chain. Therefore, *Arcobacter*'s electron transport chain would be unable to pass on the electrons derived from the organic material to TTC, which means that this redox dye remains in the unreduced colourless state. This result was to some extent anticipated as several groups such as On *et al.* (1996) and Atabay *et al.* (1998) have reported on *Arcobacter* species having a low metabolic activity, which makes them difficult to identify using biochemical tests. Furthermore, Vandamme *et al.* (1992a) observed that 67% (8/12) of *A. butzleri* strains could grow in the presence of 0.04% TTC. However, at higher concentrations, i.e. 0.1% which was used in this experiment, TTC may have an inhibitory effect which would also explain the lack of activity by this organism.

To fully understand the complex workings of arcobacters electron transport chain further experiments are required. Nevertheless, this particular aspect was outside the scope of this specific study which was concentrating on the physiology and survival of *Arcobacter* species. For this reason the work was not continued.

The results on *E. coli* will not be discussed as it was only used for comparison and would not provide any valuable information for this thesis which is based on *Arcobacter* species.

4.11. NITRATE AND NITRITE REDUCTION BY *A. butzleri* SPECIES.

Many groups such as Vandamme *et al.* (1992b) and Atabay *et al.* (1998) have observed that most *A. butzleri* strains are able to reduce nitrate in their media, but not nitrite. Additionally, due to the ability of *Arcobacter* to grow under reduced oxygen levels, the addition of nitrate or nitrite may provide this organism with an alternative electron acceptor when oxygen is absent. Due to these facts it was necessary to investigate the ability of *A. butzleri* NCTC 12481 to reduce nitrate and/or nitrite. *A. butzleri* strains Rigs 1714, Rigs 16799 and Rigs 15342 were also studied for comparison.

The preliminary spot tests (Table 3.11) showed that both *A. butzleri* NCTC 12481 and Rigs 1714 were able to reduce nitrate beyond nitrite to ammonium ions or dinitrogen gas in the BHI broths containing 0.1 mM and 1 mM of nitrate. However, at the highest nitrate concentration (5 mM) not all the nitrate was reduced past nitrite causing an accumulation of nitrite in the broth as shown by the pink colouration. The other two *A. butzleri* strains were only able to reduce nitrate to nitrite for all three nitrate concentrations. Therefore, all four stains have a partial electron transport chain that can operate with inorganic electron acceptors such as nitrate enabling it to be reduced to produce energy, whereas only *A. butzleri* NCTC 12481 and Rigs 1714 were able to reduce nitrite. These results support the findings of the other groups (Vandamme *et al.*, 1992a; Atabay *et al.*, 1998), except in this particular study two strains were able to use nitrite as an alternative terminal

electron acceptor. These results were also observed for the spot tests performed on the different growth media used in the impedance experiments (Table 3.12).

Glucose present in BHI growth media acts as an electron donor for fermentative organisms such as *E. coli*. From the TTC assay experiments (Section 4.10) it is likely that *Arcobacter* species do not rapidly metabolise carbohydrates such as glucose. This concept was supported by only small pH changes (tending towards alkaline) being detected in Section 4.3 where the effect of pH on the growth of *A. butzleri* was investigated. Therefore, in order for *Arcobacter* to use nitrate and/or nitrite as an alternative electron accepter, the organism would have to utilise proteins and amino acids. Additionally, the presence of oxygen would directly affect the rate of nitrate/nitrite reduction by *Arcobacter* spp., as oxygen is the favoured terminal electron acceptor as it creates the largest difference in redox potential, which in turn produces energy in the form of ATP. Since the broth cultures were inoculated on the bench rather than inside the anaerobic cabinet (5% CO_2 head space) oxygen would have been present in the tube. Therefore, it is accepted that these experiments were not optimised since oxygen would be the preferred electron acceptor when available. Furthermore, previous experiments (Personnel communication) have shown that since nitrite can be toxic to the bacterial cell, it was necessary to initially grow the *Arcobacter* cells on low (0.1 mM) concentrations of nitrate/ nitrite in order to induce the necessary reductase enzymes prior to growth at levels (10mM) that would sustain higher growth rates. Even though the spot test can screen strains for the ability to reduce nitrate or nitrite, the effect of their presence on the growth rate of bacteria can not be established, therefore the impedance technique was used.

Using impedance techniques the presence of nitrate/nitrite on the growth rates of the four *A. butzleri* strains were determined. Table 3.13 shows that the presence of 5 mM nitrate in the media increased the growth rate of three *A. butzleri* strains (NCTC 12481, Rigs 1714 & Rigs 16799). These elevated growth rates are probably due to oxygen being trapped in the impedance tubes whilst being set up for experimentation. This in turn meant that nitrate could not be reduced initially as the synthesis of nitrate reductase is suppressed by oxygen. Therefore, when oxygen was no longer available, nitrate was used as an alternative electron

acceptor. Thus converting electrons donated from proteins and amino acids into the necessary requirements for growth. This route is an important survival mechanism, which could explain why this organism can continue to exist in hostile environments. With the exception of Rigs 1714 the presence of nitrite caused the growth rate to be slower than in the BHI control. For example, Rigs 16799 grown in BHI medium had a doubling time (t_d) of 1.15 h, which compares to 1.72 h in the presence of nitrite. Therefore, the presence of nitrite does not improve the growth of most *A. butzleri* strains. Usually bacteria reduce nitrate past nitrite to ammonium ions or dinitrogen gas by a process called denitrification as nitrite is considered to be quite toxic. This could explain the lower growth rate for *A. butzleri* spp. when nitrite was present in the growth media. Finally, the results show that the presence of nitrate or nitrite does not increase the growth rate of Rigs 15342. The variation in nitrate/nitrite reduction between the four *A. butzleri* strains was not surprising as each particular strain shows a different level of tolerance to oxygen. This in turn will reflect on the strain's preference to use nitrate/nitrite as a terminal electron acceptor.

Both the data from the initial part of this study and current literature has shown that *A. butzleri* has similar morphological and biochemical characteristics to *Campylobacter* spp. However, *Arcobacter* have shown a greater resistance to environmental conditions such as tolerance to an aerobic environment, lower temperatures (<15°C) and desiccation. Therefore, it can be stated that *Arcobacter* species have the potential for remarkable survival under conditions non-permissive to growth compared to *Campylobacter*, thus presenting a greater risk to both humans and animals. Due to this fact it could also be stated that a certain percentage of the human gastroenteritis may be caused by *Arcobacter* spp. Nevertheless, this study has also shown that *A. butzleri* survived poorly at high temperatures, low pH, and under frozen conditions for prolonged periods. Such sensitivity to these conditions is surprising in a successful veterinary pathogen and an emerging human pathogen, as *Arcobacter* would be expected to be killed off when exposed to many harsh environments on the path from farm to folk. However, arcobacters possible success may be explained by the high contamination levels in key foods such as chicken and pork. Even though many *Arcobacter* cells will be removed or die during processing, an infectious dose may

still remain. *Arcobacter* spp. may have a low infectious dose of ~500 cells, similar to *Campylobacter* spp. Additionally, the widespread prevalence of *Arcobacter* in animals and the environment may be an important feature that could explain why this sensitive organism causes gastroenteritis and abortion in animals and most likely illness in people. Several reports (Manke *et al.*, 1998; Houf *et al.*, 2003) have commented on *Arcobacter* genetic variability, which may enable it to exploit more diverse environments than the laboratory data suggests. Although this study has provided valuable information on the growth and survival capabilities of *A. butzleri* NCTC 12481, which is important to both veterinary and food microbiology, further research is required to gain a greater understanding into this particular organism.

4.12. CYTOTOXICITY SCREENING OF *ARCOBACTER* STRAINS.

A. butzleri is a significant veterinary and possibly a human pathogen. Therefore, the second part of this project concentrated on detecting any cytotoxic effects on mammalian cell lines *in vitro*. Human *A. butzleri* isolates were screening for cytotoxicity by using the methyl tetrazolium thiazolyl blue (MTT) assay (Sladowski *et al.*, 1993).

4.12.1. PRELIMINARY CYTOTOXICITY SCREENING WITH *A. butzleri* NCTC 12481.

Preliminary experiments were carried out to establish if cell-free extracts of *A. butzleri* NCTC 12481 could exert cytotoxicity towards mammalian cells (CHO, ECV and N2a) *in vitro* when the bacterial cells were either heat-treated (45, 50, 55 and 60°C, for 3 min) or left untreated. Both the untreated and heat-treated (50, 55 & 60°C) cell-free extracts from bacteria harvested from the exponential phase (Figure 3.20a) showed that as the sample concentration increased from 2.5% to 10% the N2a cell viability decreased. Approximately 100% viability values were detected for the cell-free extracts applied at 2.5% concentration (except the 60°C sample). Consequently, the same number of N2a cells had been killed by the phosphate control as the cell-free extract and therefore, the cell-free extracts

showed very little cytotoxicity at this concentration. Nevertheless, as the treatment temperature increased the associated cytotoxicity increased as lower concentrations were required to cause a decrease in mammalian cell viability. For example, a 5% concentration of the 55°C heat-treated cell-free extract was required to produce significant cytotoxicity, whereas only 2.5% was required for the 60°C cell-free extract. Also, significant differences in cytotoxity were detected between the untreated and heat-treated (55°C and 60°C) cell-free extracts. These results suggest that as the treatment temperature increased, the induction or release of toxin(s) also increased. The most likely explanation for this effect is that the cytotoxin(s) are released due to the elevated temperatures causing cell lysis or blebbing of the cell contents. However, this was also a surprising result as most protein toxins are heat labile and would be inactivated by temperatures greater than 50°C. Both McCardell *et al.* (1986) and Lam (1993) have reported on *C. jejuni* releasing a heat stable (100°C, 10 min) cytotoxin. Due to these findings the temperature and proteolysis stability of the *Arcobacter* cytotoxin(s) was investigated (see Section 4.12.4).

The above trend observed for N2a cells was also detected for ECV and CHO mammalian cell lines. The viability of the CHO cells for the three different concentrations (2.5%, 5% & 10%) were considerably higher than those detected for either N2a or ECV. This suggests that the toxin(s) produced by *A. butzleri* NCTC 12481 had different cytotoxic capacity towards different mammalian cell lines. Johnson and Murano (2002) also observed varied levels of toxicity on CHO, HeLa and INT407 cells when exposed to extracts from *Arcobacter* strains. Therefore, the toxin(s) probably has specific cell membrane receptors and modes of action. For example, HeLa and Vero cells are extremely sensitive to toxins that inhibit protein synthesis such as *Shigella dysenteriae* and *E. coli* Stx toxins, due to the presence of Gb3 sphingoglycolipid receptor on their cell surface, whereas CHO cells lacks this particular receptor so remains unaffected by these toxins (Obrig, 1994). Additionally, the lower cytotoxicity on the CHO cells may be related to the cell-free extracts being applied to a semi-confluent monolayer, as several groups (Florin & Antillon, 1992; Mizuno *et al.*, 1994) have reported that more prominent cytotoxic affects were observed with freshly seeded CHO cells. Since freshly seeded cells provide a greater surface area for exposure compared to a

monolayer, also they are undergoing active growth and protein synthesis, thus making them more susceptible to toxins.

These cytotoxicity experiments were repeated except stationary phase cell-free extracts were used. N2a cell viability decreased with increasing treatment temperature and applied concentration. However, the cytotoxic effect was inconsistent and below the levels obtained for extracts from exponential phase cells. Furthermore, no cytotoxic affect was observed with either ECV or CHO cell lines. These preliminary experiments showed that exponential phase cell-free extracts had the greater cytotoxic capacity. This was an unexpected result as many reports have proposed that maximal toxin activity was found in cultures of *C. jejuni* and *C. coli* approaching the stationary phase (Klipstein & Engert, 1984a; Daikoku *et al.*, 1989). Solubilization experiments have shown that the toxins are produced intracellularly during active growth, but are not released until entry into the stationary phase (Daikoku *et al.*, 1989, 1990). Nevertheless, the difference in cytotoxic activity could be a growth phase-dependent phenomenon, as the release of the cytotoxin in *A. butzleri* may occur during active replication instead of being lost as the cells near death.

This preliminary set of results also showed that only a gradual decrease in cell viability of all three mammalian cell lines was detectable when the temperature of the heat treatment of the bacterial cell suspension was increased in 5°C increments from 45°C to 60°C. Therefore, a 55°C heat treatment was used in further experiments to determine the cytotoxic ability of *A. butzleri* strains, since this temperature had caused the relatively high cytotoxic affect on both N2a and ECV cell lines. Also, this temperature was used as a standard heat treatment in previous experiments (see Section 2.10).

4.12.2. DETERMINATION OF THE TOXIN LOCATION.

Since the preliminary experiments had shown that *A. butzleri* NCTC 12481 had a toxic affect on mammalian cell lines (especially N2a cells) it was necessary to study this pathogenic mechanism further. BHI spent media, cell-free extract and cell sonicate were tested for cytotoxicity, to determine if the cytotoxin(s) was released into the growth medium during incubation or if physical stresses such as

heat or sonication caused the toxin(s) to be induced or released after cell lysis. Furthermore, both exponential and stationary phase cells were used to determine a more in-depth overview of the level of cytotoxicity produced by *A. butzleri* and if the growth phase had a direct effect.

BHI spent media from *A. butzleri* NCTC 12481 cells harvested from the stationary phase (Table 3.14) did not exert a significant decrease in cell viability for any of the mammalian cell lines. This result was also observed for all the *A. butzleri* strains harvested from the exponential phase. Therefore, the BHI spent media from cells grown to either exponential or stationary phase was considered to be non cytotoxic. This suggests that *A. butzleri* did not produce cytotoxin(s) that cause a decrease in cell viability whilst growing or that the toxic activity was cell-associated and not actively exported by the bacteria. However, significant levels of cytotoxicity were detected for the BHI spent media for both CHO and ECV cell lines. The components of the BHI spent media caused the mammalian cell lines to increase proliferation and/or metabolic activity. Therefore, the cytotoxicity was shown by an increase in cell viability compared to the BHI control (>100%). This effect was also observed for CHO cells when either cell-free extracts or cell sonicates from any of the four *A. butzleri* strains (human isolates) harvested from the exponential phase were applied. Nevertheless, this type of cytotoxicity was not studied further, since most of the toxins produced by *C. jejuni* cause a decrease in cell viability and the time limitations of the project meant that this aspect could not be investigated.

Cytotoxicity was detected for both cell-free extracts and cell sonicate, with the neuronal cell line N2a and to a lesser extent the endothelial cell line ECV showing particular sensitivity to samples harvested from the exponential phase, whereas CHO cells were unaffected by the presence of the cytotoxin(s) produced by *A. butzleri* NCTC 12481. The lack of cytotoxic activity on CHO cells has consistently been observed for *Arcobacter* species by Johnson and Murano (2002), whereas *C. jejuni* enterotoxins are typically active on this particular mammalian cell line. Therefore, *Arcobacter* species produce different cytotoxins to *Campylobacter*. These results also confirm those seen previously in the preliminary cytotoxicity experiments. Thus lending further support to the notion that the cytotoxin(s)

produced by *A. butzleri* have specific mammalian cell membrane receptors and/or modes of action which affects different cell lines.

This data (Tables 3.14 & 3.15) also shows that the level of cytotoxicity increased when the cells were exposed to physical stress, especially with a 3 minute heat treatment at 55°C. Consequently, the cytotoxin(s) is released into the surrounding medium after the cells become damaged by heat or sonication, thus indicating that the cytotoxin is membrane-bound. However, slightly lower cytotoxicity levels were detected for the sonicated samples which could be related to proteolysis of the cytotoxin by protease(s), which can be solubilized by sonication (Kawaguchi *et al.*, 1989). Also, the level of toxicity produced from the samples harvested from stationary phase cells tended to be lower and less reproducible compared to parallel studies with exponential phase cells (Table 3.15). From these results it can be proposed that the cytotoxicity levels of *A. butzleri* cells decreased with age. This could possibly be due to aged cells having damaged membranes, which cause the cytotoxin to be released during the washing steps of the cell harvesting procedure. These results are further supported by no significant cytotoxicity being detected for any of the other *A. butzleri* strains (Rigs 1714, Rigs 15342 & Rigs 16799) when harvested from the stationary phase, whereas comparable cytotoxicity data was detected for all four strains harvested from the exponential phase with Rigs 15342 producing the lowest levels of cytotoxicity activity. Observations by Johnson and Murano (2002) also showed that some isolates appeared to induce less toxicity than others to the mammalian cells in culture. This phenomenon has also been described for certain *C. jejuni* strains (Welsh & McClelland, 1990, as quoted by Johnson & Murano, 2002) and is therefore not unique to the *Arcobacter* strains tested. Also, Menzl *et al.* (1996) proposed that the growth rate of bacteria was directly related to cytotoxin production, as strains with a high growth rate could produce quantitatively more cytotoxic material.

4.12.3. THE EFFECT OF GROWTH CONDITIONS ON *A. butzleri* NCTC 12481 CYTOTOXICITY.

Several groups such as Klipstein & Engert (1984b) and Goossens *et al.* (1985) observed that culture conditions like medium supplements or growth temperature influence the toxin yields in *C. jejuni*. Therefore, it was necessary to study if

growth conditions had any effect on the toxicity levels produced by *A. butzleri*. Consequently, *A. butzleri* NCTC 12481 was grown at 30°C instead of 37°C or under microaerophilic conditions unlike previous broth cultures, which had been grown under aerobic conditions.

Similar results were detected for the exponential and stationary phase cells grown at 30°C and 37°C (Tables 3.14 & 3.15 and Figure 3.21 a & b). Cytotoxicity was observed with both N2a and ECV cell lines for cell-free extracts and to a lesser extent for cell sonicate samples. Furthermore, like previous experiments, the levels of cytotoxicity produced by the stationary phase extracts were generally lower and less reproducible compared to those produced by exponential phase cultures. However, this data did show that growing the cells under a more stressful temperature such as 37°C (See Figure 3.5) increased the cytotoxin(s) production. This finding was also supported by the experiment comparing the cytotoxicity levels produced when *A. butzleri* NCTC 12481 was grown under microaerophilic or aerobic conditions, as the levels of cytotoxicity were slightly greater under the more stressful conditions of aerobic growth. This effect was more significant with cell-free extracts harvested from stationary phase cells. Therefore, changing the growth temperature and oxygen availability slightly altered the cytotoxic activity of *A. butzleri* NCTC 12481. Misawa *et al.* (1994), Bacon *et al.* (1999) and Lee *et al.* (2000) have all observed that the expression of cytotoxins is directly affected by the growth environment of *Campylobacter* species.

4.12.4. DETERMINATION OF TOXIN TYPE.

A series of experiments were conducted to establish the cytotoxin(s) heat stability and susceptibility to proteolysis. The results (Figure 3.22) showed that cytotoxin(s) was heat stable (100°C, 10 min) and resistant to pronase and trypsin treatment. This suggested that the cytotoxin(s) was either a heat and trypsin/pronase-stable protein or an LPS molecule. Therefore, to try and answer this question, a series of protein and LPS gels were performed. The protein gels showed that exponential phase samples had a well-banded profile (Figure 3.23). Nevertheless, heat and proteolytic enzyme treatment caused the protein content to decrease drastically, whereas LPS was unaffected by either treatment, as ladder-like profiles were detected for each sample (Figure 3.24). From these results it

can be proposed that *A. butzleri* NCTC 12481 produced a lipopolysaccharide, the toxic component of which is lipid A. These results were further supported as the cytotoxicity corresponded with the presence of low molecular weight LPS from exponential phase cells on silver stained polyacrylamide gels (Figure 3.25). Thus suggesting that the presence of more low molecular weight LPS molecules in exponential phase *A. butzleri* NCTC 12481 cell membranes compared to stationary phase cells, caused the levels of cytotoxicity towards mammalian cell lines to increase. This is a plausible explanation as Moran (1995) stated that the biological effects of LPS can be related to the physical properties of the molecules, particularly the fluidity of the hydrocarbon chains (which is inversely proportional to the state of order) and the supramolecular structure of the LPS or lipid A. For example, the LPS and lipid A from *C. jejuni* had higher phase-transition temperatures than *Salmonella* Minnesota and hence have lower fluidity at 37°C than *Salmonella* LPS. Consequently, the lower fluidity of *C. jejuni* LPS correlated with lower activity in biological test systems.

Since lipid A is embedded in the outer membrane, it probably causes toxicity when released from dividing cells or when the bacteria are lysed in the host body. This would also explain why heat treatment (55°C, 3 min) and sonication increased the cytotoxicity levels on N2a and ECV cell lines (Tables 3.14 & 3.15). Hitchener and Egan (1977) reported that structural injury caused by mild heat resulted in the release of LPS. About 20% of the cellular LPS was released during heating for 1 h at 48°C, which resulted in 90% of the cells being injured to such an extent that they could not grow on selective media. Furthermore, the low levels of cytotoxicity caused by the untreated samples could be due to small amounts of LPS being released from the bacterial cell surface by vigorous vortexing and centrifugation as the cells were harvested to obtain a cell-free extract. Additionally, Kelly *et al.* (2001) proposed that as *C. jejuni* cells enter the stationary phase their membranes become leaky. Therefore, another possible explanation for the low levels of cytotoxicity of *A. butzleri* in the stationary phase could be that a certain percentage of the LPS is lost during the harvesting procedure. This means that there is less LPS material left to cause cytotoxicity to the mammalian cell lines. It is not possible to release more than 50% of the total LPS, since the residual LPS is apparently more tightly bound to the inner layers of the cell envelope. Finally,

there may be selective release of low molecular weight LPS by exponential or stationary phase cells, which in turn would affect the level of cytotoxicity produced.

Furthermore, Rigs 1714 and Rigs 16799 showed similar LPS ladder-like profiles to *A. butzleri* NCTC 12481, with the LPS band intensity increasing with the samples harvested from the exponential phase and also when the samples were heat treated prior to use (Figure 12, p.275). However, LPS banding profiles were only detected for Rigs 15342 when the exponential phase cells were heat-treated (Figure 3.26). Also, LPS from stationary phase samples had a greater intensity indicating a higher concentration. This could explain the decreased toxicity observed for this particular strain compared with the other three strains (Table 3.15).

4.12.5. CYTOTOXICITY OF LPS EXTRACTED FROM *A. butzleri* NCTC 12481.

To further support the theory that the cytotoxin produced by *A. butzleri* NCTC 12481 in these experiments was LPS, LPS was extracted from exponential phase cells as well as cell-free extracts from untreated and heat-treated cells. The three mammalian cells lines (CHO, ECV & N2a) previously used (Section 4.12.1 & 4.12.2) were exposed to the LPS extracts to determine if the pure LPS produced the same cytotoxic response.

High levels of cytotoxicity were observed especially with N2a cell lines for the pure LPS extracted from whole cells and to a lesser extent cell-free extracts from heat-treated *A. butzleri* cells (Figure 3.27). No cytotoxicity was detected for the unheated cell-free extracts for any of the cell lines tested. These results were further supported by the silver stained polyacrylamide gels (Figure 3.28), which all showed LPS ladder-like profiles with increased levels of low molecular weight LPS. However, the whole cells showed increased intensity of bands compared to the other samples especially the unheated cell-free extract, thus suggesting that more LPS was obtained from the whole cell preparations. This was an expected result because more LPS is available to be removed from the whole cells, whereas only the LPS released from the cells after heat damage can be purified from the cell-free extracts.

Both Kawaguchi *et al.* (1989) and Mizuno *et al.* (1994) reported cytotoxic activity associated with the lipopolysaccaride-rich fraction of *C. jejuni*. Bacon *et al.* (1999) also reported a cytotoxin with trypsin resistance and heat stability (≤70°C, 30 min). The cytotoxic activity resulted in Hep-2, HeLa and to a lesser extent CHO cells forming vacuoles, followed by cytoplasmic blebbing, cell rounding and eventually cell death. Cytotoxin characterisation only detected a single major band on silver stained polyacrylamide gels signifying the presence of LPS. Further analysis showed that a porin-lipopolysaccharide complex was responsible for cytotoxicity. Additional treatments (i.e. 5 U of neuraminidase to cleave sialic acid residues) established that LPS did not elicit the toxic effect caused by the complex, thus suggesting that LPS is not an integral component of the cytotoxic activity, but simply plays a protective role possibly interfering with the enzymic degradation by trypsin and staining with Commassie blue stain. However, Florin and Antillion (1992) proposed that as the porins were membrane bound, some LPS may remain attached upon secretion of the cytotoxin into the culture medium. Alternatively, LPS may play a role in assisting the export of the cytotoxin and serve a protective role in shielding the bacteria from the effects of the cytotoxin. Due to these facts and the limited knowledge on LPS toxicity further investigation into *Arcobacter* LPS and toxicity are continuing at the Nottingham Trent University.

4.12.6. EFFECT OF CYTOTOXIN ON THE MORPHOLOGY OF CELL LINES.

Even though the cytotoxicity assay is both sensitive and reproducible, only changes in cell viability are detected. Therefore, the presence of cytotonic toxins or toxins that cause progressive damage for a few days before lethality occurs may be undetected by this particular method. However, many toxins exert a direct effect on the morphology of cell lines. Therefore, a morphology study was undertaken, whereby CHO, ECV and N2a cell lines were exposed to cell-free extracts from untreated and heat-treated *A. butzleri* NCTC 12481 cells.

The observation that *A. butzleri* cell-free extracts caused an increase in cell rounding of CHO cells (Table 3.16 & Figure 3.29 a, b) is in agreement with previous results using river isolates (Musmanno *et al.*, 1997). Additionally, the numbers of elongated cells decreased in the treated CHO cells. Therefore, it is unlikely that *A. butzleri* NCTC 12481 produce a cytolethal distending toxin or

cholera-like toxin which are found in *C. jejuni* and other pathogenic bacteria. Also, Johnson and Murano (2002) showed the lack of a detectable PCR product using degenerate *cdt* primers, thus lending further support to the notion that *Arcobacter* species produce a different 'cytotoxin' to CDT. In addition, Lee *et al.* (2000) reported that the rounding of CHO and HeLa cells is indicative of a toxin that inhibits actin filament formation, which suggests that *A. butzleri* cytotoxin is analogous to *Clostridium difficile* toxins A and B. Then again, some CHO rounding was observed for the 'pellet washes' sample which contains small amounts of LPS (Lee *et al.*, 2000). Therefore, the CHO cytotoxicity could be due to either a protein inhibiting toxin or LPS, but given that LPS was shown to be the cytotoxic factor produced by *A. butzleri* strains (Section 4.12.5), it is likely that LPS is responsible for this morphological change as well.

The relevance of morphological effects on ECV cells (increase in flat cells, decrease in cell process outgrowth) is uncertain, but could be involved in the invasion of ingested *A. butzleri* and subsequent bacteraemia (Wesley *et al.*, 1996; Woo *et al.*, 2001, Table 3.6 & Figure 3.29 c, d). However, the effect on morphology may have been increased, as the corresponding MTT assay showed no cytotoxicity for ECV cells, which was an unexpected result. The neuroblastoma cell line N2a also had considerable morphological changes with a large decrease (over 50%) in the outgrowth of neurites (Figures 3.29 e, f & 3.30) when exposed to cell- free extracts from heat-treated cells. This effect was also detected, but to a lesser extent with the untreated cell-free extract. These results support the cytotoxicity assay data (Table 3.15), which showed that N2a were particularly sensitive to the cytotoxic factor (most likely LPS) produced by *A. butzleri* NCTC 12481, especially after a 3 minute heat treatment at 55°C. Furthermore, the decrease in neurite growth on N2a cells would suggest that the cytotoxin affects protein synthesis in the cells, thus preventing these structures from forming. To date, very little is known on the effects of cytotoxins on N2a cell lines, so no direct comparisons can be made. However, in our laboratory the N2a cell line was established as a good general cytotoxicity model for various toxicity studies.

4.13. DETECTION OF CD14 ON MAMMALIAN CELL LINES (CHO, ECV and N2a).

The cytotoxicity and morphological results show that *A. butzleri* strains were able to exert different morphological changes and levels of cytotoxicity on the three mammalian cell lines. Also, it was shown that the cytotoxic factor produced was possibly the lipid A part of LPS. Due to these facts it was important to identify potential LPS receptors on the surface of the mammalian cells. This was achieved using antibodies raised against a common LPS receptor known as CD14.

The observations from the Western blots showed that a polypeptide band(s) of similar molecular weight to CD14 was detected in N2a and ECV and to a less extent in CHO cells (Figure 3.31). Also, an identical band was detected for the CD14 positive control (human blood). The ELISA technique provided additional support with CD14 being detected in high levels for N2a and ECV cells (Figures 3.32 & 3.33). These results suggest that CD14 are present in high concentrations on the surface of both N2a and ECV cell lines, which provides these cells with a receptor for LPS. To try and quantify the levels of CD14 on the surface of the mammalian cells flow cytometry was used (Table 3.18). The flow cytometry data also supported the Western blot and ELISA results, with all three cell lines showing the presence of CD14. The highest level of fluorescence was detected for the N2a cells, suggesting the presence of high levels of CD14 receptors. Even though consistent data was obtained, the types of antibodies used in this experiment were not appropriate as no significant levels of fluorescence were detected even for the positive blood monocyte control. Nevertheless, it was decided that this line of investigation was the best achievable outcome as no other antibodies are currently on the market, which could be used. Therefore, it is plausible that the lipid A component of released LPS bound to a CD14-like receptor, which is more abundant in N2a and ECV cell lines resulting in their greater sensitivity to the LPS fraction (Figure 3.31). Hence, the morphological changes and cytotoxicity observed in the N2a and ECV cell lines would have been triggered by this cell-surface interaction (Figure 3.28 and 3.29). The detailed structure of *A. butzleri* lipopolysaccharide is unknown to date. However, the heat-

stable antigen (referred to as lipooligosaccharide) of the related organism *C. jejuni* is being studied because of its possible role in pathogenesis and in particular neuropathogenesis (Moran & Penner, 1999).

The importance of *Arcobacter* as a causative agent of human infectious intestinal disease, in addition to being a veterinary pathogen, is debatable. Nevertheless, this study has demonstrated the production of an endotoxin by four human isolates of *A. butzleri*. As *Arcobacter* species are Gram-negative organisms it is not surprising that some cytotoxicity may be caused by the lipid A component of the lipopolysaccharide, as years of research have shown its pathogenicity in other bacteria. Therefore, these results may account, at least partially for the virulence of the organism to pigs, cattle and even humans. However, *A. butzleri* may be able to produce various toxins, with our laboratory recently identifying several strains that can produce haemolysin(s), thus providing more support to theory that this particular organism is a pathogen. Nevertheless, my study is the first report, which attempted to characterise the cytotoxic factor produced from four different *A. butzleri* strains isolated from humans. It is also the first to shown the cytotoxic activity of LPS produced by *A. butzleri*.

CHAPTER FIVE

CONCLUSIONS AND FUTURE WORK

5.0. CONCLUSIONS AND FUTURE WORK.

Arcobacter is a newly emerging food-borne pathogen that is associated with gastroenteritis and abortion in both cattle and pigs. Furthermore, links have been made to human illness in relation to food and water consumption. Therefore, research is required to develop a greater understanding into arcobacters clinical significance, pathogenicity and epidemiology. Hence, the aims of this project were to investigate the physiology and toxicology of *A. butzleri* NCTC 12481.

5.1. PHYSIOLOGICAL ASPECTS OF *A. butzleri* NCTC 12481.

- Using the impedance technique *A. butzleri* NCTC 12481 was shown to have an optimum growth range of 25-35°C and pH range from pH 6.0-8.0.
- The presence of nitrate (0.1-5 mM) in the growth media increased the growth rate of *A. butzleri* spp., whereas nitrite caused it to decrease.
- It was established that *A. butzleri* NCTC 12481 was unable to ferment or oxidise carbohydrates in their growth medium.
- Heat treatment experiments showed that exposing *A. butzleri* NCTC 12481 for 3 minutes at 55°C produced the highest percentage of survivors that were sublethally injured, but the least amount of cell death.
- Heat treatment caused cell membrane damage shown by high fluorescence values due to the uptake of DNA-binding fluorescent dyes and more UV-absorbing material being released.
- Exponential phase cells had a greater resistance to heat, shown by them having higher *D* values compared to stationary phase cells.
- Heat adaptation was detected in *A. butzleri* NCTC 12481 cells shown by an increase in viable counts when exposed to 40°C for up to 1 h prior to heat treatment (55°C, 3 min).
- Storage of *A. butzleri* cells under chilled conditions (+4°C) caused a gradual decrease in viability as exposure time increased, whereas freezing caused a large decrease (2-4 log) in viability after only 24 h in storage after which time the viability remained fairly constant.
- The cold adaptation experiments suggest that *A. butzleri* NCTC 12481 produced CAPs, which would enable this organism to tolerate low temperatures.

5.2. CYTOTOXICITY STUDIES.

- No significant cytotoxicity was detected in either cell-free extracts from stationary phase cells (except some inconsistent cytotoxicity towards N2a cells) or in the BHI spent media. Therefore, *A. butzleri* does not release

any cytotoxic factors whilst growing and as the cells age the cytotoxicity of the cultures decreased.

- Cytotoxicity was greater following heat-treatment (55°C, 3 min) or sonication of the bacterial cells, suggesting that physical stress releases the cytotoxin, indicating that it was membrane bound.
- The cytotoxin(s) was proven to be heat-stable (100°C, 10 min) and resistant to proteolytic enzymes (trypsin & protease), which is indicative of an LPS endotoxin.
- Cytotoxic activity towards N2a cells and to a less extent ECV cells was detected in cell-free extracts from exponential phase cells. It is plausible that the lipid A component of released LPS bound to a CD14-like receptor, which were more abundant on N2a and ECV cells, resulting in greater sensitivity to the LPS fraction. Furthermore, morphological changes observed in these cell lines would have been triggered by this cell-surface interaction.
- The MTT assays show that CHO cells were unaffected by the cytotoxin, which suggests that it reacts with certain receptors on the mammalian cells surface. Nevertheless, the morphology study showed cell rounding of the CHO cells. The lack of cellular distension suggests that *A. butzleri* spp. are unlikely to produce CDT cytotoxins.
- Cytotoxicity corresponds with the presence of low molecular weight LPS from exponential phase cells on silver stained polyacrylamide gels. These findings were supported by cytotoxic activity being detected by LPS extracted from both whole cells and cell-free extract of heat-treated cells.

This study has provided valuable information on the physiology of this organism, showing the potential for remarkable survival under conditions non-permissive to growth. Furthermore, my study has attempted to characterise the cytotoxic factors produced from four different *A. butzleri* strains isolated from humans. Also, it has show that LPS from *A. butzleri* has cytotoxic activity, thus determining a possible virulence factor produced by *Arcobacter* spp. Nevertheless, work needs to be continued in this field to determine a greater understanding of this particular organism.

5.3. FUTURE WORK.

Future work would include a titration experiment whereby different concentrations of LPS extracted from the *A. butzleri* isolates would be applied to mammalian cell lines to establish the amount of LPS required to cause significant cytotoxicity. In addition, a comparative study of LPS cytotoxicity will be performed, using LPS extracted from food-poisoning organisms such as *Campylobacter, Salmonella and*

E. coli, to determine if *A. butzleri* spp. produce LPS with comparable toxicity to established pathogens. Further proof of the virulence of LPS could be obtained by using periodate oxidation, which destroys the LPS molecule by breaking down the lipid A portion. Since the lipid A is reported to be the toxic part of LPS, the loss of this section would prove if the cytotoxicity produced by *Arcobacter* spp. was solely due to LPS or a combination of LPS and protein cytotoxins, which has commonly been reported for other pathogenic organisms such as *Campylobacter* spp (Wassenaar, 1997). Furthermore, it would determine if LPS purely acts as a protection mechanism for a cytotoxic protein or if it responsible for the cytotoxicity detected in this study. If the cytotoxicity is lost with this treatment, the interactions between LPS and the mammalian cells can be studied in more detail by again using periodate oxidation, which also blocks CD14, thus preventing LPS/epithelial cell interactions. Therefore, providing additional evidence that LPS causes cytotoxicity by binding to CD14 receptors, which are abundant on the surface of N2a and ECV cells. Also, it would be necessary to study the mammalian cells by electron microscopy to determine the type of effect this cytotoxin causes to the mammalian cell, as LPS causes cell lysis. In addition, this technique could be used to study vacuole formation as a recent paper (Villarruel-Lopez *et al.*, 2003) has shown that some *Arcobacter* spp. can produce a vacuolising toxin similar to a *H. pylori* cytotoxin that induces the formation of acid vacuoles in the cytoplasm of different cell lines.

However, if some cytotoxicity was detected after periodate oxidation treatment it can be proposed that *Arcobacter* spp. also produces protein cytotoxins, to study this theory further polymyxin B can be used as it binds only to LPS leaving a protein rich fraction, which could be tested on the mammalian cell lines. It is likely that *Arcobacter* spp. are able to produce more than one type of toxin as recent work at the Nottingham Trent University has shown that *A. butzleri* was able to produce haemolysins. Due to this, it would be important to study this aspect in more detail by using contact assays to establish under what conditions and the level of cytotoxicity these haemolysins produce. Finally, to complete this work the adhesive and invasive nature of the four human isolates could be studied by using a Giemsa stain, which only stains bacterial cells, thus establishing other virulence mechanisms produced by *A. butzleri* NCTC 12481.

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APPENDIX

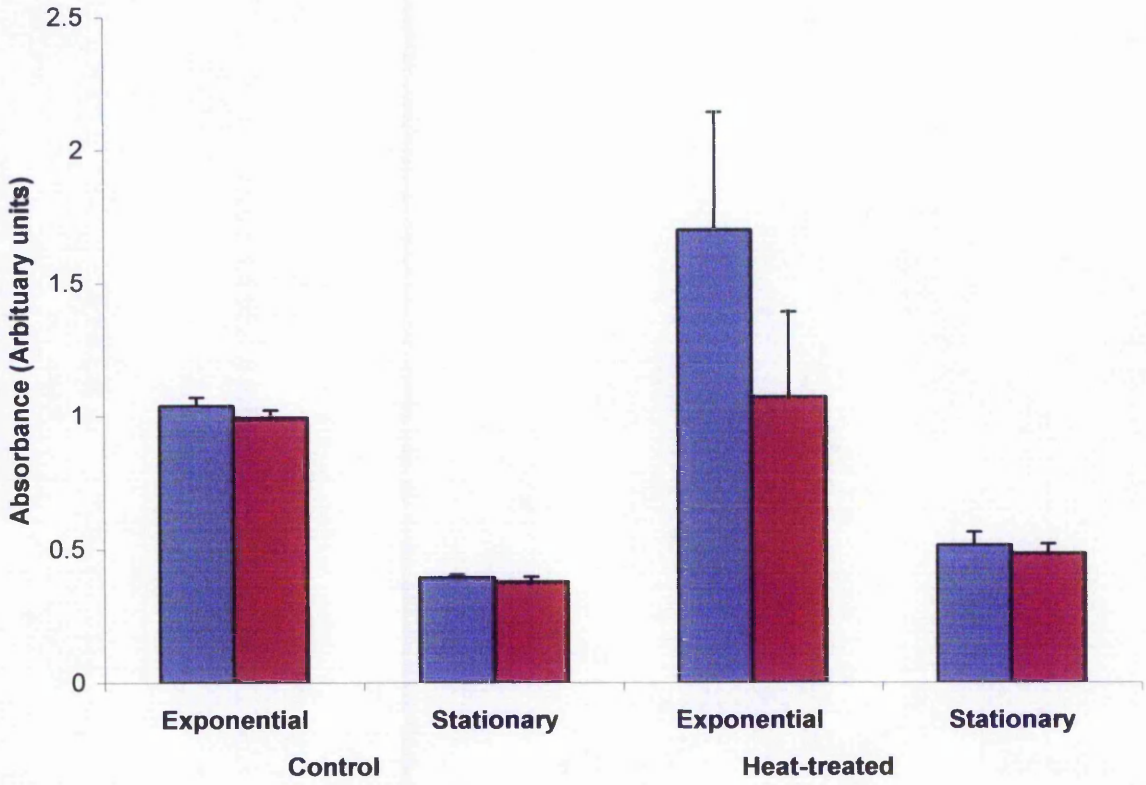


Figure 1: Cytoplasmic leakage of heat-treated exponential and stationary phase *A. butzleri* NCTC 12481. See Figure 3.10, p.145.

Error bars indicate standard error of mean where n = 3.

260 nm ( ), 280 nm ( )

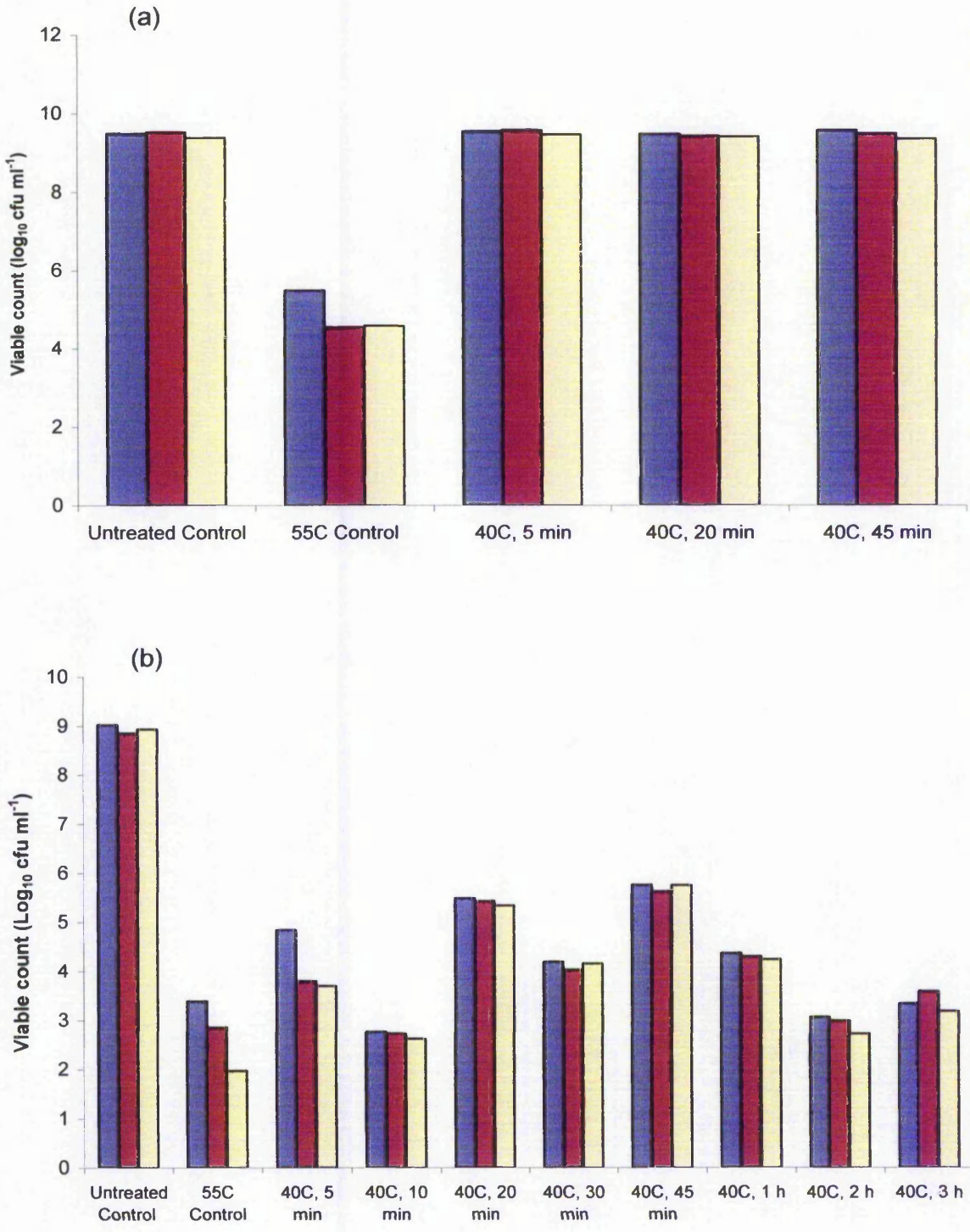


Figure 2: Heat adaptation of *A. butzleri* NCTC 12481 (a) stationary phase and (b) exponential phase by pre-exposure to 40°C.
Media: Blood agar (■), CAT (■), CCDA (□).
See Section 3.9, p.153-154.

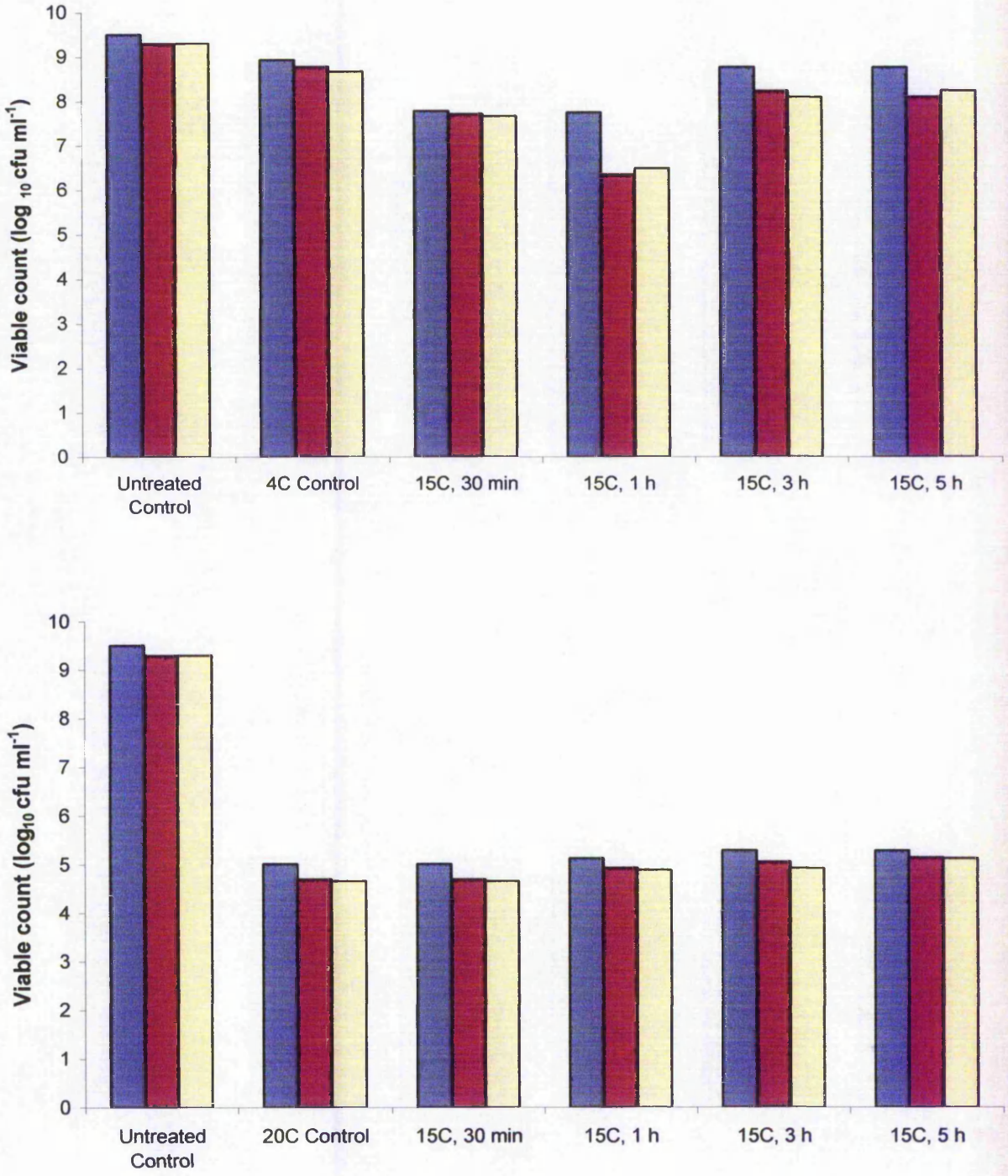
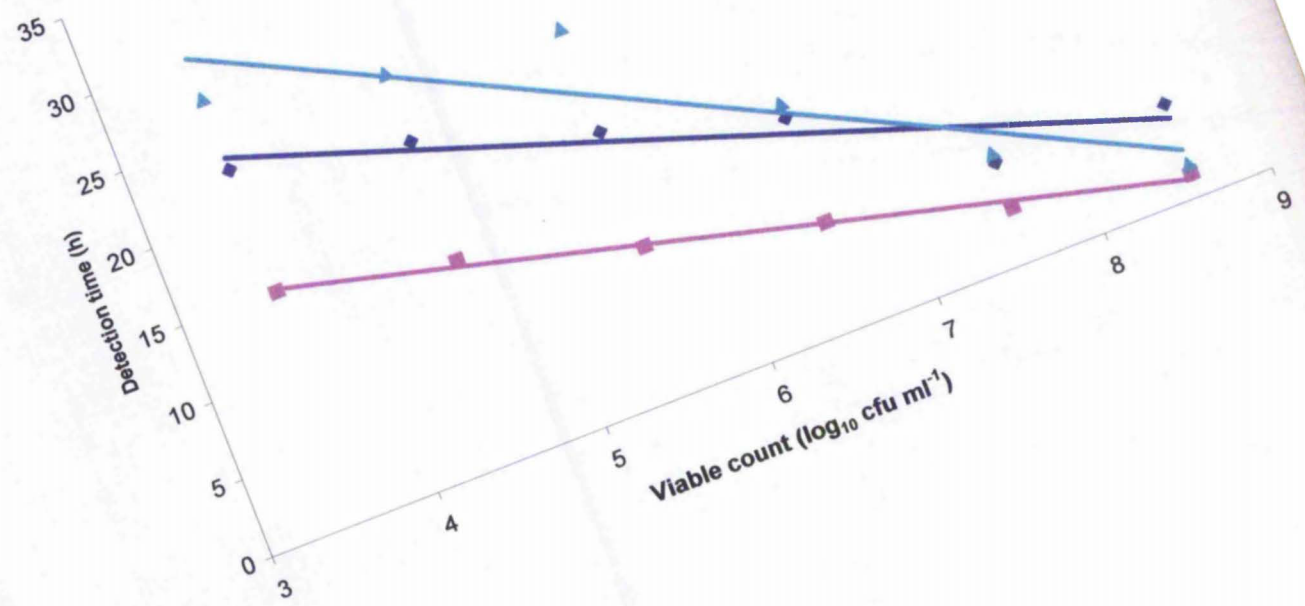
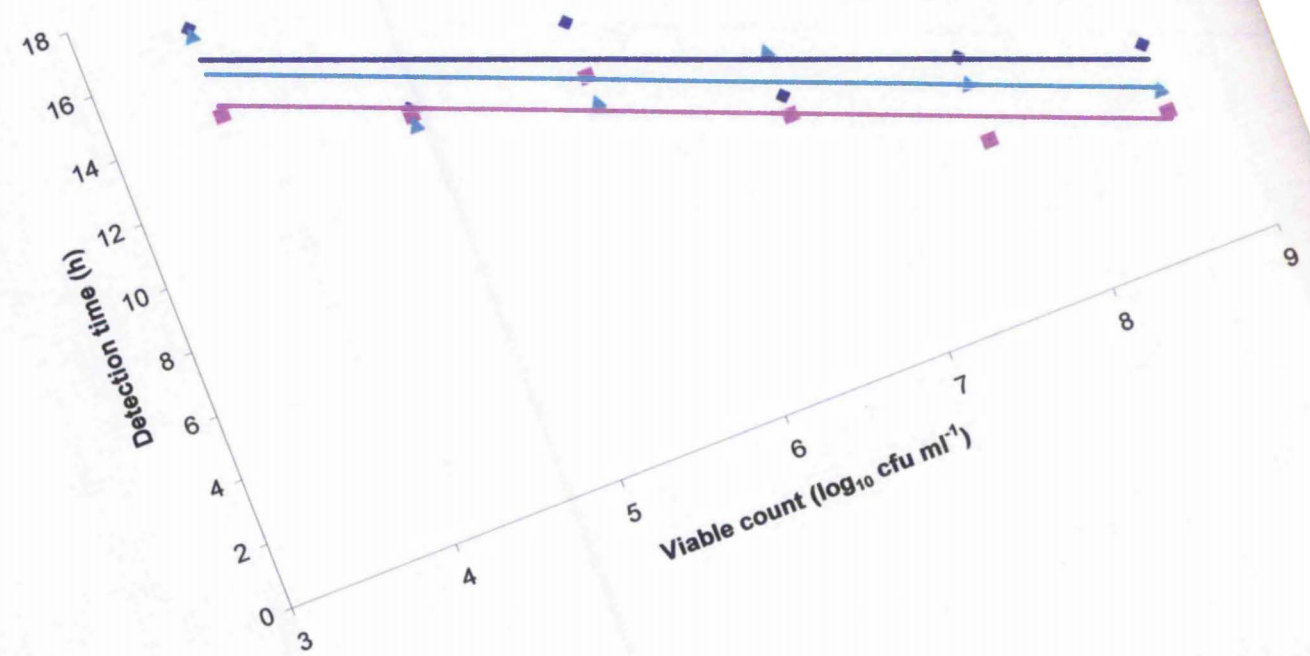


Figure 3: Cold adaptation of stationary phase *A. butzleri* NCTC 12481 cells pre-exposed at 15°C and incubated at (a) +4°C or (b) -20°C for 24 h.
Media: Blood agar (■), CAT (■), CCDA (□).
See Section 3.9, p.154-155.

(a)


Detection time (h)
0
5
10
15
20
25
30
35
3
4
5
6
7
8
9
Viable count (log_{10} cfu ml^{-1})

(b)


Detection time (h)
0
2
4
6
8
10
12
14
16
18
3
4
5
6
7
8
9
Viable count (log_{10} cfu ml^{-1})

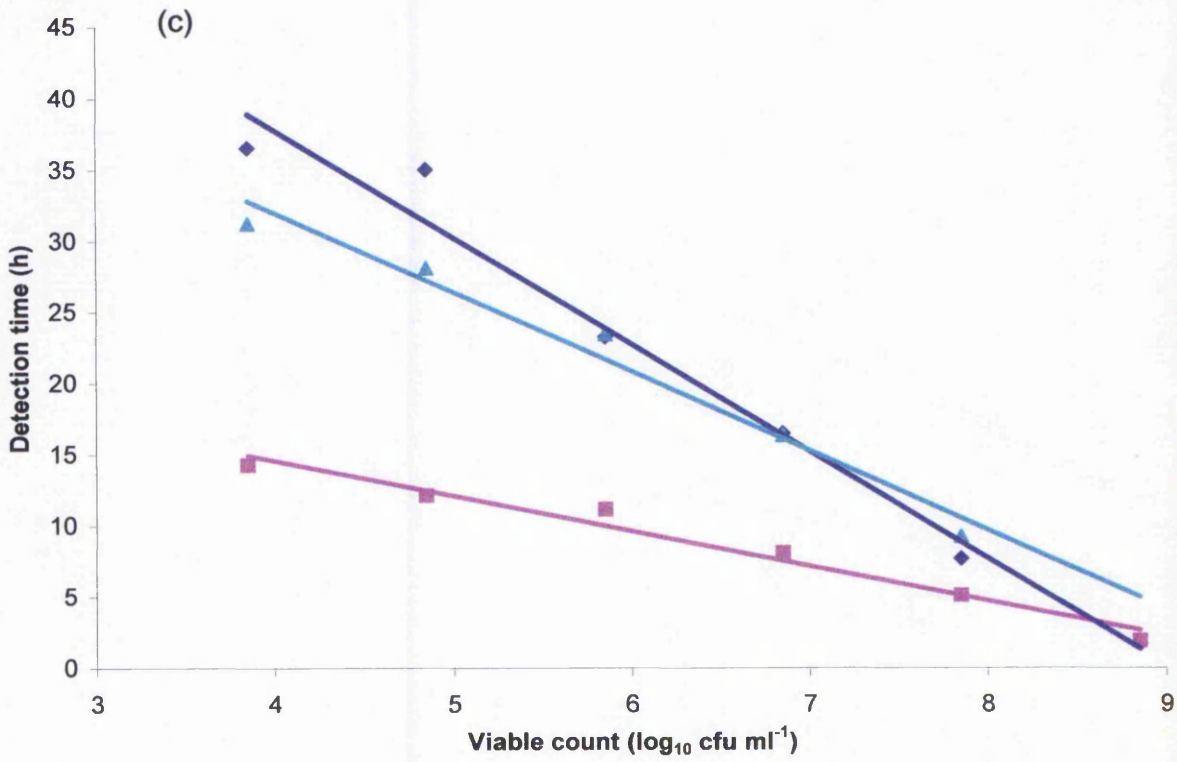


Figure 4: The effect of nitrate and nitrite on the growth of *A. butzleri* (a) Rigs 16799, (b) Rigs 15342 and (c) Rigs 1714.
Growth media: BHI broth (◆), 5 mM Nitrate broth (■), 1 mM Nitrite ▲).
See Figure 3.19, p.161.

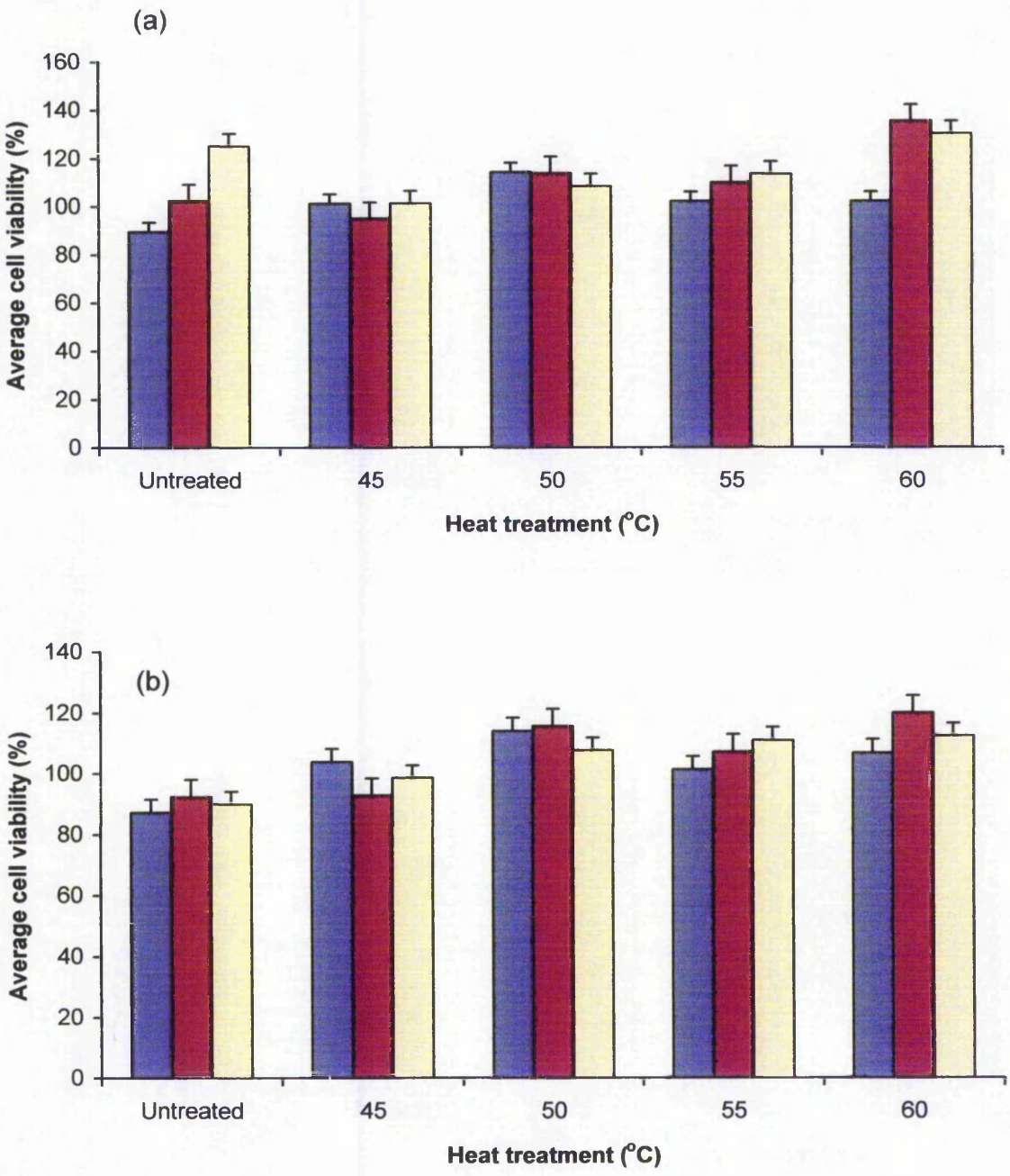


Figure 5: The effect of cell-free extract harvested from stationary phase *A. butzleri* NCTC 12481 on (a) CHO cells and (b) ECV cells. See Figure 3.20b, p.164.
Concentration of cell-free extract applied to mammalian cell lines:
2.5% (■), 5%(■) and 10%(□).
Error bars indicate standard error of mean where n = 9.

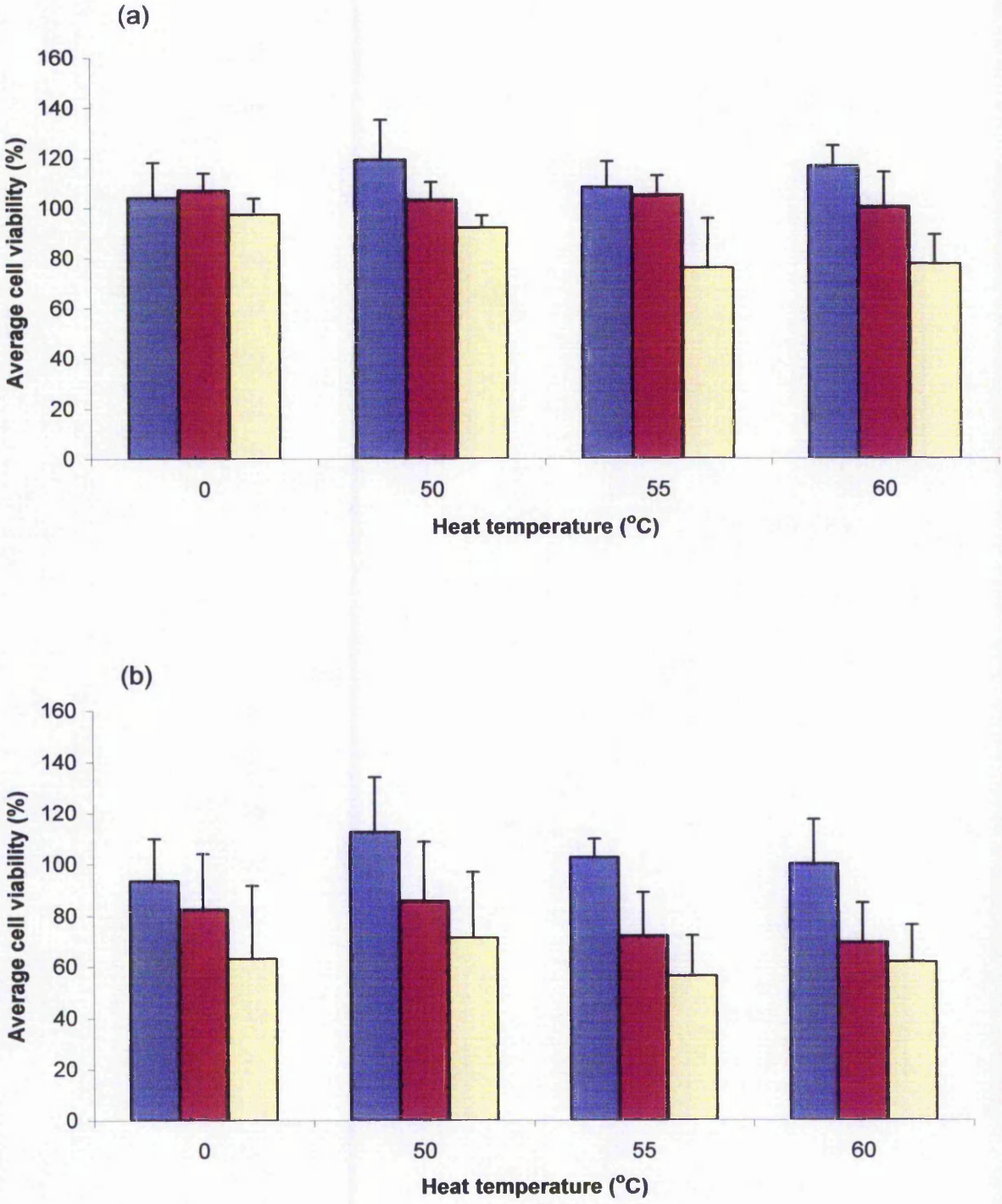


Figure 6: The effect of cell-free extract harvested from exponential phase *A. butzleri* NCTC 12481 on (a) CHO cells and (b) ECV cells. See Figure 3.20a, p.164.
Concentration of cell-free extract applied to mammalian cell lines:
2.5% (■), 5%(■) and 10%(□).
Error bars indicate standard error of mean where n = 9.

Table 1: Determination of cytotoxin location from *A. butzleri* Rigs 16799 harvested from the stationary phase. See Table 3.14, p.167.

Sample	Applied concentration	CHO		ECV		N2a	
		Not heat treated	Heat treated†	Not heat treated	Heat treated	Not heat treated	Heat treated
Spent media	2.5%‡	143.7 ± 1.2§	125.0 ± 1.6	124.2 ± 4.1	129.0 ± 3.2	85.5 ± 2.2	94.7 ± 1.33
	5%	157.0 ± 7.7	139.1 ± 5.5	125.9 ± 1.8	119.2 ± 4.6	86.0 ± 0.61	98.9 ± 3.4
	10%	155.7 ± 3.6	147.2 ± 6.8	115.7 ± 3.2	116.4 ± 1.6	104.5 ± 5.9	101.2 ± 4.9
Cell free extract	2.5%	98.7 ± 8.9	108.8 ± 6.7	99.8 ± 5.4	109.8 ± 4.8	90.1 ± 4.1	107.5 ± 3.3
	5%	90.7 ± 9.3	107.6 ± 8.1	103.0 ± 0.8	113.1 ± 2.1	98.2 ± 1.4	89.9 ± 4.9
	10%	114.2 ± 7.4	152.0 ± 3.5	119.6 ± 1.5	107.9 ± 0.2	109.0 ± 7.1	89.1 ± 6.1
Cell sonicate	2.5%	101.7 ± 2.7	105.7 ± 3.3	104.3 ± 4.1	99.5 ± 3.7	85.5 ± 1.0	87.0 ± 3.0
	5%	108.2 ± 6.3	94.4 ± 2.0	107.7 ± 3.0	98.2 ± 1.9	75.4 ± 1.1	87.5 ± 1.5
	10%	101.7 ± 1.9	93.8 ± 1.5	87.4 ± 1.1	93.8 ± 0.6	79.6 ± 1.9	85.3 ± 2.9

† = Heat-treated cells (55°C, 3 min). See Materials and Method for details.
‡ = Volume of material added to mammalian cell line (%).
§ = Standard error of mean where n = 9.

Table 2: Determination of cytotoxin location from *A. butzleri* Rigs 1714 harvested from the stationary phase. See Table 3.14, p.167.

Sample	Applied concentration	CHO		ECV		N2a	
		Not heat treated	Heat treated†	Not heat treated	Heat treated	Not heat treated	Heat treated
Spent media	2.5%‡	106.2 ± 3.9§	97.3 ± 2.0	105.3 ± 4.7	92.2 ± 4.2	113.4 ± 0.2	97.2 ± 0.74
	5%	105.6 ± 2.6	93.6 ± 1.5	101.3 ± 4.9	96.8 ± 4.2	121.9 ± 10.6	97.4 ± 4.2
	10%	89.8 ± 2.7	77.4 ± 8.9	101.0 ± 3.0	85.2 ± 3.3	92.5 ± 3.5	85.3 ± 2.3
Cell free extract	2.5%	98.7 ± 3.1	102.8 ± 2.9	103.3 ± 0.7	107.8 ± 6.0	84.9 ± 7.3	98.0 ± 4.5
	5%	103.1 ± 2.3	102.6 ± 3.7	104.7 ± 3.7	95.4 ± 4.8	92.1 ± 2.0	91.3 ± 5.1
	10%	95.5 ± 2.4	105.4 ± 1.9	104.0 ± 2.4	94.1 ± 2.3	96.7 ± 2.0	77.0 ± 4.9
Cell sonicate	2.5%	116.4 ± 3.5	111.5 ± 4.3	105.8 ± 5.1	104.9 ± 8.9	97.2 ± 5.1	108.1 ± 3.2
	5%	117.5 ± 1.7	102.9 ± 4.3	102.4 ± 6.5	95.0 ± 3.8	83.6 ± 3.4	91.5 ± 3.4
	10%	101.2 ± 0.7	102.2 ± 1.2	99.5 ± 4.7	103.9 ± 6.1	79.9 ± 1.7	102.0 ± 7.1

† = Heat-treated cells (55°C, 3 min). See Materials and Method for details.
‡ = Volume of material added to mammalian cell line (%).
§ = Standard error of mean where n = 9.

Table 3: Determination of cytotoxin location from *A. butzleri* Rigs 15342 harvested from the stationary phase. See Table 3.14, p.167.

Sample	Applied concentration	CHO		ECV		N2a	
		Not heat treated	Heat treated†	Not heat treated	Heat treated	Not heat treated	Heat treated
Spent media	2.5%‡	105.5 ± 3.8§	92.2 ± 0.7	91.3 ± 6.3	82.2 ± 4.4	104.0 ± 2.5	99.2 ± 2.46
	5%	111.1 ± 5.9	96.4 ± 1.2	97.8 ± 2.6	85.5 ± 0.6	102.3 ± 3.07	94.3 ± 0.5
	10%	96.7 ± 1.2	86.2 ± 3.4	77.6 ± 4.6	78.6 ± 5.6	100.0 ± 3.2	97.4 ± 0.2
Cell free extract	2.5%	115.6 ± 2.3	95.0 ± 3.6	102.7 ± 1.6	119.9 ± 3.8	98.3 ± 1.8	97.1 ± 6.4
	5%	112.5 ± 6.2	107.0 ± 2.7	106.1 ± 1.3	107.3 ± 2.4	95.0 ± 3.6	92.3 ± 2.1
	10%	104.5 ± 1.5	101.2 ± 6.1	118.1 ± 0.6	106.9 ± 3.0	107.0 ± 2.7	78.9 ± 1.4
Cell sonicate	2.5%	98.4 ± 3.2	96.7 ± 3.3	105.0 ± 6.0	106.0 ± 2.7	113.4 ± 0.2	97.2 ± 0.7
	5%	104.1 ± 4.9	102.8 ± 2.4	108.3 ± 6.2	102.3 ± 0.4	121.9 ± 10.7	97.4 ± 4.2
	10%	100.6 ± 4.8	97.7 ± 4.00	96.9 ± 0.7	98.6 ± 1.6	92.5 ± 3.5	85.3 ± 2.3

† = Heat-treated cells (55°C, 3 min). See Materials and Method for details.
‡ = Volume of material added to mammalian cell line (%).
§ = Standard error of mean where n = 9.

Table 4: MTT toxicity testing of spent media harvested from *A. butzleri* strains in the exponential phase. See Table 3.15, p.169.

A.butzleri strain	Applied concentration	CHO		ECV		N2a	
		Not heat treated	Heat treated†	Not heat treated	Heat treated	Not heat treated	Heat treated
NCTC 12481	2.5%‡	96.6 ± 0.8§	108.1 ± 5.6	97.3 ± 3.3	102.1 ± 4.0	86.0 ± 2.6	95.2 ± 3.9
	5%	106.9 ± 4.8	114.4 ± 5.6	107.3 ± 2.9	109.4 ± 4.7	87.6 ± 7.8	88.5 ± 5.8
	10%	98.2 ± 1.6	112.2 ± 11.5	102.8 ± 3.0	103.5 ± 1.2	84.5 ± 9.0	81.7 ± 5.6
Rigs 1714	2.5%	92.8 ± 2.0	90.6 ± 2.5	102.2 ± 2.6	96.5 ± 2.5	96.2 ± 3.5	90.5 ± 4.4
	5%	98.7 ± 3.8	89.2 ± 1.5	103.8 ± 3.9	98.4 ± 5.0	103.6 ± 2.3	102.2 ± 3.5
	10%	97.4 ± 2.5	101.4 ± 3.8	94.2 ± 5.4	93.4 ± 3.4	101.2 ± 1.1	107.9 ± 0.7
Rigs 15342	2.5%	96.5 ± 2.6	94.0 ± 1.5	99.0 ± 3.2	99.5 ± 0.3	100.4 ± 2.4	95.1 ± 4.9
	5%	104.6 ± 4.4	98.0 ± 1.3	99.0 ± 1.7	100.4 ± 2.1	85.2 ± 0.5	91.4 ± 1.0
	10%	97.3 ± 1.3	95.3 ± 1.3	100.5 ± 3.0	96.8 ± 0.2	87.5 ± 4.4	81.9 ± 2.3
Rigs 16799	2.5%	114.9 ± 6.6	106.0 ± 7.4	98.0 ± 1.5	95.0 ± 2.0	113.7 ± 1.6	101.7 ± 1.3
	5%	110.1 ± 5.1	101.8 ± 5.3	101.4 ± 3.0	93.4 ±0.6	106.0 ± 5.5	96.3 ± 3.9
	10%	81.8 ± 4.1	88.8 ± 3.7	86.3 ± 2.9	84.4 ± 2.9	101.2 ± 3.4	91.2 ± 3.2

† = Heat-treated cells (55°C, 3 min). See Materials and Method for details.
‡ = Volume of material added to mammalian cell line (%).
§ = Standard error of mean where n = 9.

Table 5: MTT toxicity testing of *A. butzleri* strains in the exponential phase, which were non heat-treated. See Table 3.15, p.169.

A.butzleri strain	Applied concentration	CHO		ECV		N2a	
		CFE¥	CS†	CFE	CS	CFE	CS
NCTC 12481	2.5%‡	_*	106.2 ± 5.6	_	113.1 ± 4.3	_	101.7 ± 3.4
	5%	_	102.6 ± 3.2	_	108.8 ± 2.7	_	87.9 ± 6.8
	10%	_	90.2 ± 1.5	_	77.6 ± 1.7	_	71.9 ± 5.6
Rigs 1714	2.5%	91.0 ± 2.6	102.8 ± 3.8	102.8 ± 2.7	89.2 ± 2.2	107.6 ± 7.8	100.8 ± 2.8
	5%	97.6 ± 4.7	105.0 ± 4.0	100.8 ± 2.9	88.8 ± 3.0	93.7 ± 5.1	97.2 ± 4.0
	10%	94.1 ± 6.7	86.8 ± 4.0	85.6 ± 2.5	90.4 ± 3.0	98.6 ± 10.9	94.7 ± 1.8
Rigs 15342	2.5%	96.5 ± 2.6	94.0 ± 1.5	99.0 ± 3.2	99.5 ± 0.3	100.4 ± 2.4	95.1 ± 4.9
	5%	104.6 ± 4.4	98.0 ± 1.3	99.0 ± 1.7	100.4 ± 2.1	85.2 ± 0.5	91.4 ± 1.0
	10%	97.3 ± 1.3	95.3 ± 1.3	100.5 ± 3.0	96.8 ± 0.2	87.5 ± 4.4	81.9 ± 2.3
Rigs 16799	2.5%	129.5 ± 4.2	116.3 ± 4.6	114.5 ± 5.3	94.3 ± 5.4	108.0 ± 5.4	98.7 ± 4.4
	5%	123.3 ± 7.5	111.1 ± 3.4	103.3 ± 2.4	99.6 ± 5.8	101.5 ± 5.0	98.4 ± 1.9
	10%	134.3 ± 8.0	126.0 ± 11.7	91.7 ± 7.1	93.6 ± 7.6	76.2 ± 7.2	89.9 ± 1.1

¥CFE = Cell-free extract from non heat-treated cells. See Materials and Method for details.
†CS = Cell sonicate from non heat-treated cells. See Materials and Method for details.
‡ = Volume of material added to mammalian cell line (%).
§ = Standard error of mean where n = 9.
* = Values presented in the Results section.

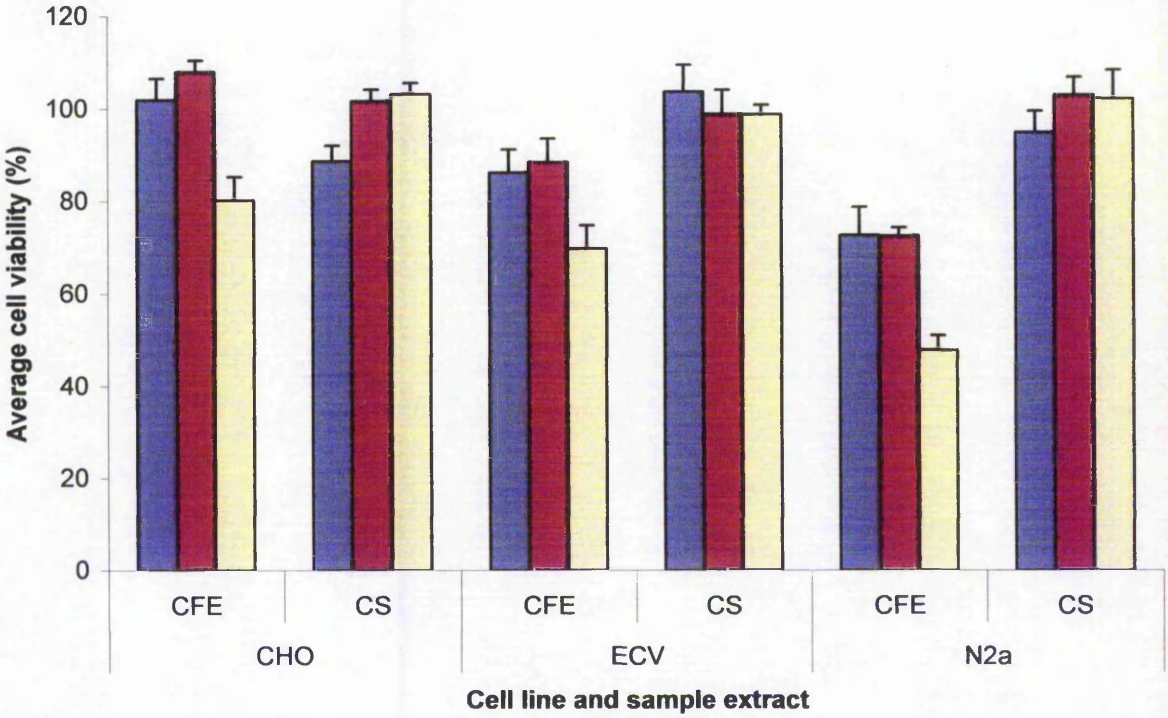


Figure 7: Cytotoxicity of heat-treated *A. butzleri* NCTC 12481 grown at 30°C under aerobic conditions (Stationary phase). See Figure 3.21a, p.173.
Concentration of cell-free extract applied to mammalian cell lines:
2.5% (■), 5%(■) and 10%(□).
Error bars indicate standard error of mean where n = 9.

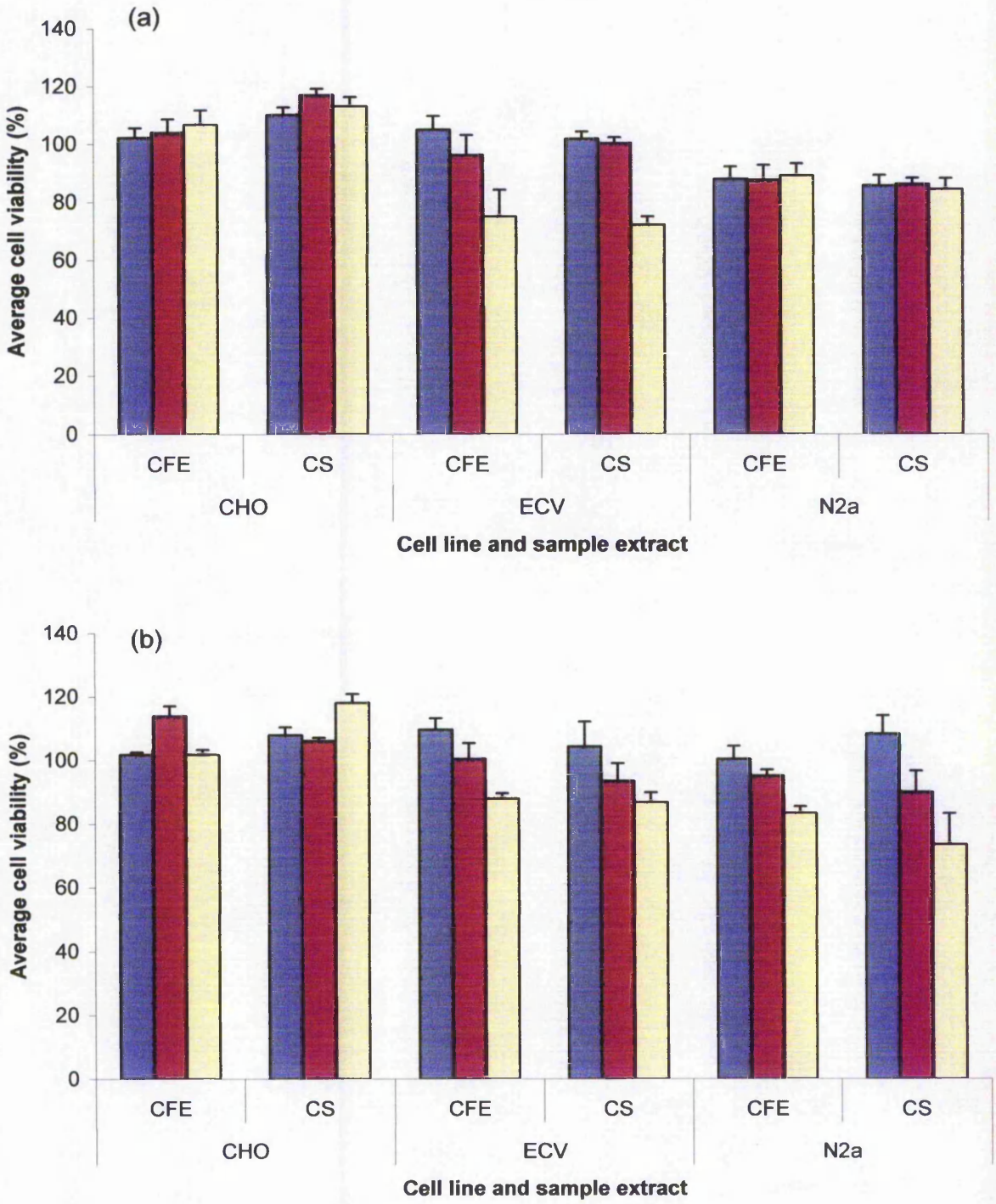


Figure 8: Cytotoxicity of non heat-treated *A. butzleri* NCTC 12481 grown at 30°C under aerobic conditions (a) exponential phase and (b) Stationary phase. See Figure 3.21a, p.173.
Concentration of cell-free extract applied to mammalian cell lines:
2.5% (■), 5%(■) and 10%(□).
Error bars indicate standard error of mean where n = 9.

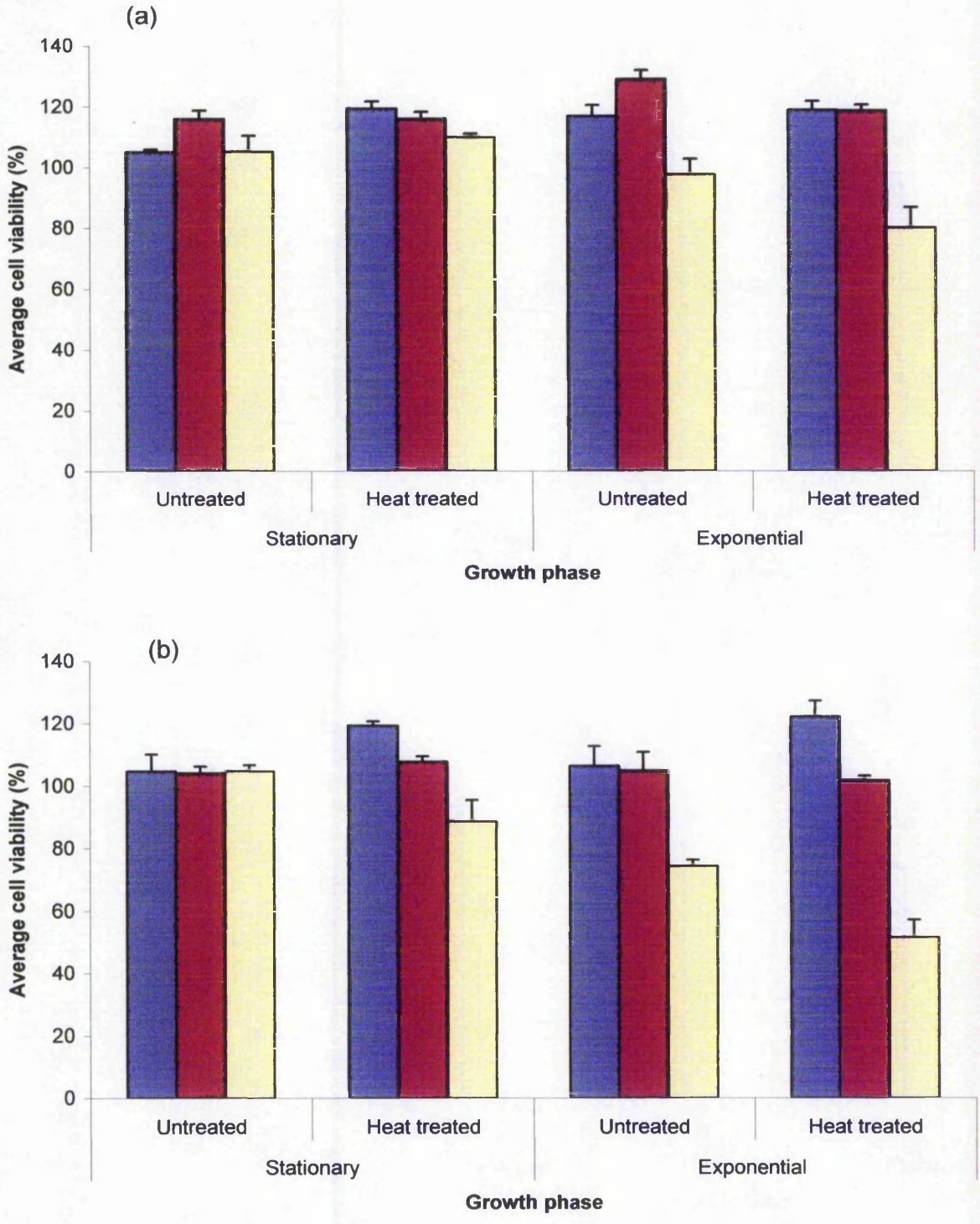


Figure 9: Cytotoxicity towards (a) CHO and (b) ECV cell lines of *A. butzleri* NCTC 12481 grown at 37°C under microaerophilic conditions. See Figure 3.21b, p.173. Concentration of cell-free extract applied to mammalian cell lines:
2.5% (■), 5%(■) and 10%(□).
Error bars indicate standard error of mean where n = 9.

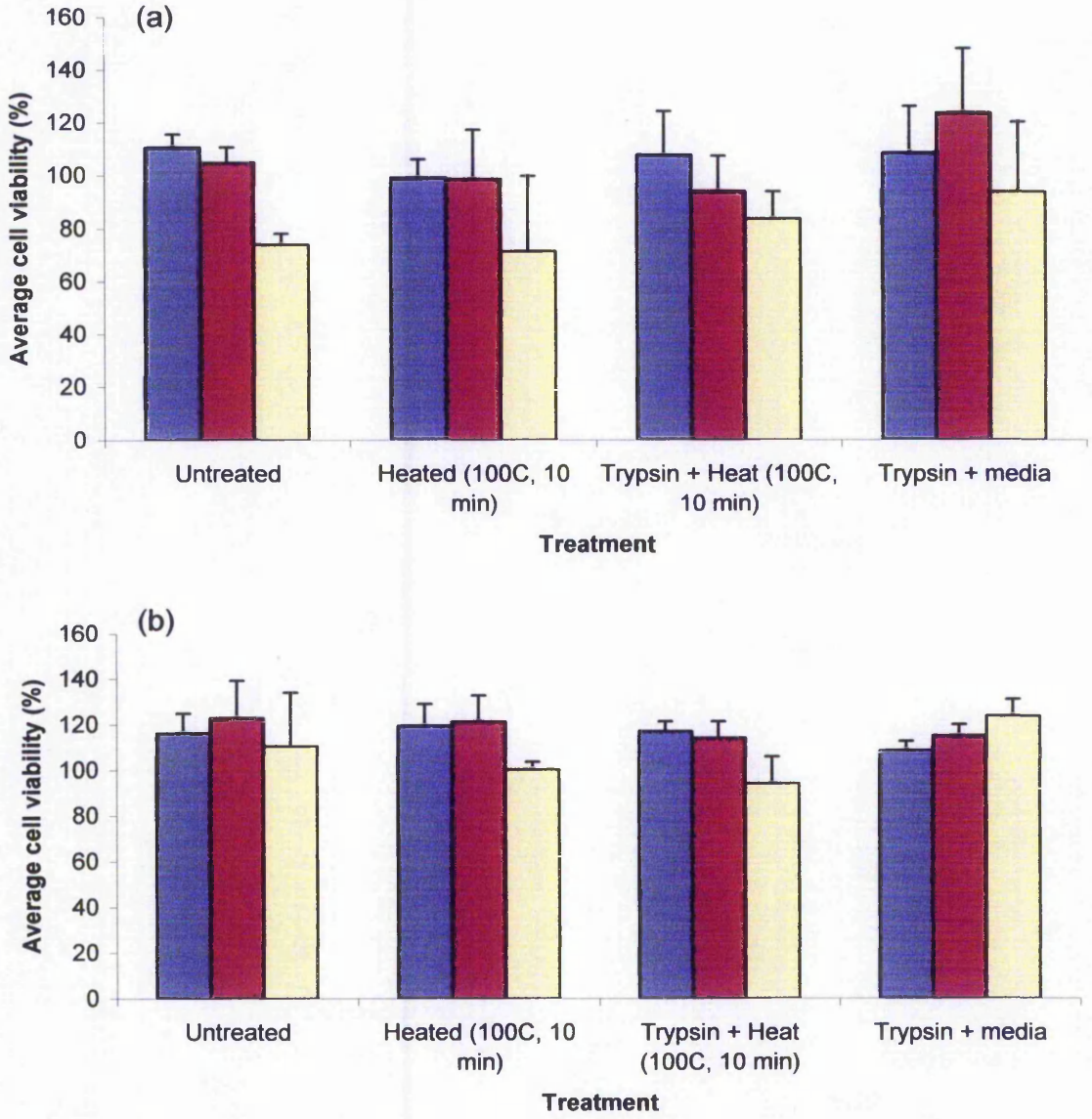
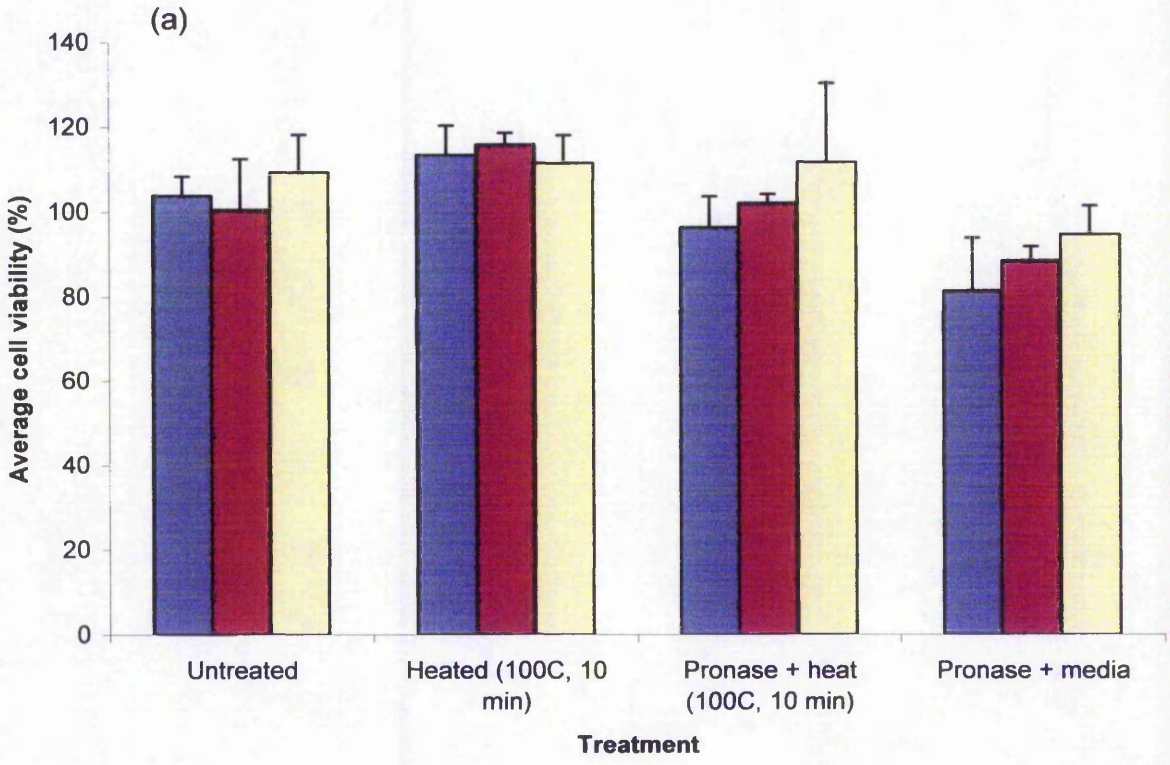
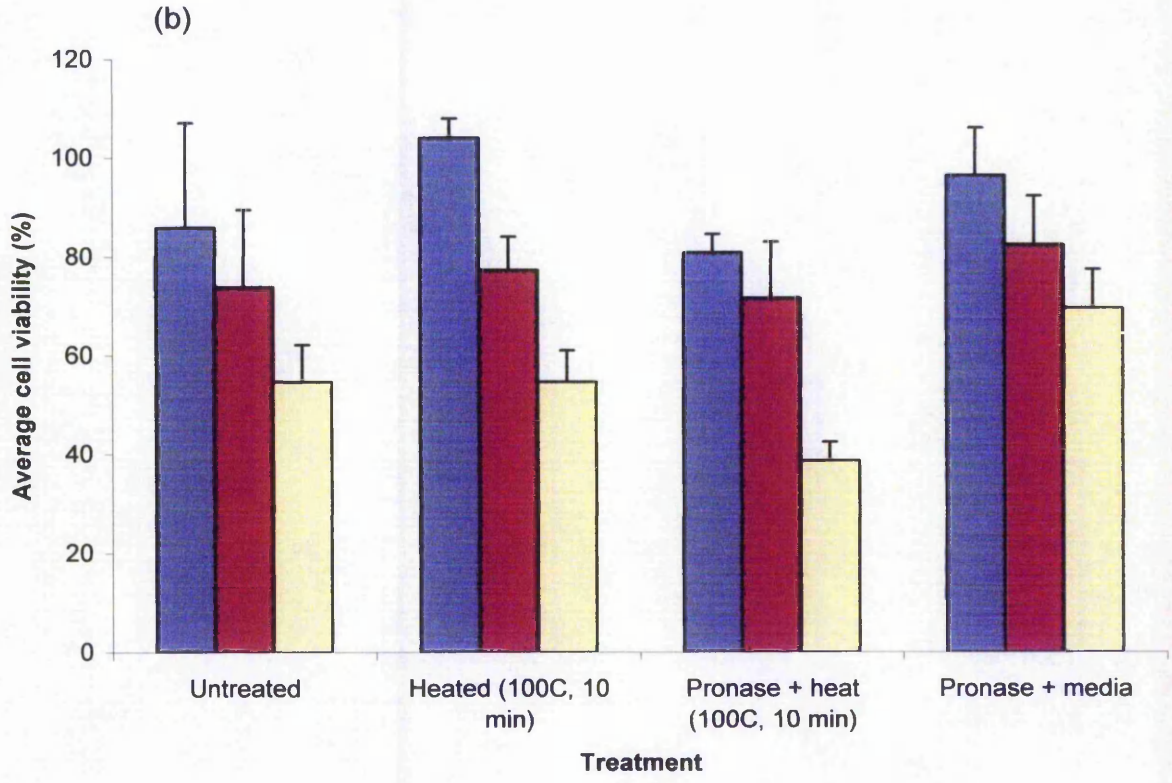


Figure 10: Heat and proteolytic (Trypsin) stability of cytotoxin(s) from *A. butzleri* NCTC 12481 on (a) CHO cells and (b) ECV cells.
Untreated control = Heat-treated (55°C, 3 min) CFE.
Heated = Heat-treated (55°C, 3 min) CFE followed by boiling (100°C) for 10 minutes. Trypsin + heat = Heat-treated (55°C, 3 min) CFE followed by incubation (30 min) with 0.25% Pronase which was deactivated by boiling (100°C) for 10 minutes. Trypsin + media = Heat-treated (55°C, 3 min) CFE followed by incubation (30 min) with 0.25% Pronase which was neutralised by mixing with DMEM media containing 10% foetal calf serum.
Concentration of cell-free extract applied to mammalian cell lines:
2.5% ( ), 5%( ) and 10%( ).
Error bars indicate standard error of mean where n = 9. See Figure 3.22 p.174.


(a)
140
120
100
80
60
40
20
0
Average cell viability (%)
Untreated
Heated (100C, 10 min)
Pronase + heat (100C, 10 min)
Pronase + media
Treatment


(b)
120
100
80
60
40
20
0
Average cell viability (%)
Untreated
Heated (100C, 10 min)
Pronase + heat (100C, 10 min)
Pronase + media
Treatment

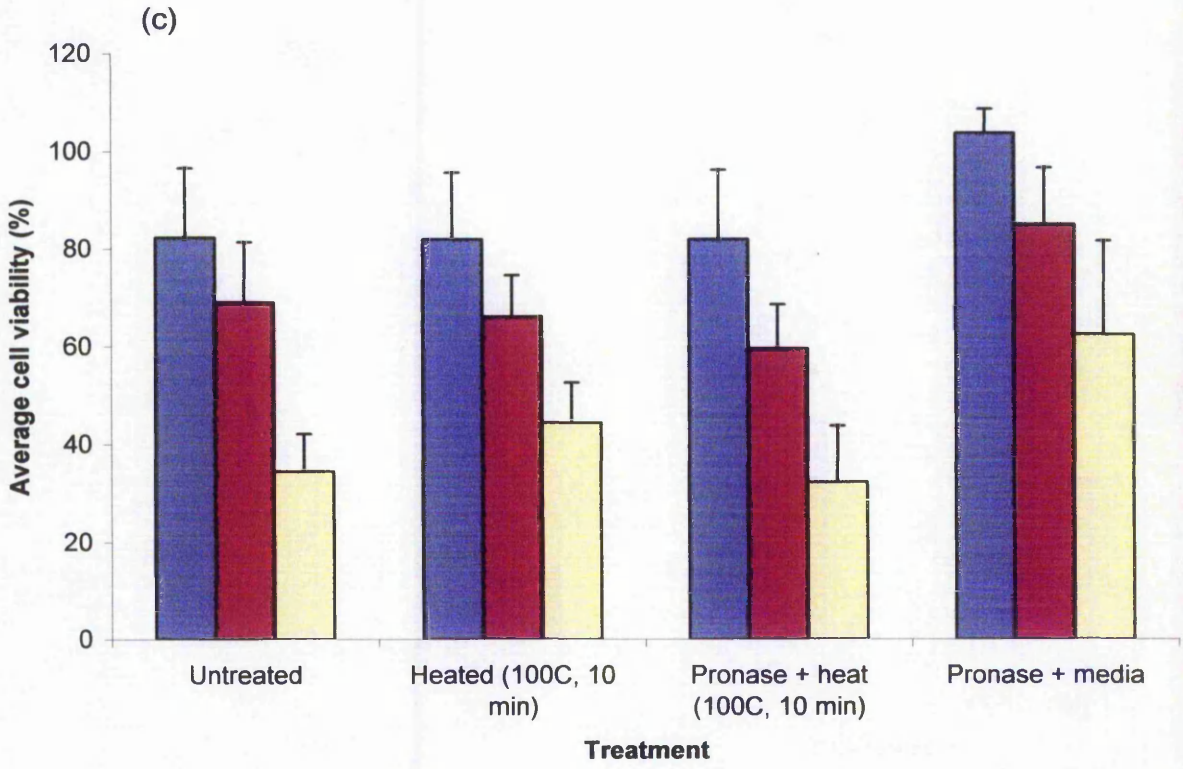


Figure 11: Heat and proteolytic (Pronase) stability of cytotoxin(s) from *A. butzleri* NCTC 12481 on (a) CHO cells, (b) ECV cells and (c) N2a cells.
Untreated control = Heat-treated (55°C, 3 min) CFE.
Heated = Heat-treated (55°C, 3 min) CFE followed by boiling (100°C) for 10 minutes.
Pronase + heat = Heat-treated (55°C, 3 min) CFE followed by incubation (30 min) with 0.25% Pronase which was deactivated by boiling (100°C) for 10 minutes.
Pronase + media = Heat-treated (55°C, 3 min) CFE followed by incubation (30 min) with 0.25% Pronase which was neutralised by mixing with DMEM media containing 10% foetal calf serum.
Concentration of cell-free extract applied to mammalian cell lines:
2.5% ( ), 5%( ) and 10%( ).
Error bars indicate standard error of mean where n = 9.
See Figure 3.22, p174.

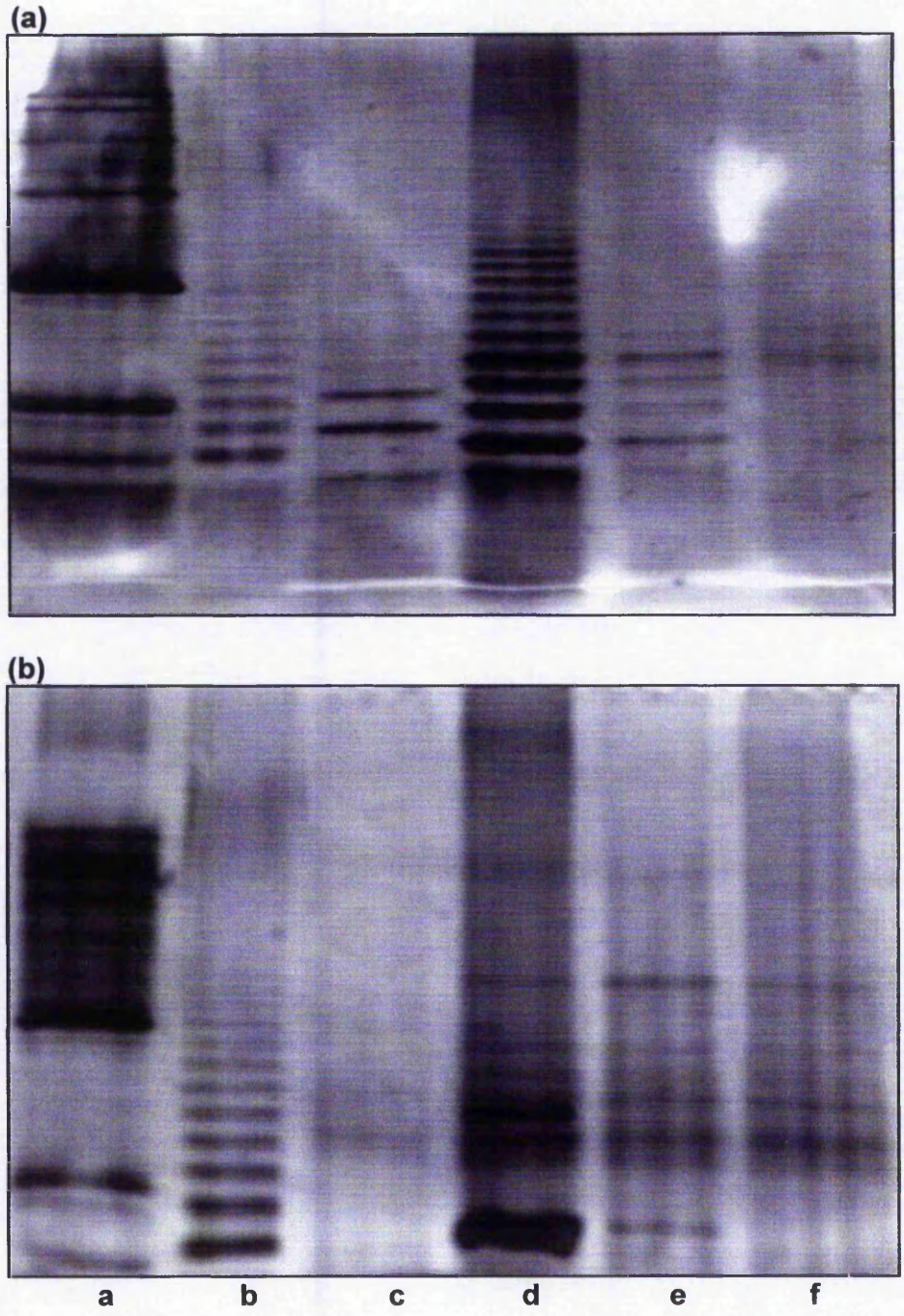


Figure 12: Detection of lipopolysaccharide fractions from *A. butzleri* (a) Rigs 1714 and (b) Rigs 16799. See Section 3.12.4, pp.179-181.
Arcobacter heat-treated and non heat-treated cell-free extracts prepared from exponential phase (c, d) and stationary phase (e, f) cells prior to separation by polyacrylamide gel electrophoresis as described in Material and Methods. Shown is a silver stained gel of lipopolysaccharide from (b) *Salmonella* Typhimurium (marker), (c) cell-free extract from non heat-treated exponentially growing cells, (d) cell-free extract from heat-treated exponentially growing cells, (e) cell-free extract from non heat-treated stationary phase cells, (f) cell-free extract from heat-treated stationary phase cells. Lane (a) shows silver stain molecular marker used to check the staining procedure.

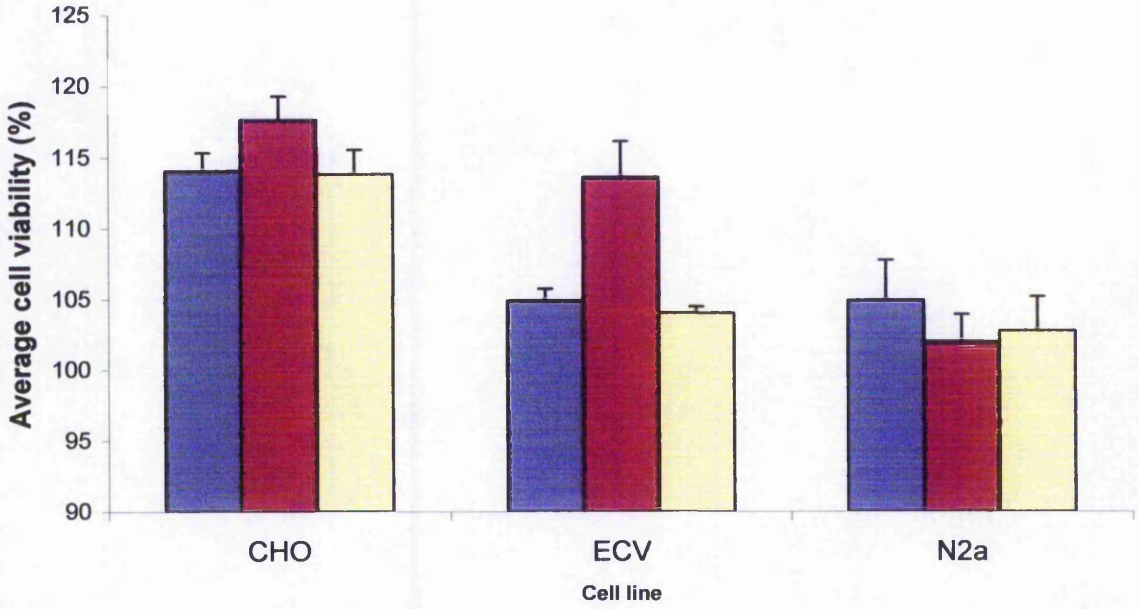


Figure 13: Cytotoxicity of LPS extracted from untreated cell-free extracts of *A. butzleri* NCTC 12481 in the exponential phase.
Concentration of LPS extract applied to mammalian cell lines:
2.5% (■), 5% (■) and 10% (□).
Error bars indicate standard error of mean where n = 9.
See Figure 3.27 a & b, p.183.

Table 6: Morphological changes induced by the presence of the cell-free extract from non heat-treated *A. butzleri* NCTC 12481 (Exponential phase) after forty eight hours. See Table 3.16, p.185.

Cell free extract (%)	CHO morphologies			ECV morphologies		
	Round†	Flat	Elongated	Round	Flat	Process
2.5%	13.3	59.4	28.7	14.4	49.3	36.3
2.5% Control	20.7	51.8	27.3	21.0	50.3	22.8
10%	14.8	63.9	21.3	19.7	58.6	21.7
10% Control	13.4	65.7	28.2	19.8	52.4	27.3

† = For a more detailed description on cell line morphologies see Materials and Methods Section 2.18.7.

Table 7: Morphological changes induced by the presence of the cell-free extract from heat-treated *A. butzleri* NCTC 12481 (Exponential phase) after twenty four hours. See Table 3.16, p.185.

Cell free extract (%)	CHO morphologies			ECV morphologies		
	Round†	Flat	Elongated	Round	Flat	Process
2.5%	9.5	69.7	18.8	14.7	67.3	23.8
2.5% Control	11.9	63.9	24.5	13.1	71.2	15.8
10%	9.6	73.5	16.9	14.0	62.2	23.8
10% Control	19.3	53.0	25.8	13.3	70.5	14.2

† = For a more detailed description on cell line morphologies see Materials and Methods Section 2.18.7.

Table 8: Morphological changes induced by the presence of the cell-free extract from non heat-treated *A. butzleri* NCTC 12481 (Exponential phase) after twenty four hours. See Table 3.16, p.185.

Cell free extract (%)	CHO morphologies			ECV morphologies		
	Round†	Flat	Elongated	Round	Flat	Process
2.5%	10.5	74.8	15.3	8.5	63.3	28.2
2.5% Control	10.9	69.6	19.5	15.0	66.3	18.6
10%	9.1	73.9	17.3	9.4	56.6	34.0
10% Control	15.7	62.7	21.6	16.2	56.6	23.8

† = For a more detailed description on cell line morphologies see Materials and Methods Section 2.18.7.

Table 9: Morphological changes induced by the presence of the cell-free extract from heat-treated *A. butzleri* NCTC 12481 (Stationary phase) after either twenty four or forty eight hours. See Table 3.16, p.185.

Exposure time (h)	Cell free extract (%)	CHO morphologies			ECV morphologies		
		Round†	Flat	Elongated	Round	Flat	Process
24	2.5%	13.9	54.0	32.0	28.6	44.3	26.6
	2.5% Control	17.9	49.7	33.1	27.5	48.7	23.3
	10%	14.8	62.1	23.7	26.1	53.8	20.3
	10% Control	14.0	54.4	31.4	30.1	50.2	19.7
48	2.5%	15.6	60.7	23.8	20.7	42.1	37.2
	2.5% Control	14.9	60.2	25.0	25.0	40.3	34.7
	10%	17.7	55.0	27.5	22.8	40.5	36.6
	10% Control	15.0	60.5	23.3	26.7	40.5	32.2

† = For a more detailed description on cell line morphologies see Materials and Methods Section 2.18.7.

Table 10: Morphological changes induced by the presence of the cell-free extract from non heat-treated *A. butzleri* NCTC 12481 (Stationary phase) after either twenty four or forty eight hours. See Table 3.16, p.185.

Exposure time (h)	Cell free extract (%)	CHO morphologies			ECV morphologies		
		Round†	Flat	Elongated	Round	Flat	Process
24	2.5%	12.3	60.6	27.2	18.4	51.4	30.9
	2.5% Control	15.0	59.2	26.1.1	23.5	47.2	28.0
	10%	17.6	57.3	24.7	22.9	48.9	28.2
	10% Control	20.0	50.3	29.7	29.2	44.2	26.9
48	2.5%	20.7	56.1	23.0	17.5	48.7	33.8
	2.5% Control	16.7	61.2	23.8	20.7	40.1	39.2
	10%	19.0	59.0	22.4	22.8	41.1	37.6
	10% Control	19.6	54.5	25.9	20.0	39.0	41.0

† = For a more detailed description on cell line morphologies see Materials and Methods Section 2.18.7.

Table 11: Frequency of neurite outgrowth by cell-free extract of *A. butzleri* NCTC 12481 in exponential phase. See Figure 3.30, p.187.

Treatment	Applied concentration (%)	Frequency of neurite outgrowth after 24 h (%)		Frequency of neurite outgrowth after 48 h (%)	
		Bacterial CFE	PBS control	Bacterial CFE	PBS control
Untreated	2.5	19.2	26.0	18.5	38.5
	10	23.0	33.5	15.1	34.1
Heat treated	2.5	23.1	52.9	-*	-
	10	29.6	48.7	-	-

* = Values presented in the Results section. See Figure 3.26.

Table 12: Frequency of neurite outgrowth by cell-free extract of *A. butzleri* NCTC 12481 in stationary phase. See Figure 3.30, p.187.

Treatment	Applied concentration (%)	Frequency of neurite outgrowth after 24 h (%)		Frequency of neurite outgrowth after 48 h (%)	
		Bacterial CFE	PBS control	Bacterial CFE	PBS control
Untreated	2.5	41.4	47.6	27.7	30.7
	10	36.9	43.7	23.5	35.5
Heat treated	2.5	41.3	50.2	42.2	49.7
	10	28.2	34.3	26.3	33.0

Table 13: MTT toxicity testing of *A. butzleri* NCTC 12481 cell-free extracts in the morphology study after 24 h (Exponential phase). See Table 3.17, p. 188.

Cell line	Applied concentration (%)	Untreated CFE	Heat treated CFE
CHO	2.5%	117.0 ± 2.2	123.9 ± 2.3
	10%	133.0 ± 2.4	107.6 ± 11.1
ECV	2.5%	109.8 ± 3.6	110.7 ± 6.0
	10%	100.8 ± 4.2	93.3 ± 1.7
N2a	2.5%	100.0 ± 10.2	107.8 ± 5.5
	10%	77.3 ± 10.2	111.7 ± 5.7

Table 14: MTT toxicity testing of *A. butzleri* NCTC 12481 cell-free extracts in the morphology study after 24 h (Stationary phase). See Table 3.17, p.188.

Cell line	Applied concentration (%)	Untreated CFE	Heat treated CFE
CHO	2.5%	110.3	118.5
	10%	125.8	71.2
ECV	2.5%	105.5	94.5
	10%	111.9	89.1
N2a	2.5%	115.6	104.1
	10%	106.3	107.4

Table 15: MTT toxicity testing of *A. butzleri* NCTC 12481 cell-free extracts in the morphology study after 48 h (Stationary phase). See Table 3.17, p.188.

Cell line	Applied concentration (%)	Untreated CFE	Heat treated CFE
CHO	2.5%	111.4	113.4
	10%	121.4	112.7
ECV	2.5%	106.9	114.2
	10%	134.0	120.4
N2a	2.5%	103.6	85.7
	10%	83.9	110.3