RISK FACTORS ASSOCIATED WITH THE EXPOSURE OF NEONATES TO ACINETOBACTERS THROUGH THE INGESTION OF INFANT FORMULA IN NICUs

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ABSTRACT

Acinetobacter is gaining importance as a pathogen in intensive care units (ICUs) due to the numerous outbreaks reported and the severity of the infections caused. Of particular interest in this project are neonates whose immune systems have not fully developed and affected by their prematurity, low birth weight, or various other conditions. The vulnerability of these neonates combined with the multidrug resistance of the clinical isolates of this organism poses a real threat to neonates. The understanding of its pathogenicity is still at an elementary stage despite the clinical evidence of nosocomial infections, and the concern of the FAO-WHO (2006) regarding neonatal health due to the consumption of powdered infant formula (PIF). The ingestion of contaminated infant formula is a particularly unexplored mechanism of acquiring *Acinetobacter* infections. Therefore, this research work aimed to analyse the potential risk factors associated with the consumption of a contaminated infant formula administered enterally. The detection of the organism in PIF was examined. A selective chromogenic medium was designed, primarily for this purpose, and evaluated against other specific media. In addition, the desiccation persistence in infant formula was assessed. Biofilm formation inside nasogastric feeding tubes, survival in gastrointestinal fluids, and subsequent interactions with the host, as consequences of the ingestion of a feed containing *Acinetobacter*, were also investigated. Strains were finally screened for the presence of phospholipase genes. Based on the main findings, *Acinetobacter* showed the ability to persist over an extended storage period (2 years) in desiccated infant formula and recover after reconstitution. Bacterial cells in the contaminated formula formed biofilms and multiplied over time inside enteral feeding tubes causing all the subsequent fresh feeds to be also contaminated. When exposed to simulated gastrointestinal fluids, these cells did not show signs of complete viability loss except at an acidity level of pH 2.5 (lower than the normal neonatal stomach pH). Strains also successfully attached to colonic epithelial cells (Caco-2), and the majority were subsequently able invade the host cells. Twenty-four hour survival within the Caco-2 cells was also shown. Furthermore, *Acinetobacter* demonstrated the capacity to evade the bactericidal activity of macrophages, persist, and moreover multiply within these phagocytic cells. Genes encoding for the cytotoxic phospholipase enzymes were also detected in all the clinical isolates of this organism. In conclusion, *Acinetobacter* was established to possess multiple virulence factors that can potentially contribute to its pathogenicity once ingested.

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ABBREVIATIONS

AIF	Apoptosis inducing factor
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
ARDRA	Amplified 16S ribosomal DNA restriction analysis
ASP	Acid shock protein
ATCC	American type culture collection
ATR	Acid tolerance response
BAA	Benzyl-arginine arylamidase
BB	Baumann broth
BIMP	Bacterial integral membrane proteins
BPW	Buffered peptone water
CAC	Codex Alimentarius Commission
CCUG	Culture collection of the University of Göteborg
CFU	Colony forming unit
CHDL	Carbapenem-hydrolysing oxacillinase
COSHH	Control of substances hazardous to health
DC	Dendritic cell
DDA	Druggan-Dimmer agar
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSM	Deutsche sammlung von mikroorganismen
DT	Doubling time
EAEC	Enteroaggregative *Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EE	Enterobacteriaceae enrichment
EFSA	European Food Safety Authority
ESBL	Extended-spectrum β-lactamase
FH	Factor H
FAO	Food and Agriculture Organisation
FBS	Fetal bovine serum

FDA	Food and Drug Administration
IAMCC	Institute of Applied Microbiology culture collection
ICU	Intensive care unit
IF	Infant formula
IROMP	Iron regulated outer membrane proteins
LAM	Leeds *Acinetobacter* medium
LPS	Lipopolysaccharide
MATE	Multidrug and toxic compound extrusion
MBL	Metallo-β-lactamase
MDR	Multidrug resistance
MEM	Minimum essential medium
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
MOI	Multiplicity of infection
MPN	Most probable number
MTT	Methyl-thiazolyldiphenyl-tetrazolium
NA	Nutrient agar
NCDO	National collection of dairy organisms
NCIMB	National collection of industrial and marine bacteria
NCTC	National collection of type cultures
N-AHL	N-acylhomoserine lactones
NHS	Normal human serum
NICU	Neonatal intensive care unit
NIPH	National Institute of Public Health
NLS	Nuclear localization signal
NTUCC	Nottingham Trent University culture collection
OCC	Oxoid culture collection
OD	Optical density
OmpA	Outer membrane protein A
OMV	Outer membrane vesicle
PBS	Phosphate buffered saline
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction

PH	Potential hydrogen
PIF	Powdered infant formula
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PR	Percentage recovery
QMC	Queen's Medical Centre
RABIT	Rapid automated bacterial impedance technique
RND	Resistance-nodulation-cell Division
ROS	Reactive oxygen species
SEIP	Service des Enterobactbries de 1'Institut Pasteur
SIRS	Systematic inflammatory response syndrome
SM-TSA	Skimmed milk-tryptic soy agar
TAE	Tris-acetate EDTA
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TTD	Time to detection
VRBGA	Violet red bile glucose agar
WHO	World Health Organization

CHAPTER 1. GENERAL MATERIALS & METHODS

1.1 Safety considerations

The experiments were carried out in compliance with the Health and Safety Code of Practice for Microbiology containment level 2. Risks associated with all the materials and procedures described in this project were assessed according to the Control of Substances Hazardous to Health (COSHH) regulations.

1.2 Bacterial cultures

All the bacterial strains used in this project, their sources, and culture collection designations are listed in **Table 1.1**. The relevant research studies applied for these strains are also indicated.

1.3 Stock culture preparation and storage

All the strains were grown overnight on tryptic soy agar (TSA) and then mixed with Microbank^{TM} cryobeads (Pro-LAB Diagnostics, PL.170) for long-term storage at -70^{o}C. Replicate stock cultures were also kept at -20^{o}C in 4% glycerol broth.

1.4 Identification and characterization of bacteria

1.4.1 Gram staining

Bacterial smears were prepared by emulsifying a portion of a colony in sterile distilled water on a dry clean slide. Smears were air dried, and then passed through a Bunsen burner flame. Heat-fixed smears were flooded with 1% crystal violet and left for 2½ min. Next, they were rinsed with tap water before they were flooded with 5% iodine and left for 2 min. Excess iodine was removed by rinsing with tap water. Decolorization step was then carried out using 50:50% ethanol:acetone, which was removed by tap water afterwards. Finally, the smears were counterstained with 2% safranin for 10 sec, then rinsed and blot dried with blotting paper. For visualization under the microscope, cells were covered with immersion oil and examined at the magnification of 1000x. Gram-positive bacterial cells appeared blue/purple, whereas the Gram-negative cells appeared pink/red.

1.4.2 Oxidase test

This was performed in accordance with the manufacturer's instructions (Fluka Analytical, 40560). Several bacterial colonies were smeared using a sterile loop on a paper saturated with alpha-naphthol and N,N-dimethyl-1,4-phenylene diamine. A positive oxidase reaction was determined by development of a purple colour in approximately 10 sec. *Pseudomonas aeruginosa* ATCC 15442 was used as a positive control.

1.4.3 Identification using biochemical profiles

API 20E (bioMerieux, 20120) and API 20NE kit (bioMerieux, 20050) were used to phenotypically identify presumptive Enterobacteriaceae as well as non-glucose fermentative isolates recovered on VRBGA respectively. Suspensions of overnight cultures in saline (Oxoid, BR0053G) were adjusted to 0.5 MacFarland standard then used to inoculate the standardized biochemical test strips. Inoculation, incubation, and interpretation were all carried out as according to the manufacturer's instructions. Identification profiles were obtained via Apiweb™ online database (bioMerieux SA, France).

1.4.4 Identification using 16S rDNA gene sequencing

FTA® Elute Micro Cards (Whatman International Ltd., WB120410) were used to send DNA samples of the bacteria to Accugenix Inc. (Newark, USA) for partial 16S rDNA gene sequencing. Colonies from overnight cultures on TSA plates were suspended in phosphate buffered saline (PBS). Forty microliter volumes of this suspension were dispensed onto FTA Elute Micro Cards, and then allowed to dry completely for at least 3h at room temperature before sealing. The cards were stored at room temperature prior to dispatch.

1.5 Enumeration of bacterial cells

1.5.1 Miles and Misra method

Bacterial cultures were serially diluted, then 20µl of the dilutions were placed on air dried TSA. This was done in triplicate and the average number of colonies between 3 and 30 was calculated following incubation. This average was then multiplied by both 50 and the dilution factor in order to determine the number of colony forming units per ml of the original sample.

1.5.2 Spread plate method

Serially diluted cultures (0.1ml) were pipptted onto the surface of triplicate agar plates and spread using a sterile glass spreader. The samples were allowed to soak in and then incubated. Only the plates that had colonies between 30 and 300 colonies were selected for the calculation. The number of colony forming units per ml of the sample was averaged and finally divided by the relevant dilution factor.

1.6 Preparation of media

1.6.1 Baumann broth (BB)

This medium was designed by Baumann (1968) for the enrichment of *Acinetobacter* isolates from water and soil. It contained 2g/l KNO_3 (Sigma-Aldrich, P6083), 2g/l CH_3COONa (Sigma-Aldrich, S8750), and 0.2g/l $MgSO_4.7H_2O$ (Sigma-Aldrich, 63138), which were added to 0.04M KH_2PO_4-Na_2HPO_4 buffer (Sigma-Aldrich, P5655-S7907) (pH 6.0), and 20ml/l Hutner's vitamin free mineral base.

The mineral base was described by Cohen-Bazire *et al.* (1957) and consisted of 10g/l $N(CH_2COOH)_3$ (Sigma-Aldrich, N9877) which was dissolved in distilled water and neutralized with 7.3g of KOH (Sigma-Aldrich, 221473) before adding the remaining salts. These were 14.45g/l $MgSO_4.7H_2O$, 3.34g/l $CaCl_2.2H_2O$ (Sigma-Aldrich, C5080), 9.25mg/l $(NH_4)_6Mo_7O_{24}.4H_2O$ (Sigma-Aldrich, A1343), 99mg/l $FeSO_4.7H_2O$ (Sigma-Aldrich, F8048), and was dissolved in 950ml distilled water and 50ml Metals ''44''. The

Metals ''44'' contained (per 100ml) 250mg Na_2.EDTA (Sigma-Aldrich, E1644), 1.1g $ZnSO_4.7H_2O$ (Sigma-Aldrich, 20635), 500mg $FeSO_4.7H_2O$, 154mg $MnSO_4.xH_2O$ (Sigma-Aldrich, 229784), 39.2mg $CuSO_4.5H_2O$ (Sigma-Aldrich, C8027), 24.8mg $Co(NO_3)_2.6H_2O$ (Sigma-Aldrich, 230357), and 17.7mg $Na_2B_4O_7.10H_2O$ (Sigma-Aldrich, B9876). The components were dissolved in 100ml distilled water.

1.6.2 Buffered peptone water (BPW)

According to the manufacturer's instructions, 20g of BPW (Oxoid, CM509) was dissolved in 1litre of distilled water, and sterilized at 121°C for 15 min in 9ml aliquots. It was then cooled to room temperature before use.

1.6.3 CHROMagar *Acinetobacter* medium

As per the instructions of CHROMagar (CHROMagar Microbiology, France), the medium was prepared as follows: 32.8g/l of the ready-made base (AC092 (B)) was suspended in distilled water. One millilitre of the liquid supplement (AC092 (S)) was then added to the base. Afterwards, the medium was heated and brought to boil while stirring regularly. When cooled to 55°C, the agar was dispensed into Petri dishes and allowed to set. Dry plates were then stored at 4°C for up to a week. Quality control strains comprised *A. baumannii* ATCC 19606^T, which grew on the medium as red colonies, and *E. faecalis* NCIMB 775^T as the positive and negative quality control strains respectively. The latter replaced *Enterococcus faecalis* ATCC® 29212 recommended by the medium provider, due to unavailability, but was correctly inhibited.

1.6.4 Dimmer-Druggan agar (DDA)

The medium contained 12 g/l tryptone (LAB M, MC005), 1g/l yeast extract (LAB M, MC001), 5g/l soy peptone (LAB M, MC003), 0.5g/l ferric ammonium citrate (Sigma-Aldrich, F5879), 0.4g/l DHF-riboside (Glycosynth, 50180), and 10 g/l agar No. 2 (LAB M, MC006). These components were mixed with 1 litre of distilled water and autoclaved at 121°C for 15 min. At 50-55°C, 20mg/l lincomycin (Sigma-Aldrich, 62143) and 2mg/l aztreonam (Sigma-Aldrich, A6848) were mixed with the medium (pH7) before dispensed into Petri dishes and allowed to solidify.

1.6.5 Enterobacteriaceae Enrichment broth (EE)

The manufacturer's instructions were followed by adding 43.5g to 1 litre of distilled water. The medium (Oxoid, CM0317) was distributed in 250ml bottles which was then brought to boil and heated for 30 min until dissolved at 100°C. Once dissolved, it was cooled rapidly under flowing cold water before use.

1.6.6 Glycerol broth

Four percent glycerol broth was prepared as described by Gibson and Khoury (1986). Fourty millilitre of glycerol (Sigma-Aldrich, 49781) was mixed with 30g/l tryptone soya broth (Merck, 105459), 5g/l glucose (Merck, 346351), and 20g/l skimmed milk powder (Oxoid, LP0031). The volume was then made up to 1000ml with distilled water and sterilised by autoclaving at 115°C for 20 min.

1.6.7 Leeds *Acinetobacter* medium (LAM)

The medium was prepared as according to Jawad *et al.* (1994). The formula included 10g/l bacteriological agar No. 2 (LAB M, MC006), 5g/l neutralized soy peptone (LAB M, MC003), 15g/l acid casein hydrolysate (Merck, 102245), 5g/l sodium chloride (Sigma-Aldrich, S7653), 5g/l sucrose (Sigma-Aldrich, S7903), 5g/l D-(-)-fructose (Sigma-Aldrich, F0127), 1g/l L-phenylalanine (Sigma-Aldrich, P2126), 5g/l D-mannitol (Sigma-Aldrich, M4125), 0.02g/l phenol red (Sigma-Aldrich, P3532), and 0.4g/l ferric ammonium citrate (Sigma-Aldrich, F5879). The pH of the complete medium was 7. All the ingredients were dissolved in distilled water by heating, then autoclaved for 15 min at 121°C. Once cooled to 50-55°C, 10mg/l vancomycin (Sigma-Aldrich, 861987), 50mg/l cephradine (Sigma-Aldrich, C8395), and 15mg/l cefsulodin (Sigma-Aldrich, C8145) were added to the medium and mixed before dispensing into Petri dishes.

1.6.8 Modified M9 medium with casamino acids

The medium was prepared by adding 6.0g/l Na_2HPO_4 (Sigma-Aldrich, S7907), 5.0g/l casamino acids (Fisher-Scientific, BP1424), 3.0g/l KH_2PO_4 (Sigma-Aldrich, P5655), 1,0g/l NH_4Cl (Sigma-Aldrich, A0171), and 0.5g/l NaCl (Sigma-Aldrich, S7653) to

distilled water. In contrast to the original recipe, the 10ml of 20g/l glucose solution was not included. The volume was then made up to 997ml. The ingredients were mixed thoroughly and the pH adjusted to 6.0 instead of 7.0. The medium was autoclaved at 121°C for 15 min at a pressure of 15 psi. The medium was left to cool before aseptically adding 1.0ml filter-sterilized solutions of 10mg/10ml thiamine.HCl (Sigma-Aldrich, T4625), 246.5g/l $MgSO_4.7H_2O$ (Sigma-Aldrich, 63138), and 14.7g/l $CaCl_2$ (Sigma-Aldrich, C5670). The medium was mixed thoroughly and distributed into baffled flasks.

1.6.9 Saline solution

Saline solution was used for diluting bacterial cultures and was prepared as Oxoid (BR0053) instructed. One sodium chloride tablet was dissolved in 500ml of distilled water. This 0.85% saline solution was then sterilised by autoclaving at 121°C for 15 min.

1.6.10 Tryptic soy agar (TSA)

According to Oxoid's instructions, 40 grams of TSA (CM0131) were suspended in 1 litre of distilled water and sterilised by autoclaving for 15 min at 121°C. The medium was allowed to cool to around 60°C before dispensing into sterile petri dishes.

1.6.11 Tryptic soy broth (TSB)

Tryptic soy broth was prepared in accordance with the instructions of Merck (105459). Thirty grams of TSB was dissolved in 1 litre distilled water. Aliquots (10ml) of TSB were dispensed into Universal bottles, which were then autoclaved for 15 min at 121°C. The broth was allowed to cool to room temperature before used.

1.6.12 Violet red bile glucose agar (VRBGA)

As per the manufacturer's instructions, 38.5g of VRBGA (Merck, 1.10275) was dissolved in 1 litre of distilled water. It was then brought to the boil to dissolve completely. Once dissolved, it was mixed, and then dispensed into Petri dishes.

Table 1.1 List of bacterial strains and their associated research studies.

Species	Strain number	Source/Culture collection designation	Associated study					
			1	2	3	4	5	6
A. baumannii	1095	QMC	√	√	√	√	√ *	
A. baumannii	1096	QMC	√	√	√	√	√ *	
A. baumannii	1098	QMC	√	√	√	√	√ *	
A. baumannii	1099	QMC	√	√	√	√	√	√
A. baumannii	1102	NCTC 12156^T	√	√	√	√	√	√
A. baumannii	1109	QMC	√				√ *	
A. baumannii	1110	QMC	√				√ *	
A. baumannii	1111	QMC	√				√	√
A. baumannii	1112	QMC	√				√*	
A. baumannii	1113	QMC	√				√ *	
A. baumannii	1114	QMC	√				√ *	
A. baumannii	1115	QMC	√				√ *	
A. baumannii	1116	QMC	√				√ *	
A. baumannii	1117	QMC	√				√ *	
A. baumannii	1118	QMC	√				√ *	
A. baumannii	1119	QMC	√				√ *	
A. baumannii	1120	QMC	√				√ *	
A. baumannii	1121	QMC	√				√ *	
A. baumannii	1122	QMC	√				√ *	
A. baumannii	1123	QMC	√				√ *	
A. baumannii	1124	QMC	√				√ *	
A. baumannii	1125	QMC	√				√ *	
A. baumannii	1126	QMC	√				√ *	
A. baumannii	1127	QMC	√				√ *	
A. baumannii	1183	PIF			√		√	√
A. baumannii	1440	ATCC 17978			√		√	√
A. calcoaceticus	418	Food	√	√	√	√	√	√
A. calcoaceticus	1097	NCTC 7844	√	√	√	√	√	√
A. calcoaceticus	1103	NCTC 12983^T	√	√				
Acinetobacter gensp. 3	415	Food	√	√	√	√	√	√
Acinetobacter gensp. 3	1182	PIF			√		√	√
A. haemolyticus	1337	NCTC 10305^T	√					
A. junii	1335	NCTC 10307^T	√					
A. johnsonii	1336	NCTC 10308^T	√					
A. lwoffii	1338	NCTC 5866^T	√					
A. radioresistens	1340	ATCC 43998^T	√					
A. schindleri	1339	ATCC BAA-618^T	√					
A. ursingii	1334	ATCC BAA-617^T	√					
Bacillus cereus	1170	ATCC 11778	√					
Bacillus sp.	N33	PIF	√					
Bacillus sp.	N94	PIF	√					
B. subtilis	702	NCIMB 10106	√					
B. subtilis	1248	NCIMB 10108	√					

Species	Strain number	Source / Culture collection designation	Associated study					
			1	2	3	4	5	6
Burkholderia cepacia	627	NCTC 10661	√					
Citrobacter freundii	624	NCTC 9750	√					
Citr. koseri	48	SMT 319					√	
Citr. koseri	224	ATCC 27028 - OCC 270	√					
Citr. koseri	225	ATCC 25409	√					
Citr. koseri	226	ATCC 25410 - OCC 254	√					
Citr. koseri	826	ATCC 25408	√					
Cronobacter sakazakii	1	ATCC 29544 - NCTC 11467	√					
C. sakazakii	2	ATCC 12868	√					
Enterobacter cloacae	49	ATCC 13047 - NCTC 10005	√					
Ent. cloacae	50	OCC		√				
Ent. cloacae	218	ATCC 23355 - OCC 1062	√					
Ent. cloacae	597	Textile surfactant solution	√	√				
Ent. hormaechei	790	Neonatal feeding tube		√				
Enterococcus faecalis	1172	NCIMB 775^{T}	√					
Escherichia coli	100	NCIMB 9517	√					
E. coli	605	NCH	√					
E. coli	571	ATCC 8196	√					
E. coli	800	ATCC 43888	√					
E. coli	1247	NCTC 10418	√					
E. coli	1230	HB 101					√	
Klebsiella pneumoniae	68	NCDO 711	√					
K. pneumoniae	541	NCIMB 10341	√					
K. pneumoniae	1013	Neonatal feeding tube	√					
Lactococcus lactis	1171	NCIMB 6681	√					
Lactobacillus plantarum	574	NCIMB 6376	√					
Pseudomonas aeruginosa	117	NCTC 8602	√					
P. aeruginosa	401	ATCC 15442	√					
P. aeruginosa	570	NCTC 6749	√					
P. luteola	133	PIF	√					
Salmonella Enteritidis	358	NCTC 3046					√	
Serratia liquefaciens	579	AES medium	√					
S. marcescens	763	NCTC 1377	√					
S. marcescens	834	OCC 217	√					
S. marcescens	835	OCC 219	√					
Staphylococcus aureus	1167	NCTC 6571	√					
Staph. aureus	1168	NCTC 4163	√					
Staph. aureus	152	NCTC 7447	√					
Staph. aureus	616	NCTC 10788	√					
Staphylococcus sp.	N50	PIF	√					
Staphylococcus sp.	N76	PIF	√					
Staph. epidermidis	671	NCTC 7944	√					

1 Development and evaluation of *Acinetobacter* medium for the detection of the organism in PIF, 2 Desiccation survival study, 3 Biofilm formation study, 4 Gastrointestinal survival study, 5 *In vitro* infection model studies, 6 Genes detection study. ATCC American Type Culture collection, HB Hybrid Bacterium, NCDO National Collection of Dairy Organisms, NCIMB National Collection of Industrial and Marine Bacteria, NCTC National Collection of Type cultures, NCH Nottingham City Hospital, NTU Nottingham Trent University, OCC Oxoid Culture Collection, PIF Powdered Infant Formula, QMC Queen's Medical Centre, ^{T} Type strain, √* strain was only included in the preliminary experiments.

CHAPTER 2. OVERVIEW ON *ACINETOBACTER* SPP.

2.1 Characteristics of *Acinetobacter* spp.

Acinetobacter species are Gram-negative/variable, strictly aerobic, non-spore forming, non-motile, oxidase-negative, catalase-positive, and non-fermentative (Mahon *et al.*, 2011). Cells appear as short rods during the log phase and display a coccobacillary shape in the stationary phase (Allen and Hartman, 2010). Due to the morphological similarity, they are often confused with *Achromobacter*, *Moraxella*, and *Neisseria* (King *et al.*, 2009). However, *Acinetobacter* can be differentiated by its negative reaction of the oxidase test (de la Maza *et al.*, 2004). The organism grows at 20°C to 37°C with the optimum growth temperature between 33°C and 35°C. Growth at 44°C has been recorded for *A. baumannii* species (Bouvet and Grimont, 1986). *Acinetobacter* spp. are also known for their ability to utilise a variety of compounds as sources of carbon and energy, and to grow easily on general culture media (Allen and Hartman, 2010). Their smooth non-pigmented colonies on these media may appear mucoid as they are generally encapsulated (Allen and Hartman, 2010).

2.2 Current taxonomy and species identification

The genus *Acinetobacter* belongs to the Moraxellaceae family within the Gammaproteobacteria of the Proteobacteria (Rossau *et al.*, 1991), and currently comprises of thirty-six species (named and unnamed) **(Table 2.1)** delineated based on DNA-DNA hybridization, which is considered as the gold standard for species identification. The nomenclature of the unnamed genomic species (gensp.) was hindered by the lack of phenotypic identification criteria that could differentiate and assign them to a specific group (Gerner-Smidt, 1992; Gerner-Smidt *et al.*, 1991). In addition, the small number of strains in these genomic species did not allow a generalisation to be made in terms of their phenotypic diversity. Another taxonomy issue for this organism is the close genomic relatedness of some species as represented by the *A. calcoaceticus* - *A. baumannii* (Acb) complex (Bouvet and Jeanjean, 1989). In addition to these two species, the complex includes gensp. 3, 13TU, as well as the DNA-hybridization groups labelled 'close to 13TU' and 'between 1 and 3'. *Acinetobacter* gensp. 10 and 11 are also believed to be closely related (Bouvet and Jeanjean, 1989). Species belonging to this complex cannot all be accurately distinguished from each other (Gerner-Smidt *et al.*, 1991). Commercial phenotypic systems such as API20 NE, and VITEK 2, which are routinely used for

species identification of other bacteria in diagnostic laboratories, are unreliable for *Acinetobacter* in this respect (Bernards *et al.*, 1995; Bernards *et al.*, 1996; Horrevorts *et al.*, 1995; Loubinoux *et al.*, 2003). Although some species are correctly identified by these systems, others such as *Acinetobacter* gensp. 3 and 13TU, as well as *A. schindleri*, and *A. ursingii* can be misidentified (Dortet *et al.*, 2006; Gerner-Smidt *et al.*, 1991). This is partly due to the limited species database for *Acinetobacter*, and since the included biochemical tests are not tailored for acinetobacters considering their limited fermentation activities (Gerner-Smidt *et al.*, 1991).

Genotypically, a range of identification methods, based on the comparative analysis of DNA sequences and the analysis of DNA fragments separated electrophoretically, have been used. The DNA fragment based-identification techniques include tRNA intergenic spacer fingerprinting (Vaneechoutte *et al.*, 2009), ribotyping (Gerner-Smidt, 1992), amplified fragment length polymorphism (AFLP^{TM}) (Nemec *et al.*, 2010), and amplified 16S ribosomal DNA restriction analysis (ARDRA) (Nemec *et al.*, 2011). The last two methods have been extensively validated and hence widely recognized as reference procedures for species identification of this organism (Peleg *et al.*, 2008). The second identification approach commonly applied involves the analysis of similarities between 16S rDNA sequences where those lower than 98.7-99.0% are designated to different species (Janda and Abbott, 2007; Stackebrandt and Evers, 2006). Values above this threshold may or may not mean the organisms belong to the same species. DNA-DNA hybridization would then be used for confirmation in this case (Stackebrandt and Evers, 2006). The above-mentioned methods are useful for species identification and suitable for reference laboratories (Peleg *et al.*, 2008). However, they are laborious and non-practical for routine day-to-day diagnostic microbiology laboratories.

Table 2.1 Delineation of *Acinetobacter* species.

Named species	Genomic species	Type strain	Reference
A. baumannii	2	ATCC 19606^T	(Bouvet and Grimont, 1986; Tjernberg and Ursing, 1989)
A. baylyi	-	DSM 14961^T	(Carr *et al.*, 2003)
A. beijerinckii	-	NIPH 838^T	(Nemec *et al.*, 2009)
A. berezinae	10	ATCC 17924^T	(Bouvet and Grimont, 1986; Nemec et al., 2010; Tjernberg and Ursing, 1989)
A. bouvetii	-	DSM 14964^T	(Carr *et al.*, 2003)
A. brisouii	-	DSM 18516^T	(Anandham *et al.*, 2010)
A. calcoaceticus	1	ATCC 23055^T	(Bouvet and Grimont, 1986; Tjernberg and Ursing, 1989)
A. gerneri	-	DSM 14967^T	(Carr *et al.*, 2003)
A. grimontii	-	DSM 14968^T	(Carr *et al.*, 2003)
A. guillouiae	11	ATCC 11171^T	(Bouvet and Grimont, 1986; Nemec et al., 2010; Tjernberg and Ursing, 1989)
A. gyllenbergii	-	NIPH 2150^T	(Nemec *et al.*, 2009)
A. haemolyticus	4	ATCC 17906^T	(Bouvet and Grimont, 1986; Tjernberg and Ursing, 1989)
A. johnsonii	7	ATCC 17909^T	(Bouvet and Grimont, 1986; Tjernberg and Ursing, 1989)
A. junii	5	ATCC 17908^T	(Bouvet and Grimont, 1986; Tjernberg and Ursing, 1989)
A. lwoffii	8, 9	ACTC 15309^T	(Bouvet and Grimont, 1986; Tjernberg and Ursing, 1989)
A. nosocomialis	13TU	NIPH 2119^T	(Nemec et al., 2011; Tjernberg and Ursing, 1989)
A. parvus	-	NIPH 384^T	(Nemec *et al.*, 2003)
A. pittii	3	ATCC 19004^T	(Bouvet and Grimont, 1986; Nemec et al., 2011; Tjernberg and Ursing, 1989)
A. radioresistens	12	ATCC 43998^T	(Bouvet and Grimont, 1986; Nishimura et al., 1988; Tjernberg and Ursing, 1989)
A. rudis	-	DSM 24031^T	(Vaz-Moreira *et al.*, 2011)
A. schindleri	-	NIPH 1034^T	(Nemec *et al.*, 2001)
A. soli	-	JCM 15062^T	(Kim *et al.*, 2008)
A. tandoii	-	DSM 14970^T	(Carr *et al.*, 2003)
A. tjernbergiae	-	DSM 14971^T	(Carr *et al.*, 2003)
A. towneri	-	DSM 14962^T	(Carr *et al.*, 2003)
A. ursingii[a]	-	NIPH 137^T	(Nemec *et al.*, 2001; Nemec et al., 2008)
A. venetianus	-	ATCC 31012^T	(Vaneechoutte et al., 2009; Vaneechoutte *et al.*, 1999)
	6	ATCC 17979	(Bouvet and Grimont, 1986; Tjernberg and Ursing, 1989)
	13BJ, 14TU	ATCC 17905	(Bouvet and Jeanjean, 1989; Tjernberg and Ursing, 1989)
	14BJ	CCUG 14816	(Bouvet and Jeanjean, 1989)
	15BJ	SEIP 23.78	(Bouvet and Jeanjean, 1989)
	16	ATCC 17988	(Bouvet and Jeanjean, 1989)
	17	SEIP Ac87.314	(Bouvet and Jeanjean, 1989)
	15TU	LUH 1090	(Tjernberg and Ursing, 1989)
	Between 1 and 3	LUH 1469	(Gerner-Smidt and Tjernberg, 1993)
	Close to 13TU	LUH 1472	(Gerner-Smidt and Tjernberg, 1993)

The genus *Acinetobacter* is currently comprised of 27 named and 9 unnamed species delineated based on DNA-DNA hybridization. DNA-DNA hybridization groups were termed as genomic species and were assigned numbers if species names were not originally given. The designated numbers of genomospecies 13-15 described by two independent studies were extended with BJ and TU to avoid confusion. [a] it has also been called *A. septicus*, - non-specified genomic species, ATCC American Type Culture Collection, CCUG Culture Collection; University of Göteborg, DSM Deutsche Sammlung von Mikroorganismen, JCM Japan Collection of Microorganisms, LUH Leiden University Medical Centre, NIPH National Institute of Public Health, SEIP Service des Enterobacteries de l'Institut Pasteur, ^{T} type strain.

2.3 Clinically important *Acinetobacter* species

The most commonly isolated *Acinetobacter* species from hospitals belong to the Acb complex, particularly *A. baumannii* (Turton *et al.*, 2010). Other species including *A. baylyi*, *A. beijerinckii*, *A. bereziniae*, *A. guillouiae*, *A. gyllenbergii*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. lwoffii*, *A. nosocomialis* (gensp. 13TU), *A. parvus*, *A. pitti* (gensp. 3), *A. radioresistens*, *A. schindleri*, and *A. ursingii* have also been found in clinical specimens. However, they are less frequently encountered in association with hospital-acquired infections (Chen *et al.*, 2007b; Dortet *et al.*, 2006; Gundi *et al.*, 2009; Loubinoux *et al.*, 2003; Nemec *et al.*, 2011; Nemec *et al.*, 2010; Turton *et al.*, 2010; van den Broek *et al.*, 2009).

2.4 Natural habitats and transmission of *Acinetobacter* spp.

Although *Acinetobacter* spp. are ubiquitously present in nature (water and soil) (Baumann, 1968), there is a misconception that the environment is the natural habitat of the entire *Acinetobacter* genus. *A. ursingii* and *A. schindleri*, for example, have only been isolated from clinical materials (Dortet *et al.*, 2006; Nemec *et al.*, 2001). The bacterium can also exist in humans as a commensal of the skin, throat, nose, ear, axilla, toe web, perineum, and groin (Berlau *et al.*, 1999; Seifert *et al.*, 1997). In fact, acinetobacters comprise part of the skin flora of healthy humans (Peleg *et al.*, 2008; Wilson, 2005). An epidemiological survey of the skin and mucosal membranes of non-hospitalised individuals revealed that 43% were colonised with *Acinetobacter* (Seifert *et al.*, 1997). *A. lwoffii*, *A. johnsonii*, *A. junii*, and *Acinetobacter* gensp. 3 were isolated from 58, 20, 10, and 6% of the samples respectively. In another study, an *Acinetobacter* carriage rate of 44% in healthy persons was also reported (Berlau *et al.*, 1999). At 61%, *A. lwoffii* was the most prevalent species followed by *Acinetobacter* gensp. 15BJ (12%). *A. radioresistens* and *Acinetobacter* gensp. 3 were recovered at 8 and 5% respectively (Berlau *et al.*, 1999). Compared to the 43% colonisation of non-hospitalised individuals, that of patients in a regular ward was much higher (75%) (Seifert *et al.*, 1997). Faecal carriage with *A. johnsonii* and *Acinetobacter* gensp. 11 has been previously detected in 25% of healthy humans (Dijkshoorn *et al.*, 2005). *A. baumannii* was also found in 0.8% of the samples (Dijkshoorn *et al.*, 2005). Another study reported 77% rectal colonization with *A. baumannii*, while that of the pharynxes or axillae was 75% (Ayats *et al.*, 1997).

Although *A. baumannii* are occasionally found on the skin of healthy individuals (Jawad *et al.*, 1998a), the skin carriage of this organism increases following hospitalization (Gerner-Smidt, 1994). Marchaim *et al.* (2007) investigated re-admitted cases with previous history of hospitalization and who had been colonised and/or infected with multidrug-resistant (MDR) *A. baumannii*. The isolation of this organism was traced back to 17.5 months on average since the patients were hospitalized. Approximately 55% of the recent carriers (<10 days) had more than one positive culture of MDR *A. baumannii*. Five of thirty patients with remote isolation history were also positive for the organism cultured from the pharynx and skin but not from other surveillance body sites (endotracheal aspirates, wounds, rectum, and nose). Isolates obtained from different sites in the same patient were identified as clonal in all cases except one (Marchaim *et al.*, 2007).

It has been suggested that hospitals are the primary reservoir for *A. baumannii* in particular (Zeana *et al.*, 2003). The hands of the health-care personnel and contaminated surfaces have been identified as the sources of direct and indirect transmission of the organism in the clinical setting (Dijkshoorn *et al.*, 2007; Go *et al.*, 1994; Roberts *et al.*, 2001). *Acinetobacter* are believed to persist under desiccated conditions on inanimate hospital surfaces, generally, longer than other Gram-negative organisms such as *Escherichia coli* and other Enterobacteriaceae (Hirai, 1991; Jawad *et al.*, 1996; Pettit and Lowbury, 1968; Wendt *et al.*, 1997). The organism can be isolated from the surfaces of the components of patients' rooms (e.g. pillows and mattresses, and from dust inside dialysis machines and mechanical ventilators (Bernards *et al.*, 2004; Mottar *et al.*, 2006; Sherertz and Sullivan, 1985; Weernink *et al.*, 1995). Almost all the medical devices have been considered a potential reservoir for *Acinetobacter* outbreaks. Common inanimate sources of cross-contamination includes blood pressure cuffs (Bureau-Chalot *et al.*, 2004), pressure transducers (Beck-Sague *et al.*, 1990), temperature and oxygen probes (Snelling *et al.*, 1996), humidifiers (Ebenezer *et al.*, 2011; Schloesser *et al.*, 1990), ventilator tubing (Cefai *et al.*, 1990; Struelens *et al.*, 1993), mouthpieces of ventilation masks (Stone and Das, 1986), resuscitation bags (Hartstein *et al.*, 1988). Parenteral nutrition and intravenous fluids have also been identified as foci for *Acinetobacter* outbreaks (De Vegas *et al.*, 2006; Ng *et al.*, 1989). The prolonged survival on inanimate hospital surfaces combined with inefficient disinfecting procedures may enhance the existence of this organism in the hospital environment and the propensity for epidemic spread. In other words, contamination of these surfaces can serve as a dry vector for the

re-occurrence of outbreaks. The transmission of *Acinetobacter* between patients may also collectively contribute to the spread of acquired multidrug resistance and hence infections that are difficult to treat (D'Agata *et al.*, 2000; Webster *et al.*, 2000).

2.5 Virulence determinants

It is well established that *Acinetobacter* is an opportunistic organism that has the ability to be pathogenic and cause infections. The degree of pathogenicity (virulence) may vary between strains of the same species and depends on a combination of factors involving the bacterium, the host, and the interaction between them. The virulence of a bacterial strain is promoted by the virulence determinants and partly contributes to the outcomes of the infection associated. In the next part, an overview of the major virulence factors of *Acinetobacter* is given in terms of their relevance to the pathogenicity of the organism. These factors described in the literature are mainly reported in *A. baumannii* which has been the most extensively researched species compared to other *Acinetobacter* species due to its clinical significance as a multidrug resistant pathogen. However, this does not eliminate the potential pathogenicity or the virulence of the other species, nor reflects a sole interest of this research project on *A. baumannii*.

2.5.1 Antibiotic resistance determinants

In comparison to other *Acinetobacter* species, *A. baumannii* has gained a wide reputation as a problematic nosocomial MDR pathogen. Resistance to all commonly used antibiotics against Gram-negative bacteria has become a frequent issue in clinical practice (Livermore *et al.*, 2008; Morgan *et al.*, 2009; Rossolini and Mantengoli, 2008). It has been proposed that the presence of MDR *A. baumannii* in a clinical sample indicates a potential outbreak-causing strain (Koeleman *et al.*, 2001).The range of resistance mechanisms exhibited by this species has considerably limited the effective therapeutic options available for patients' treatment (Neonakis *et al.*, 2011). Drug resistance of this organism is enhanced by its ability to acquire and integrate foreign genetic elements, such as integrons, transposons, and plasmids, from other bacteria in the same environment (Giamarellou *et al.*, 2008; Vila and Pachon, 2008). Genes which are associated with multidrug resistance and believed to be acquired from other Gram-negative species have been found in a number of large genomic islands (AbaR1, R2, R3 and R5) in the genomes

of MDR isolates (Adams *et al.*, 2008; Fournier *et al.*, 2006; Iacono *et al.*, 2008). Those resistant to three or more classes of drugs are regarded as multidrug resistant and have been increasingly reported worldwide (Doi *et al.*, 2009; Fournier and Richet, 2006; Ko *et al.*, 2007; Maviglia *et al.*, 2009; Paterson, 2006; Zavascki *et al.*, 2010). Collectively, the morbidity and mortality resulting from infections caused by such strains are on the increase (Paterson, 2006; Peleg *et al.*, 2008; Rello *et al.*, 1993; Zavascki *et al.*, 2010).

The spread of multidrug resistance has forced the use of antimicrobial agents that are not otherwise preferred as the first or optimal treatment option (Gordon and Wareham, 2010). Polymyxin B and E (colistin) for example should be infrequently used due to associated renal toxicity (Neonakis *et al.*, 2011). However, these antibiotics have currently been resorted to in the absence of new alternative antibiotics (Neonakis *et al.*, 2011). Alarmingly, resistance of *A. baumannii* to colistin which is considered to be the only antibacterial agent effective against MDR *A. baumannii* strains has also emerged (Fernandez-Reyes *et al.*, 2009; Gales *et al.*, 2006; Ko *et al.*, 2007; Nation and Li, 2009; Souli *et al.*, 2008). In addition, the emergence of non-susceptible *A. baumannii* isolates has compromised the use of carbapenems, which used to be the usual antibiotics of choice for this organism's infections (Bassetti *et al.*, 2008; Maragakis and Perl, 2008; McDonald, 2006; Vila and Pachon, 2008).

The major mechanisms of resistance identified in *A. baumannii* include the production of the naturally occurring β-lactamase enzymes (chromosomal AmpC cephalosporinase and intrinsic oxacillinase OXA-51-like cluster) (Alsultan *et al.*, 2009; Heritier *et al.*, 2005; Koh *et al.*, 2007; Peleg *et al.*, 2008). AmpC cephalosporinases hydrolyse amino-penicillins and cephalosporins but not ceftazidime when the associated gene is expressed at a basal level. Nevertheless, resistance to ceftazidime develops when a strong transcriptional promoter (the insertion element; IS*Aba*1) is present upstream of the bla_{ampC} gene to modulate its expression (Corvec *et al.*, 2003; Heritier *et al.*, 2006; Segal *et al.*, 2004). In addition, the intrinsic oxacillinase enzymes strongly hydrolyse carbapenems under the overexpression of the $bla_{\text{OXA-51-like}}$ gene containing the IS*Aba*1element (Turton *et al.*, 2006).

As well as the intrinsic β-lactamases, *A. baumannii* may acquire clavulanic acid-inhibited class A β-lactamases (extended-spectrum β-lactamases; ESBLs), which can cause resistance to broad-spectrum cephalosporins (Bergogne-Bérézin, 2010; Gordon and

Wareham, 2010). These enzymes include the CTX-M, GES, PER, SCO, SHV, TEM, and VEB families (Gordon and Wareham, 2010). *A. baumannii* may also acquire β-lactamases belonging to the class B (metallo-β-lactamases; MBLs), or to the class D (oxacillinases) (Poirel and Nordmann, 2006). MBLs are active against all carbapenems and β-lactams, excluding aztreonam, while some oxacillinases (carbapenem-hydrolysing oxacillinases; CHDLs) may not always be effective on meropenem (Gordon and Wareham, 2010; Poirel and Nordmann, 2006). The production of oxacillinases is the commonest enzymatic mechanism of carbapenem resistance (Gordon and Wareham, 2010). These enzymes are particularly encoded by genes of the $bla_{\text{OXA-23}}$, $bla_{\text{OXA-40}}$ and $bla_{\text{OXA-58}}$-like lineage (Poirel and Nordmann, 2006). Resistance to penicillins can also be conferred by these oxacillinase enzymes (Heritier *et al.*, 2005). Aminoglycoside resistance is found to be caused by aminoglycoside modifying enzymes (phosphotransferases, nucleotidyltransferases, and acetyltransferases) in *A. baumannii* (Seward *et al.*, 1998). Other enzymes such as 16S rRNA methyltransferases also confer high resistance to this class of drugs (Doi *et al.*, 2007; Yu *et al.*, 2007).

The intrinsic antimicrobial resistance of *A. baumannii* may be caused partly by the presence of relatively few porins in its outer membrane compared to other Gram-negative bacteria (Sato and Nakae, 1991). The lack of outer membrane proteins (OMPs), which perform as porins for the transport of substances across the outer membrane, decreases the permeability of the membrane and may accordingly enhance antimicrobial resistance. β-lactam resistance has been frequently associated with the absence of a 29 kDa protein, a 33-36 kDa protein, and a 43 kDa protein from *A. baumannii* (del Mar Tomas *et al.*, 2005; Dupont *et al.*, 2005; Siroy *et al.*). In addition, the colistin-resistant *A. baumannii* ATCC 19606^{T} has been found to down-regulate proteins such as OmpA in the presence of this antibiotic (Fernandez-Reyes *et al.*, 2009).

Removal of antimicrobial agents by active multidrug efflux systems represents a substantial contribution to antibiotic resistance (Gordon and Wareham, 2010). Narrow-spectrum pumps of the major facilitator superfamily (MFS) are, for example, implicated in minocycline (*TetB*), tetracycline (*TetA*, *TetB*), and chloramphenicol (*CmlA*) resistance (Fournier *et al.*, 2006; Huys *et al.*, 2005). The resistance–nodulation–cell division (RND) type of pumps, namely AdeABC and AdeIJK, has broad substrate specificity and has been identified in *A. baumannii*. The overexpression of AdeABC results in resistance to tetracyclines, erythromycin, chloramphenicol, β-lactams, and aminoglycosides (Marchand

et al., 2004). The AdeIJK pump contributes with AdeABC to tigecycline resistance (Damier-Piolle *et al.*, 2008). The overexpression of another efflux pump (AbeM), which belongs to the multidrug and toxic compound extrusion (MATE) family of transporters, has also been implicated in the resistance of *A. baumannii* to trimethoprim, chloramphenicol, erythromycin, kanamycin, gentamicin, and quinolones (Su *et al.*, 2005). In addition, a MDR family of bacterial integral membrane proteins (BIMP) comprising an AbeS efflux system contributes to chloramphenicol, macrolides, and quinolones resistance (Srinivasan *et al.*, 2009). Identified resistance against polymyxins in *A. baumannii* involves mutations in a two-component regulator (*PmrA/B*), which modifies lipid A in the lipopolysaccharide membrane (Adams *et al.*, 2009). Mutations in DNA gyrase and topoisomerase IV have accounted for fluoroquinolone resistance (Vila *et al.*, 1997). The production of a putative dihydrofolate reductase (DHFR) and a ribosomal protection protein, encoded by *TetM*, confer resistance to trimethoprim and tetracycline respectively (Mak *et al.*, 2009; Ribera *et al.*, 2003). β-lactam resistance due to downregulation of penicillin-binding proteins (PBPs) is another characterized mechanism for the antibiotic resistance of *A. baumanii* (Fernandez-Cuenca *et al.*, 2003). Generally, several resistance mechanisms may exist in MDR isolates and act against the same class of antibiotics which can represent a difficulty in selecting suitable therapeutic agents (Gordon and Wareham, 2010).

2.5.2 Autoinducer molecules

Most bacteria possess a major regulatory system that involves the production and response to signal molecules as a means of intercellular communication (Camilli and Bassler, 2006). Gene expression, in such systems, is dependent on cell density and so it is known as quorum sensing. Depending on population density, small diffusible molecules such as N-acylhomoserine lactones (N-AHL/AHL) self-generate and consequently induce the expression of genes (Bassler, 2002; Fuqua *et al.*, 1994). Several virulence genes, for example those responsible for toxin production, surface adhesion, and motility, are controlled by AHLs in Gram-negative pathogens (Antunes *et al.*, 2010; Bosgelmez-Tinaz *et al.*, 2005). This regulatory mechanism may also contribute to the auto-induction of several virulence traits in *Acinetobacter*. However, information regarding the quorum sensing signals modulating the physiology of *Acinetobacter* and its pathogenesis in humans is limited.

Both environmental and clinical *Acinetobacter* spp. isolates are able to produce one to five quorum sensing molecules for the activation of AHL biosensors (Bosgelmez-Tinaz *et al.*, 2005; Gonzalez *et al.*, 2009; Gonzalez *et al.*, 2001; Niu *et al.*, 2008; Vallenet *et al.*, 2008). Cell density-mediated communication has been found to control the surface twitching motility (Clemmer *et al.*, 2011) and biofilm development of *A. baumannii* (Antunes *et al.*, 2011a; Gaddy and Actis, 2009b; Niu et al., 2008; Sarkar and Chakraborty, 2008). Sarkar and Chakraborty (2008) showed that inhibition of quorum sensing results in significant inhibition of cell growth and biofilm formation of *A. junii* strain BB1A. The quorum sensing pathway is mediated by the *aba*I autoinducer synthase, which encodes for the AHL molecule, and is anticipated to be the only autoinducer synthase encoded by *A. baumannii* (Niu *et al.*, 2008). The deletion mutation of this gene impairs the production of detectable AHL signals and consequently biofilm formation by *A. baumannii* strain M2. This is in agreement with the study by Gaddy and Actis (2009b), where inactivation of *abaI* caused the biofilm of *A. baumannii* M2 strain to be reduced by 30– 40% in comparison to the isogenic parental strain. Biofilm maturation was restored when the AHL was exogenously added to the *abaI* mutant (Gaddy and Actis, 2009b). Antunes *et al.* (2011a) specified that this gene is required for biofilm maturation during the later stages of the development since there were no differences between the biofilm of the *abaI*::Km mutant and the wild type at 8h of development while variations were apparent after 16 and 24 h.

2.5.3 Capsule formation

Although exopolysaccharide has generally been recognised as an important virulence factor in Gram-negative bacteria (Russo *et al.*, 2010), little is known about its contribution to the pathogenesis of *Acinetobacter*. Vidal *et al.* (1996) noticed an amorphous matrix, surrounding *A. baumaniii* cells adhering to a glass coverslip, which resembled the exopolysaccharide exhibited by other biofilm-forming bacteria. Although adherence of clinical isolates of *A. baumannii* to rat bladder and tracheal tissues was observed to be independent of the exopolysaccharide (Ruiz *et al.*, 1998; Sepulveda *et al.*, 1998), the K1 capsule is required for the survival of *A. baumannii* strain AB307-0294 in human serum and also for its optimal growth in human ascites fluid (Russo *et al.*, 2010). Both the human serum and ascites fluid contain complement, which acts as a major defense mechanism against bacterial pathogens. The presence of the capsule prevents the

complement from accessing the bacterial cell wall and blocks the induction of the alternate complement activation pathway (Bergogne-Bérézin *et al.*, 2010). In addition, the capsule has been shown to be necessary for the existence of *A. baumannii* in a rat soft tissue infection model where the complete clearance of the capsule-negative mutants was a strong indication of the capsule's involvment in the protection of this organism (Russo *et al.*, 2010).

2.5.4 Extracellular enzymes

Acinetobacter produces a range of extracellular enzymes that are believed to contribute to the virulence of the bacterium. The urease enzyme has been shown to assist *A. lwoffii* strains in the colonization of the achlorhydric or hypochlohydric stomach and induce inflammation as a result (Rathinavelu *et al.*, 2003). The ability to also cause damage to tissue lipids has been associated with the activity of esterases that hydrolyses the short-chain fatty acids at the ester bond (Poh and Loh, 1985). Antunes *et al.* (2011a) identified the presence of several haemolysin-related genes and two phospholipase C genes in four *A. baumannii* strains whose genomes have been sequenced (SDF, ATCC 17978, ACICU, and AYE). The β-haemolytic activity of these strains was demonstrated on 5.0% horse blood agar plates but not when sheep erythrocytes were used. The production of the enzyme phospholipase C, which has been reported to enhance the toxicity of *A. baumannii* to epithelial cells (Camarena *et al.*, 2010), was also identified in all of the above mentioned strains (Antunes *et al.*, 2011a). Another phospholipase enzyme (phospholipase D) has also been found to be important for the invasion of epithelial cells, and the pathogenesis of *A. baumannii* in a murine model of pneumonia, as well as resistance to 40% human serum (Jacobs *et al.*, 2010).

2.5.5 Fimbriae/Pili

To establish an infection, bacteria have to first establish themselves by adhering to the mucosal surface of the host. This generally involves the interaction between bacterial surface structures called adhesins such as fimbriae or pili, and the surface of the host cell namely the complementary receptors. Sepulveda *et al.* (1998) identified fimbrial structures in *A. baumannii* strains which were able to adhere to rat bladder tissue. Thin

fimbrial-like extensions on the surface of *A. baumannii* that enabled the organism to firmly attach to the membrane surface of the human bronchial epithelial cells NCI-H (292) have also been observed by scanning electron microscopy (Lee *et al.*, 2006). Electron microscopy also revealed the presence of both thick and thin fimbriae assemblies in *A. calcoaceticus* RAG-1 (Rosenberg *et al.*, 1982). Deletion of the *acuA* gene encoding for the main protein involved in the production of the thin fimbriae caused *Acinetobacter* sp. strain BD413 to lose the fimbriae and consequently the attachment to erythrocytes and polystyrene (Gohl *et al.*, 2006).

Acinetobacter lacks flagellar-based motility to migrate and form biofilms. Instead, long filaments evenly spread around the cell surface are believed to be needed for the initiation and biofilm development on abiotic surfaces. These structures have been suggested to be type I pili utilised solely for surface adherence (Tomaras *et al.*, 2003). Tomaras *et al.* (2003) demonstrated the pili involvement by identifying an attachment-deficient mutant of *A. baumannii* ATCC 19606^T which did not have appendages on the cell surface. A six-gene operon (*csuA/BABCDE*), encoding a chaperone-usher system, was shown responsible for pili assembly/secretion and subsequent biofilm formation on plastic surfaces (Tomaras *et al.*, 2003). This was subsequently confirmed where all the genes required for a *csuA/BABCDE* chaperone-usher pili assembly system were detected in *A. baumannii* ATCC 19606^T as well as eight other clinical isolates forming biofilms on plastic (McQueary and Actis, 2011). Comparative genomic analysis of *A. baumannii* strains 17978 and AYE showed the existence of operons similar to that described in the 19606 type strain (Gaddy and Actis, 2009a; Smith *et al.*, 2007; Vallenet *et al.*, 2008). In addition, these two strains harbor three additional loci coding for secretion functions, which were possibly related to the assembly of pili and adhesion (Smith *et al.*, 2007; Vallenet *et al.*, 2008). Another study showed that the *A. baumannii* ACICU strain also contains a locus coding for a putative chaperone-usher secretion system, but with a different number of genes. Their order appeared to differ from that identified in the 19606^T strain (Iacono *et al.*, 2008). In comparison to the above strains, similar *csu* loci were absent from the genome of *A. baumannii* SDF and *Acinetobacter baylyi* ADP1 (Vallenet *et al.*, 2008).

2.5.6 Lipopolysaccharides (LPSs)

The hydrophobic anchor domain (Lipid A) forming the core oligosaccharide structure of LPS, in the outer membrane of Gram-negative bacteria, is generally regarded as the most inflammatory/toxic part of LPS (Luke *et al.*, 2010). In some cases of *A. baumannii* infections, severe systemic inflammatory reactions can be triggered and result in symptoms such as systemic inflammatory response syndrome (SIRS) and septic shock (Aguirre-Avalos *et al.*, 2010; Asati *et al.*, 2011; Leung *et al.*, 2006). It has been proposed that LPS (endotoxin) might be associated with the exaggerated host response and consequently the fulminant course of a disease (Leung *et al.*, 2006). Erridge *et al.* (2007) concluded that endotoxins derived from one clinical isolate of *Acinetobacter* gensp. 9 and five of *A. baumannii* were highly potent in stimulating the innate immune response in human monocytic cells through TLR2 and TLR4 inflammatory signalling which may correlate with the pathology of *Acinetobacter* infections.

In addition to the initiation of over-inflammatory reactions, LPS has been shown to be involved in the resistance of *Acinetobacter* to the lytic impact of complement in human serum. According to Garcia *et al.* (2000), 9 of 16 clinical *A. baumannii* isolates showing resistance to normal human serum were rendered susceptible to serum when the production of LPS was reduced by prior ethylene diamine tetraacetic acid (EDTA) treatment. Protection against normal human serum has been critically associated with the expression of full-length LPS on the surface of *A. baumannii* 307-0294 *in vitro* (Luke *et al.*, 2010). In addition, its contribution to the organism's survival in a rat soft tissue infection model *in vivo* was recognized by mutagenesis (Luke *et al.*, 2010). The gene encoding for the glycosyltransferase involved in LPS core biosynthesis in *A. baumannii* is believed to be highly conserved among clinical isolates (Luke *et al.*, 2010).

2.5.7 Outer membrane protein A (OmpA)

OmpA is believed to be the most abundant protein on the cell surface of *A. baumannii* and the most potent cytotoxic molecule in this bacterium (Jin *et al.*, 2011). It does not only act as a major transmembrane porin facilitating the transport or permeability of small molecules such as antibiotics across the membrane but also plays a multi-factorial role in the pathogenesis of this organism (Jyothisri *et al.*, 1999; Lee *et al.*, 2007a; Vashist and

Rajeswari, 2006). It is essential for the adherence of *A. baumannii* ATCC 19606^T to human alveolar epithelial cells (A549) and *Candida albicans* filaments (Gaddy *et al.*, 2009b). It is also believed to be a major contributor to the invasion of epithelial cells according to the significantly less invasion (95.0%) observed with the OmpA-mutant as opposed to wild-type bacteria (Choi *et al.*, 2008c). Gaddy *et al.* (2009b) on the other hand suggested that OmpA is not essential for the attachment of *Acinetobacter* to abiotic surfaces and partially contributes to the development of robust biofilms on plastic. Analysis of electron microscopy images following OmpA inactivation showed clear alterations of the *A. baumannii* 19606^T cell wall, possibly due to the destabilization of the outer membrane. According to Gaddy *et al.* (2009b), the exact role played by this surface protein in the attachment and biofilm formation on abiotic surfaces is, however, unclear.

In addition to its major role in the direct binding of *A. baumannii* to the host epithelial cells, OmpA also imposes cytotoxic effects on these targeted cells. Induction of host cell apoptosis has been shown to be OmpA-dependent where its deletion mutant lacked the ability to cause the same impact (Jin *et al.*, 2011). According to Jin *et al.* (2011), the induction of host cell death by OmpA is mediated by the delivery of this protein via outer membrane vesicles (OMVs) to host cells. The mechanism of the OmpA-mediated apoptosis in the early stage of *A. baumannii* ATCC 19606^T infection can be initiated through entering the mitochondria and nuclei of the invaded cells (Choi *et al.*, 2008b; Choi *et al.*, 2005). Localisation of the OmpA (Omp 38) in the mitochondria of human laryngeal cells (HEp-2) leads to mitochondrial disintegration, which subsequently induces the release of the apoptosis inducing factor (AIF) and cytochrome c. The release of these proapoptotic molecules mediates AIF-dependent and caspase-dependent apoptosis. The caspase cascades and the translocation of apoptosis-inducing factors to the nuclei eventually result in host cell death (Choi *et al.*, 2005). Besides the effect of OmpA on the viability of the host cells through mitochondrial targeting, cell death can occur through nuclear damage (Choi *et al.*, 2008b). *In vitro*, this surface protein was found to directly bind to eukaryotic cells, translocate to the nuclei, and consequently induce their death mediated by the carried monopartite nuclear localization signal (NLS). The NLS sequences are believed to be conserved in the OmpAs of the Acb complex (Choi *et al.*, 2008b). Recombinant OmpA (rOmpA), secreted *in vitro*, displayed a DNAse I-like enzymatic activity that degrades chromosomal DNA when translocated into the nuclei of host cells (Choi *et al.*, 2008a). *In vivo*, embryonic death of the frog *Xenopus laevis*, due to

inability to develop normal embryogenesis, was seen following the injection of the nucleus of frog embryos with this surface protein (Choi *et al.*, 2008a). The disruption of the mucosal lining as a consequence of the death of the epithelial cells may ultimately allow the access of the bacterium or its products to the deep tissues (Choi *et al.*, 2005). A study conducted by Choi *et al.* (2008c) showed that the wild type *A. baumannii* ATCC 19606^T strain produced severe lung pathology in a murine pneumonia model and was capable of disseminating into the bloodstream in a high bacterial load, which was rarely detected with the OmpA-deficient mutant.

One of the common diseases caused by *A. baumannii* is bacteraemia. A key part of the innate immune defense against such pathogens in the blood is known to be the complement system (Kim *et al.*, 2009). OmpA has been shown to be important in enabling *A. baumannii* to evade the direct complement-mediated killing. *A. baumannii* isolates obtained from bacteraemic patients appeared to be resistant to 40% normal human sera (NHS) (Kim *et al.*, 2009). Their mechanism of evading the complement-mediated killing (lysis) was through the surface acquisition of factor H (FH), necessary for the activation of this defense pathway. Factor H interacted with the OmpA (Omp 38) as well as those with molecular sizes of 32, and 24 kDa. OmpA proved particularly important in acquiring the complement regulator FH as shown by the high sensitivity of the OmpA-negative mutant to NHS compared to the wild type strain (Kim *et al.*, 2009). Furthermore, OmpA can induce apoptosis and necrosis of dendritic cells (DCs) by targeting the mitochondria and consequently triggering the production of reactive oxygen species (ROS) (Lee *et al.*, 2010). The death of these cells may impair T cell biology and hence affect adaptive immune responses against *A. baumannii* (Lee *et al.*, 2010).

2.5.8 Siderophores

Secretion of the high affinity iron-chelators (siderophores) and their specific cell surface receptor (iron regulated outer membrane proteins; IROMPs), provide bacterial pathogens with chelated iron essential for their living in otherwise iron-limited conditions such as those within the host during the infection process. They are hence considered as major virulence factors in the pathogenicity of organisms (Eijkelkamp *et al.*, 2011). Infections caused by *Acinetobacter* are indicative of the existence of mechanisms of acquiring iron from their environment. Septicemic patients, due to *A. calcoaceticus*, were found to have

a specific antibody response to IROMPs in their convalescent sera (Smith and Alpar, 1991). Several studies have also reported the production of siderophores in *A. calcoaceticus* (Nudel *et al.*, 2001; Smith and Alpar, 1991; Smith *et al.*, 1990) and *A. haemolyticus* ATCC 17906 (Okujo *et al.*, 1994). Likewise, *A. baumannii* strains have the ability to survive and grow under iron-deficient conditions by secreting siderophores, which may in turn enhance their chances of colonizing the human host (Actis *et al.*, 1993; Echenique *et al.*, 1992; Eijkelkamp *et al.*, 2011; Goel and Kapil, 2001; Goel *et al.*, 1998).

So far, three gene clusters for siderophore biosynthesis have been identified in *A. baumannii*. The first siderophore characterized was in *A. baumannii* ATCC 19606^T and was named acinetobactin (Yamamoto *et al.*, 1994). *A. baumannii* ATCC 19606^T and four of twelve other strains from the same species were found to produce this novel siderophore comprising both the hydroxamate and catechol functional groups (Yamamoto *et al.*, 1994). The biosynthesis and transport of the acinetobactin is believed to be associated with a cluster of 18 genes found in this organism (Mihara *et al.*, 2004). Iron acquisition proteins with siderophore receptors were expressed in the outer membrane of *A. baumannii* ATCC 19606^T when cultured under iron-chelated conditions (Nwugo *et al.*, 2011). Some of these proteins differ from the acinetobactin receptor protein indicating the presence of different siderophore utilization pathways in addition to the acinetobactin-mediated system as a means of acquiring iron. The acinetobactin gene cluster has been found in all the currently sequenced *A. baumannii* strains, namely AB307-294, AB0057, AYE, ACICU, and ATCC 17978 but not in the non-clinical SDF isolate (Adams *et al.*, 2008; Antunes *et al.*, 2011b; Iacono *et al.*, 2008; Smith *et al.*, 2007; Vallenet *et al.*, 2008). The non-human isolate SDF was found to have significantly lower capacity to resist iron starvation than the clinical *A. baumannii* isolates (Antunes *et al.*, 2011a). In addition, SDF was the only strain not to show an iron-chelating activity when grown in an iron-depleted environment as opposed to all the clinical isolates (Antunes *et al.*, 2011a). Recent genome analysis of *A. baumannii* SDF, AYE, ATCC 17978, ACICU, ATCC 19606^T, as well as another 50 genotypically diverse clinical isolates of the same species have revealed the coding potential for the utilization/expression of two siderophores other than the siderophore acinetobactin (Antunes *et al.*, 2011a), which support previous reports suggesting the production of more than one siderophore by this species (Dorsey *et al.*, 2003; Dorsey *et al.*, 2004; Mihara *et al.*, 2004; Zimbler *et al.*, 2009). The two siderophores include one conserved in all *A. baumannii* strains currently sequenced apart

from the *A. baumannii* SDF, as well as another siderophore found only in ATCC 17978 (Antunes *et al.*, 2011b) and whose cluster has previously been identified (Zimbler *et al.*, 2009). This is in agreement with another study indicating that iron-uptake systems found in *A. baumannii* can vary between different clinical isolates (Dorsey *et al.*, 2003). The fact that several iron-uptake systems are widely distributed among diverse *A. baumannii* clinical isolates underline the importance of these systems and their potential role in the pathogenicity of this bacterium.

2.6 *Acinetobacter* infections and risk factors

The various virulence factors described above offer some explanation to the clinical relevance of *Acinetobacter* in response to hospitals reports, which have named the organism as the cause of a wide spectrum of hospital-acquired infections such as endocarditis, Keratitis or dophthalmitis, meningitis, skin and wound infections, urinary tract infections, nosocomial and community-acquired pneumonia, and blood stream infections (Garnacho-Montero *et al.*, 2005; Gaynes and Edwards, 2005; Levy *et al.*, 2005; Metan *et al.*, 2007; Rizos *et al.*, 2007; Wisplinghoff *et al.*, 2004). Those caused by *A. baumannii* represent about 10% of all nosocomial infections (Dijkshoorn *et al.*, 2007; Joly-Guillou, 2005; Peleg *et al.*, 2008). According to the voluntary surveillance scheme adopted by the Health Protection Agency (2011), the total number of *Acinetobacter* spp. bacteraemia reports, in England, Wales, and Northern Ireland, was 1139 in 2006 and 779 in 2010. The attributable mortality rate of *Acinetobacter* blood stream infections is reported to be 7.8% (Blot *et al.*, 2003), while Lee *et al.* (2007b) described a higher percentage (21.8%) associated with MDR *A. baumannii*. With respect to *A. baumannii* overall infections, a systematic review of cohort and matched case-control studies revealed that attributable mortalities ranged from 10% to 43% in intensive care units (Falagas *et al.*, 2006).

According to Fournier and Richet (2006), the majority of *Acinetobacter* infections occur in critically-ill patients treated in ICUs. Infections associated with this organism in these settings are mostly severe and septic shock can be incurred as a result (Taccone *et al.*, 2006). The risk of mortality to these patients has been shown to be 4-fold greater than uninfected patients (García-Garmendia *et al.*, 2001). They are particularly vulnerable as they are likely to be immunocompromised due to advanced age, prolonged stays in

intensive care units, prior antibiotic therapy, need for mechanical ventilation or invasive devices, severe underlying diseases, surgery or arterial and venous annulations, or cord blood transplantation (Bergogne-Berezin and Towner, 1996; Jung *et al.*, 2010; Saavedra *et al.*, 2002; Torres *et al.*, 1990). Risk factors predisposing *Acinetobacter* infections also include low birth weight (< 2500g), as defined by the World Health Organization (World Health Organization WHO, 2011), and prematurity (gestation age < 37 week). According to the national statistics on births in England and Wales, the number of live births in 2009 was 706,248; of those 7.2% had a low birthweight (< 2,500 grams) and 1.2% had a very low birthweight (< 1,500 grams) (Office for National Statistics, 2009).

Acinetobacter is known to be one of the organisms that cause severe infections in neonatal intensive care units (NICUs). Outbreaks of pneumonia, skin infections, urine infections, bacteraemia, septicaemia, and meningitis, due to this bacterium, have been reported worldwide (Anyebuno and Newman, 1995; Bernards *et al.*, 1997; Chan *et al.*, 2007; de Beaufort *et al.*, 1999; Horrevorts *et al.*, 1995; Mader *et al.*, 2010; McDonald *et al.*, 1998; Mishra *et al.*, 1998; Morgan and Hart, 1982; Oto *et al.*, 1991; Pillay *et al.*, 1999; Saleem *et al.*, 2010; Schloesser *et al.*, 1990; Siegman-Igra *et al.*, 1993; Simmonds *et al.*, 2009; Touati *et al.*, 2009; Vivanco and Figueroa, 1991; von Dolinger de Brito *et al.*, 2005). In these reports, outbreaks of blood stream infections have been described to last from 1 day to 30 months, involving up to 53 neonates in a single outbreak. Although *A. baumannii* has been isolated, the causative agents were not limited to this species and included *A. calcoaceticus*, *A. junii*, *A. ursingii*, *Acinetobacter* gensp. 14, *Acinetobacter* gensp. 3, as well as *Acinetobacter* whose species identities were not specified. Of note is that some of the isolates obtained showed multidrug-resistance (Pillay *et al.*, 1999; Simmonds *et al.*, 2009; von Dolinger de Brito *et al.*, 2005). While successful treatment was achieved with some cases, others failed. A retrospective investigation recorded 47% crude mortality of neonatal *Acinetobacter* sp. infections during a five-year period (Saleem *et al.*, 2010). Seventy percent of the neonates died within 4 days of obtaining positive *Acinetobacter* cultures. Furthermore, Pillay *et al.* (1999) described 22% attributable mortality during a 16-day outbreak of MDR *Acinetobacter* spp. infections in 9 pre-term neonates. These neonates were presented with clinical symptoms of sepsis, pneumonia, disseminated intravascular coagulopathy, and abdominal distension. Contaminated catheters and suction bottles were presumed to be the source of the outbreak. Mishra *et al.* (1998) retrospectively described an outbreak that occurred over five months and involved 53

septicemia cases including full-term neonates. Three neonates had meningitis complications while five experienced necrotising enterocolitis. Bleeding manifestations were observed in six additional cases. The overall mortality was 13.9%.

Neonates in ICUs often require supporting medical devices and antibiotic treatment (Westerbeek *et al.*, 2006). The intensive care treatment combined with their immature immune system may render them highly vulnerable for colonisation and possibly subsequent infections when exposed to opportunistic organisms such as *Acinetobacter*. This could be particularly alarming considering that common antibiotics may not be effective against the MDR strains. The exposure of these neonates to this bacterium is often through the respiratory route, or skin at the insertion site of an intravenous line, catheter, or surgery. Another possible risk of acquiring *Acinetobacter* is through ingestion. Although it has not been highlighted before, the potential risks of this exposure deserves a great deal of understanding and assessment. These may not only be local gastrointestinal infections but also systematic infections where other organs and tissue might be involved especially if the bacterium has the potential ability to translocate from the immature gastrointestinal system of the neonate. In their meeting in 2006, FAO-WHO called for research on sources and vehicles, including PIF, of infections by organisms of concern to neonatal health and among them was *Acinetobacter*. In response to this call, the aim defined in the next section was set for this project.

AIMS & OBJECTIVES

The overall aim of this research project was to characterise the risk factors of neonatal exposure to *Acinetobacter* spp. through the administration of infant formula via the nasogastric tract. This was achieved according to the following objectives:

1- Develop a new chromogenic-selective *Acinetobacter* medium.

2- Screen powdered infant formula for *Acinetobacter* contamination using the developed medium and other selective media.

3- Examine the long-term persistence of *Acinetobacter* desiccated in infant formula.

4- Investigate biofilm formation of *Acinetobacter* inside enteral (nasogastric) feeding tubes using practices similar to those applied in the healthcare and inspect the contamination magnitude of subsequent fresh enteral feeds.

5- Examine the effect of different physiological elements of the gastrointestinal system on the viability of *Acinetobacter*.

6- Characterise the pathogenicity of *Acinetobacter* and its attributes.

7- Screen for selected virulence genes in *Acinetobacter*.

CHAPTER 3. DETECTION OF *ACINETOBACTER* IN PIF USING DIFFERENTIAL-SELECTIVE MEDIA

3.1 INTRODUCTION

Although *Acinetobacter* has previously been isolated from dehydrated infant formula (Cawthorn *et al.*, 2008; Chap *et al.*, 2009; Miled *et al.*, 2010), its prevalence in PIF has not been examined before. The U.S. Food and Drug Administration (Food and Drug Administration FDA) method, adopted in 2002 for screening PIF, was designed to detect of *Cronobacter sakazakii* and is not specific to *Acinetobacter*. The method principally involved the resuscitation of the sub-lethally injured bacteria followed by an enrichment stage, the detection on agar media, and eventually biochemical testing. In this mixed culture environment of the PIF, the predominant microflora may outgrow the minorities that are present in smaller numbers during the resuscitation and enrichment steps. This may consequently decrease the chances of picking the fewer colonies recovered on the agar plates leading to a false negative result. Therefore, the detection of *Acinetobacter* whether in PIF, in the manufacturing, or clinical setting, from mixed microbial populations requires an isolation method which is both specific and sensitive. Selective/differential media in this respect are of great use as they offer additional simplicity, specificity and quick identification over the solely selective media.

A differential/selective medium for the isolation of *Acinetobacter* was first described by Mandel *et al.* (1964). The medium consisted of soy peptone, lactose, pancreatic digest of casein, bile salts, sodium chloride, maltose, agar, and bromocresol purple as a pH indicator. In (1983), Holton modified Mandel's medium by substituting desiccated ox bile for bile salts. *Acinetobacter* spp. were distinguished by their pink colonies with mauve background on both Mandel and Holton's media. Two years later, MacFaddin (1985) replaced the maltose in Mandel's agar with sucrose, and removed the bromocresol purple and lactose. In addition, mannitol, fructose, phenol red, and phenylalanine were added. Cefsulodin, ampicillin, and vancomycin were also incorporated into the medium as the antibacterial agents. Colonies with pale lavender colour and yellow background were regarded as potential *Acinetobacter*. This medium was named Herellea agar (MacFaddin, 1985). Another selective *Acinetobacter* medium was designed by Flint in (1993) and was based on the addition of di-ammonuim hydrogen orthophosphate as an inorganic source of nitrogen, and ethanol as a carbon source. The selective agents were ethanol, bile salts, cycloheximide and tetracycline. A differential selective medium, similar to that of Holton, was then developed and known as Leeds *Acinetobacter* medium (LAM) (Jawad *et al.*,

1994). LAM contained agar, neutralized soy peptone, acid casein hydrolysate, sodium chloride, sucrose, D-(-)-fructose, L-phenyalanine, D-mannitol, and ferric ammonium citrate. Instead of inhibitory bile salts, three antibiotics (cefsoludin, vancomycin, and cephradine) were included. The differential indicator was phenol red which changes to mauve when the pink *Acinetobacter* colonies grow and produce highly alkaline end-products. According to Jawad, *et al.* (1994), LAM was more selective than Herellea and Holton's agar media that were either poorly selective or too inhibitory.

The most recent medium available for the detection of *Acinetobacter* spp. is CHROMagar *Acinetobacter* . It is also the first chromogenic medium, specific for this organism, on the market. It is designed to permit the growth and the differentiation of *Acinetobacter* spp. from other Gram-negative bacteria (CHROMagar Microbiology, 2008). Some Enterobacteriaceae may grow and produce blue to metallic blue colonies while some other non-fermenters such as *Stenotrophomonas* spp. and *Pseudomonas* spp. appear red pigmented similar to *Acinetobacter*. The medium contains mineral salts, yeast extract and peptone, as well as a non-specified chromogen. A liquid supplement containing growth and regulation factors is also added. The components of this supplement have not been reported. Another different selective mixture can also be added if MDR-*A. baumannii* is the target strain (CHROMagar Microbiology, 2008).

With chromogenic media, the detection and differentiation of organisms is based on the presence of characteristic enzyme activities shown by these organisms (Manafi, 1996, & 2000; Manafi and Kneifel, 1990; Manafi and Kremsmaier, 2001; Orenga *et al.*, 2009). Principally, synthetic enzymatic substrates are incorporated into the media and the hydrolysis of these substrates by specific enzymes results in color changes of the bacterial colonies that are positive for this activity. 5-bromo-4-chloro-3-indolyl-b-D-ribofuranoside (x-riboside) is a novel chromogenic substrate synthesized for the differentiation and identification of Gram-negative bacteria and evaluated by Butterworth *et al.* (2004). Hydrolysis of this substrate in air, by the β–ribosidase enzyme, results in blue-green complexes that are intense and do not diffuse in the medium. Another novel chromogen named DHF-riboside (3′,4′-dihydroxyflavone-4′-β-D-ribofuranoside) can also be used for the detection of β-ribosidase through the formation of black colonies.

Based on the application of chromogens (Butterworth *et al.*, 2004) and also on antibiotic sensitivity testing, a chromogenic-selective *Acinetobacter* medium named Druggan-

Dimmer Agar (DDA) was developed in collaboration with Dr Patrick Druggan (Oxoid Thermofisher, UK). It was aimed primarily for investigating the prevalence of *Acinetobacter* spp. in PIF especially with the limited choice of specialised agar for this organism. However, it could be potentially applied for clinical and environmental sampling for a robust and quick presumptive identification of the bacterium. A modified enrichment broth was also designed to encourage the recovery and growth of *Acinetobacter* spp. during the survey of 63 PIF products collected from 17 different countries. Isolates were cultured on both the DDA and LAM media for comparison. These agar media were also characterized and compared to the new CHROMagar *Acinetobacter* medium, which had not been introduced to the market prior to the survey starting.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of the test media

Description of the media used in this study is detailed in **Chapter 1**.

3.2.2 Development of the DDA medium

The final formula of the DDA medium described in **Chapter 1** was developed according to the following stages:

Stage 1: Formulation of the basal DDA medium.
Stage 2: Antibiotic sensitivity testing to choose a selective antimicrobial agent for the medium.
Stage 3: Evaluation of the DDA medium during a survey of 63 PIF products.
Stage 4: Modification of the DDA medium following further antibiotic sensitivity testing.
Stage 5: Characterization of the DDA medium in comparison to other *Acinetobacter* media.

3.2.2.1 Formulation of the basal DDA medium

The basal DDA medium was designed by Patrick Druggan (Oxoid Thermofisher, UK) and consisted of 12g/l tryptone, 1g/l yeast extract, 5g/l soy peptone, 0.15g/l sodium deoxycholate, and agar 10g/l. The agar plates were then streaked with thirty-five *Acinetobacter* strains to check their recovery on the medium.

During preliminary studies, the positive and negative recovery of *Acinetobacter* strains on the basal DDA medium was examined in the presence of 0.15g/l sodium deoxycholate to inhibit Gram-positive bacteria. In order to enhance the growth of some strains, sodium chloride (NaCl) was also added at 1 and 5g/l. Before selecting a suitable chromogen, the appearance of one *Pseudomonas aeruginosa*, four Enterobacteriaceae, and sixteen *Acinetobacter* strains on DDA containing X-riboside (0.1g/l), and DHF-riboside (0.4g/l) was separately observed. The latter substrate was combined with 0.5g/l ferric ammonium citrate.

3.2.2.2 Antibiotic susceptibility testing and selectivity of the basal DDA medium

To select an appropriate selective antibacterial agent for the DDA medium, the antibiotic sensitivity of a number of *Acinetobacter* and non-*Acinetobacter* strains, on the basal DDA medium, was tested in accordance with the British Society for Antimicrobial Chemotherapy guidelines using the agar dilution method (BSAC, 2001). The antibiotics employed and their spectrum of activity are shown in **Table 3.1**. Antibiotic solutions were prepared in the appropriate solvents and stored at -20°C for a maximum of 5 days before use. Temocillin was prepared and added to the agar medium on the same day. Once the DDA medium was sterilized, it was allowed to cool to 50°C before adding the antibiotics. Antibiotic susceptibility tests were performed in duplicate/triplicate. Growth on antibiotic-free TSA and DDA media was also included. The surface of the agar plates was dried before inoculation. Overnight cultures (150µl) of the test strains in TSB were dispensed into a microtitre tray. A replicator was used to inoculate around 2µl of the bacterial suspensions onto TSA and DDA plates. The inocula were then allowed to be absorbed into the agar before incubation for 24h at 30°C and 37°C, for *Acinetobacter* and the Enterobacteriaceae respectively. Other organisms were also incubated at suitable temperatures. The control strain was *Escherichia coli* NCTC 10418.

Table 3.1 List of the antibiotics tested and their spectrum of activity.

Antibiotic	Target organisms
Aztreonam	Gram negatives
Cefotaxime	Gram-positives and Gram-negatives
Cefsulodin	Gram-positives and Gram-negatives
Ceftazidime	Gram-positives and Gram-negatives
Ciprofloxacin	Gram-positives and Gram-negatives
Enoxacin	Gram-positives and Gram-negatives
Lincomycin	Gram-positives
Lomefloxacin	Gram-positives and Gram-negatives
Netilmicin	aerobic Gram-negatives
Norfloxacin	Gram-positives and Gram-negatives
Pipemidic acid	Gram-positives and Gram-negatives
Temocillin	Gram-positives and Gram-negatives
Vancomycin	Gram-positives

3.2.2.3 Survey of powdered infant formula

A total number of 63 PIF products **(Table 3.2)**, aimed at neonates from birth to the age of 6 months, were collected from 17 different countries, and were screened for contamination with *Acinetobacter* using the procedure described in **section 3.2.2.9.** Briefly, each PIF sample was analysed by reconstituting 25g of the powder in 225ml BPW, followed by the enhancement of bacterial growth in an appropriate enrichment broth medium. Enriched cultures were then inoculated onto DDA and LAM for a comparative assessment.

Table 3.2 List of powdered infant formula products screened for the presence of *Acinetobacter* spp.

Product number	PIF product	Country of manufacture
1	NESTLE NAN 1	Germany
2	ULKER HERO BABY 1	Germany
3	MILUPA CONFORMIL 1	Germany
4	NUTRICIA BEBELAC 1	Germany
5	SAHHA INFANT FORMULA 1	Jordan
6	SAHHA PREMA 1	Jordan
7	SAHHA COMFORT	Jordan
8	NESTLE NIDINA CONFORT 1	Germany
9	PULEVA PEQUES 1	Spain
10	HERO BABY INICIA	Spain
11	HERO SUNAR	Czech Republic
12	NUTRICIA NUTRILON	Czech Republic
13	SNOW BRAND, STEP 1	Japan
14	ANMUM INFACARE RUMUSAN BAYI	New Zealand
15	FRISOLAC 1 GOLD	Holland
16	DULAC MAXI-Q INFANT FORMULA	New Zealand
17	NESTLE LACTOGEN 1	Philippines
18	NUTRICIA BEBELAC INFANT FORMULA	New Zealand
19	ENFALAC A+ INFANT FORMULA MILK STEP 1	Thailand
20	DUMEX MAMEX GOLD INFANT FORMULA STEP 1	New Zealand
21	NESTLE NAN 1 INFANT FORMULA	Holland
22	SIMILAC NEOSURE IQ	Spain
23	DUTCH LADY INFANT FORMULA STEP 1	Holland
24	MY BOY ELDOLZAN 1	Holland
25	NESTLE GUIGOZ 1	Switzerland
26	BEBELAC 1	Holland
27	ORGANIC INFANT FORMULA	Korea
28	NUSEIN GOAT MILK INFANT FORMULA	Korea
29	INFANT FORMULA CHOICE	Korea
30	EMERALD PREMIUM	Korea
31	SCIENCE 1	Korea
32	GOAT MILK INFANT FORMULA	Korea
33	BABY SCIENCE QT	Korea
34	PREMIUM 1	Korea
35	PREMIUM 2	Korea
36	PREMIUM FORMULA XO	Korea
37	GOAT MILK BASED ORGANIC INFANT FORMULA	Korea
38	EMERALD	Korea
39	MOJUNG	Korea
40	SU 1	Korea
41	SCIENCE 2	Korea
42	IQ	Korea
43	EXTRA SELECTION	Korea
44	SU 2	Korea
45	SUPER PREMIUM	Korea
46	PREMIUM	Korea
47	MILUPA APTAMIL 1 PREMIUM	Portugal
48	NESTLE NAN 1 H.A. PREMIUM	Portugal
49	NESTLE NIDINA 1 H.A.	Portugal
50	NEOLATTE 1	Italy
51	ABBOTT ISOMIL 1	Holland
52	ABBOTT SIMILAC ® ADVANCE	Ireland
53	NESTLE NAN H.A. 1	Switzerland
54	PROMIL GOLD (FOLLOW-ON FORMULA)	Ireland
56	BEBELAC 1	Holland
57	GALLIIA 1	France
58	NUTRIBEN	France
59	ABBOTT ISOMIL 1	Holland
60	FRISOLAC 1 GOLD	Holland
61	APTAMIL1	Turkey
62	MODILAC EXPERT HA	France
63	LACTUM 1	Turkey

The PIF products were designed for neonates from birth to the age of 6 months and were collected from 17 different countries to investigate the presence of *Acinetobacter*.

3.2.2.4 Optimization of the enrichment stage prior to the survey of PIF

In order to evaluate the prevalence of *Acinetobacter* in PIF, an enrichment broth was required. Preliminary experiments were conducted to choose an efficient enrichment broth to promote the growth of *Acinetobacter* present in PIF following their resuscitation stage in buffered peptone water (BPW). The growth of *Acinetobacter* spp. in Enterobacteriaceae enrichment (EE) broth, modified M9, and Baumann broth (BB) (Baumann, 1968) was evaluated. First, viable counts of overnight cultures in BPW were determined by the Miles & Misra method. One millilitre of the overnight cultures was then transferred to the enrichment broths before incubation for 18h at 30°C. Next, the plate count (Miles & Misra) was performed and the percentage recovery in each broth was then calculated in relation to BPW. This procedure was performed once.

The next step was optimizing the incubation conditions in terms of aeration. To do so, overnight cultures of 13 *Acinetobacter* strains were inoculated (1:10) into the chosen enrichment broth (M9), which was then incubated statically and in a rotary shaker (280 rpm) at 30°C for 18h. Cell counts were subsequently determined by Miles & Misra, and the percentage recovery of the strains with/without vigorous aeration was obtained comparatively to the viable counts in BPW (conducted once).

3.2.2.5 Survey procedure

To ensure aseptic handling of the PIF samples, the surface of weighing boats was disinfected with 70% (v/v) ethanol and then dried. A spatula was also flame sterilized and allowed to cool before 25g of each sample was added to 225ml of sterile BPW. Samples were then incubated overnight at 30°C. The following day, an aliquot (0.1 ml) of these reconstituted samples was transferred into 9.9ml of the modified M9 broth in baffled flasks, which were next then incubated overnight at 30°C in a rotary shaker at 280 rpm.

Following the 24h incubation of the M9 broths in a rotary shaker, 0.1ml was streaked on DDA and LAM media. The plates were incubated at 30°C for 48h. Growth on the two media was then recorded and the colour of colonies noted. Presumptive Enterobacteriaceae strains appeared black on DDA plates, whereas presumptive *Acinetobacter* and Gram-positive strains were non-pigmented on this medium (Butterworth *et al.*, 2004). All the isolates were next subcultured on TSA for 24h at 30°C.

Once the isolates from PIF were grown on TSA plates, the morphological appearance was observed and compared with control *Acinetobacter* strains grown on TSA. In addition, they were provisionally identified under the microscope using Gram staining. Since *Acinetobacter* can be Gram-variable, all Gram-positive coccobacilli and Gram-negative rod shaped isolates were further identified. Additionally, all the Gram-negatives were tested for the oxidase reaction. Subsequently, the oxidase-negative isolates were plated on VRBGA, which is semi-selective for Enterobacteriaceae. Although the growth of non-enteric and Gram-positive organisms is normally inhibited due to the presence of bile salts and crystal violet, some *Acinetobacter* strains can form small colonies (2mm in diameter) on this medium. Once presumptive Enterobacteriaceae or *Acinetobacter* spp. were identified, API 20E biochemical profiles (bioMerieux Ltd., UK) were used for primary identification. The isolates that did not grow on VRBGA were considered as non-enteric. Therefore, the appropriate API kit to use was API 20NE for Gram-negative non-Enterobacteriaceae (oxidase-negative) strains including *Acinetobacter* spp. For further confirmation of the identification, isolates of interest (Gram-negatives including *Acinetobacter*) were sent to Accugenix Inc. (Newark, USA) for partial 16S rDNA gene sequencing.

3.2.2.6 Evaluation and modification of the DDA medium

The performance of DDA medium, following the addition of 10mg/l lincomycin, was evaluated in comparison to LAM during the survey of PIF products. In light of the results, the selectivity of the DDA medium was further optimized by increasing the concentration of lincomycin to 20mg/l lincomycin and incorporating aztreonam (2mg/l) into the medium.

3.2.2.7 Characterization of DDA and other selective *Acinetobacter* media

This part of the study was composed of a comparative assessment of DDA, CHROMagar *Acinetobacter,* and LAM in terms of selectivity, sensitivity, percentage recovery (PR) and the detection limit.

3.2.2.7.1 Selectivity and sensitivity

Bacterial cultures were grown in TSB for 18h (stationary phase) at 30 or 37°C, as appropriate. Their turbidity was then adjusted to McFarland Standard 0.5 (~ $1.5x10^8$ cfu/ml) before being diluted to 10^5 cfu/ml in sterile saline. Inocula were simple streaked onto the three test media and the control (TSA) using a sterile 10µl loop. Plates were incubated at 37°C for 24h. Based on presence or absence, the selectivity of the media was expressed as the percentage of the negative recovery of non-*Acinetobacter* strains. In terms of sensitivity, the percentage of the positive number of *Acinetobacter* that grew was recorded.

3.2.2.7.2 Percentage recovery (PR)

Percentage recovery was analysed by performing the Miles and Misra plate count method on all media plus TSA. TSA was used as a non-selective control medium. Recoveries <50% were deemed a failure for the selective medium.

$$PR = \frac{\text{Viable count on selective agar}}{\text{Viable count on TSA}} \times 100$$

3.2.2.7.3 Detection limit

The detection limit was assessed by the lowest number of *Acinetobacter* each medium could detect. Three *Acinetobacter* strains (1096, 415, and 418) were grown overnight (18) at 37°C in sterile ready-to-feed formula (Cow & Gate 1). The next day, fresh Cow & Gate 1 was spiked with 1000, 100, 10, 1, 0.1 cfu/ml of *Acinetobacter* cultures. Using the spread plate method, the viable counts were determined by aseptically spreading 0.1ml of the spiked ready to feed formula on the test media. The plates were incubated at 37°C for 24h before enumeration.

3.3 RESULTS

3.3.1 Formulation of the basal DDA medium

The basal DDA medium containing tryptone, yeast extract, soy peptone, sodium deoxycholate, and agar was initially evaluated for the recovery of thirty-five *Acinetobacter* strains **(Table 3.3)**. The majority (94.3%) of strains were able to grow. Exceptions were two type strains; *A. radioresistens* (ATCC 43998^T) and *A. calcoaceticus* (NCTC 12983^T). However, the removal of sodium deoxycholate, which was originally incorporated into the medium to inhibit the growth of Gram-positive bacteria, allowed the recovery of the two sensitive strains **(Table 3.3)**. The addition of 2 and 5g/l NaCl into the medium did not enhance the growth of these two strains as they remained inhibited **(Table 3.4)**. Sodium deoxycholate was consequently excluded from the basal medium.

The colour exhibited by the colonies of *Acinetobacter* in comparison to other Gram-negative bacteria was assessed in the presence of the chromogen X-riboside and DHF-riboside. One *P. aeruginosa*, four strains from the family Enterobacteriaceae, and 16 *Acinetobacter* strains were streaked onto the DDA medium. Any pigmentation was recorded and checked with the descriptions reported by Butterworth *et al.*, (2004). Colonies of those from the Enterobacteriaceae showed blue-green pigmentation in the presence of the X-riboside substrate. Conversely, *Acinetobacter* and *Pseudomonas* were non-pigmented **(Table 3.5)**. This non-pigmentation was also observed with *Acinetobacter* when DHF-riboside was added. The remaining non-*Acinetobacter* strains appeared black on the medium **(Table 3.5)**.

Table 3.3 Positive and negative recovery of *Acinetobacter* strains on DDA with and without the addition of 0.15g/l sodium deoxycholate.

Species	Strain number	DDA medium	
		with SD*	without SD
A. baumannii	1095	+++	+++
A. baumannii	1096	+++	+++
A. baumannii	1098	+++	+++
A. baumannii	1099	+++	+++
A. baumannii	1102	+++	+++
A. baumannii	1109	+++	+++
A. baumannii	1110	+++	+++
A. baumannii	1111	+++	+++
A. baumannii	1112	+++	+++
A. baumannii	1113	+++	+++
A. baumannii	1114	+++	+++
A. baumannii	1115	+++	+++
A. baumannii	1116	+++	+++
A. baumannii	1117	+++	+++
A. baumannii	1118	+++	+++
A. baumannii	1119	+++	+++
A. baumannii	1120	+++	+++
A. baumannii	1121	+++	+++
A. baumannii	1122	+++	+++
A. baumannii	1123	+++	+++
A. baumannii	1124	+++	+++
A. baumannii	1125	+++	+++
A. baumannii	1126	+++	+++
A. baumannii	1127	+++	+++
A. calcoaceticus	418	+++	+++
A. calcoaceticus	1097	+++	+++
A. calcoaceticus	1103	---	+++
Acinetobacter gensp. 3	415	+++	+++
A. haemolyticus	1337	+++	+++
A. junii	1335	+++	+++
A. johnsonii	1336	+++	+++
A. lwoffii	1338	+++	+++
A. radioresistens	1340	---	+++
A. schindleri	1339	+++	+++
A. ursingii	1334	+++	+++

Sodium deoxycholate (0.15g/l) was originally incorporated into the basal DDA medium to inhibit the growth of Gram-positive bacteria. The majority (94.3%) of strains were able to grow in its presence, while 100.0% was achieved following its removal. * Sodium deoxycholate, +++ triplicate positive recovery, --- triplicate negative recovery.

Table 3.4 Positive and negative recovery of *Acinetobacter* strains on DDA containing 2 and 5g/l NaCl.

Species	Strain number	DDA (+ NaCl)	
		2g/l	5g/l
A. baumannii	1095	+++	+++
A. baumannii	1096	+++	+++
A. baumannii	1098	+++	+++
A. baumannii	1099	+++	+++
A. baumannii	1102	+++	+++
A. baumannii	1109	+++	+++
A. baumannii	1110	+++	+++
A. baumannii	1111	+++	+++
A. baumannii	1112	+++	+++
A. baumannii	1113	+++	+++
A. baumannii	1114	+++	+++
A. baumannii	1115	+++	+++
A. baumannii	1116	+++	+++
A. baumannii	1117	+++	+++
A. baumannii	1118	+++	+++
A. baumannii	1119	+++	+++
A. baumannii	1120	+++	+++
A. baumannii	1121	+++	+++
A. baumannii	1122	+++	+++
A. baumannii	1123	+++	+++
A. baumannii	1124	+++	+++
A. baumannii	1125	+++	+++
A. baumannii	1126	+++	+++
A. baumannii	1127	+++	+++
Acinetobacter gensp. 3	415	+++	+++
A. calcoaceticus	418	+++	+++
A. calcoaceticus	1097	+++	+++
A. calcoaceticus	1103	---	---
A. haemolyticus	1337	+++	+++
A. junii	1335	+++	+++
A. johnsonii	1336	+++	+++
A. lwoffii	1338	+++	+++
A. radioresistens	1340	---	---
A. schindleri	1339	+++	+++
A. ursingii	1334	+++	+++

Sodium chloride (2 and 5g/l) was included in the basal DDA medium (containing sodium deoxycholate) to enhance the growth of the sensitive strains. However, these strains remained inhibited. +++ triplicate positive recovery, --- triplicate negative recovery.

Table 3.5 Colony colour of *Acinetobacter* and other Gram-negative organisms cultured on DDA containing X-riboside and DHF-riboside.

Strain	Strain number	Colony colour on DDA medium	
		with X-riboside	with DHF-riboside
A. baumannii	1098	Off-white	Off-white
A. baumannii	1099	Off-white	Off-white
A. baumannii	1102	Off-white	Off-white
A. baumannii	1095	Off-white	Off-white
A. baumannii	1096	Off-white	Off-white
A. calcoaceticus	418	Off-white	Off-white
A. calcoaceticus	1097	Off-white	Off-white
A. calcoaceticus	1103	Off-white	Off-white
Acinetobacter gensp. 3	415	Off-white	Off-white
A. haemolyticus	1337	Off-white	Off-white
A. johnsonii	1336	Off-white	Off-white
A. junii	1335	Off-white	Off-white
A. lwoffii	1338	Off-white	Off-white
A. radioresistens	1340	Off-white	Off-white
A. schindleri	1339	Off-white	Off-white
A. ursingii	1334	Off-white	Off-white
Enterobacter cloacae	597	Blue-green	Black
Escherichia coli	605	Blue-green	Black
Klebsiella pneumoniae	1013	Blue-green	Black
Pseudomonas aeruginosa	1169	Off-white	Black
Serratia liquefaciens	579	Blue-green	Black

The chromogens X- and DHF-riboside were compared to enable better differentiation of *Acinetobacter* from other Gram-negative bacteria. All *Acinetobacter* strains exhibited non-pigmented colonies compared to the other Gram-negative bacteria in the presence of these substrates. Differentiation between the *P. aeruginosa* 1169 and *Acinetobacter* strains was achieved with DHF-riboside based on the black pigmentation produced by the former organism.

3.3.2 Antibiotic sensitivity testing

Acinetobacter strains were tested for antibiotic sensitivity at concentrations thought to be required for minimum inhibition. The antibiotics and the susceptibilty results are presented in **Table 3.6**. In general, all *A. baumannii* strains tended to have similar antibiotic resistance trends which were greater than those of non-*A. baumannii* strains. *Acinetobacter* gensp. 3 (415) was sensitive to 16.7% (2/12) of the antibiotics tested, while *A. calcoaceticus* 418 showed 41.7% (5/12) sensitivity. Similar to the food isolate 418, the clinical *A. calcoaceticus* 1097 was susceptible to the same antibiotics except 0.5mg/l Netilmicin. Seventeen of the twenty-four *A. baumanii* isolates, including the type strain (1102), showed resistance to 100.0% (12/12) of the antibiotics included. The only sensitivity shown by the remaining *A. baumanii* strains (7/25) was to 256mg/l vancomycin. The type cultures of *A. haemolyticus*, *A. lwoffii*, and *A. schindleri* showed particular sensitivity and were inhibited by 58.3% (7/12), 75.0% (9/12), and 91.6 % (11/12) of the antibiotics respectively **(Table 3.6)**. *A. schindleri* was the only strain whose recovery was affected by the presence of aztreonam (4mg/l) in DDA medium **(Table 3.6)**.

Antibiotics (aztreonam, ceftazidime, ciprofloxacin, norfloxacin, and temocillin) which resulted in a maximum inhibition of 3 *Acinetobacter* strains and known for their activity against Gram-positive bacteria were further tested against a sample of these organisms **(Table 3.7)**. Temocillin inhibited 5 of the 6 Gram-positive bacteria while none were inhibited by ceftazidime. In contrast, the presence of 0.5mg/l norfloxacin prevented the growth of *Bacillus cereus*, *Enterococcus faecalis*, and *Lactobacillus plantarum* which was the only Gram-positive strain to be also sensitive to ciprofloxacin (0.05mg/l). Apart from the temocillin, none of the antibiotics mentioned above showed high selectivity against the examined Gram-positive organisms **(Table 3.7)**. However, due to the unstability of temocillin (Dr. Patrick Druggan, personal communication), it was deemed appropriate to investigate another choice of antibiotics. Subsequently, a range of lincomycin concentrations from 10mg/l to 260mg/l was next tested against 7 Gram-positive strains as well as 34 acinetobacters to ensure both high sensitivity and selectivity of the medium under development **(Table 3.8)**. At 10mg/l lincomycin, there was a 100% recovery of the 34 *Acinetobacter* strains with a 100% inhibition of the 7 Gram-positive strains. The increase of the antibiotic concentration to 20mg/l prevented the growth of *A. radioresistens*. At 60mg/l *A. lwofii* was also unable to grow. Therefore, lincomycin

(10mg/l) was incorporated into the preliminary formula of the DDA as the selective agent against Gram-positive bacteria. The medium was then evaluated during a survey of PIF products for the presence of *Acinetobacter* spp.

The PIF analysis was an opportunity to assess the differentiation characteristics of the DDA as a chromogenic medium using not only Gram-negative strains from culture collections as described earlier but also natural PIF isolates for an in-use comparison. In addition, uninhibited Gram-negative isolates that might be found in PIF could be clinically significant and important to report. For these reasons, no antibiotics against the non-*Acinetobacter* Gram-negative organisms were added to the DDA medium at this stage.

Table 3.6 Antibiotic sensitivity of *Acinetobacter* spp. on the DDA medium.

Species	Strain number	Control medium		Aztr.	Cefo.	Cefs.	Cefta.	Cipr.	Eno.	Lom.	Net.	Nor.	Pip.	Tem.	Van.
		TSA	DDA	4mg/l	2mg/l	64mg/l	1mg/l	0.05mg/l	2mg/l	0.25mg/l	0.5mg/l	0.5mg/l	64mg/l	100mg/l	256mg/l
A. baumannii	1095	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++ -
A. baumannii	1096	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1098	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1099	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1102	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1109	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1110	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1111	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	---
A. baumannii	1112	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	---
A. baumannii	1113	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1114	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1115	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1116	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	---
A. baumannii	1117	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1118	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	---
A. baumannii	1119	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1120	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	---
A. baumannii	1121	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1122	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1123	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1124	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	---
A. baumannii	1125	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1126	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. baumannii	1127	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-- +
A. calcoaceticus	418	+++	+++	+++	+++	+++	+++	+++	---	---	---	+++	---	+++	---
A. calcoaceticus	1097	+++	+++	+++	+++	+++	+++	+++	---	---	+++	+++	---	+++	---
Acinetobacter gensp. 3	415	+++	+++	+++	+++	+++	+++	+++	-- +	---	+++	+++	+++	+++	+++
A. haemolyticus	1337	+++	+++	+++	---	---	+++	+++	---	---	+++	+++	---	---	---
A. johnsonii	1336	+++	+++	+++	+++	+++	+++	+++	---	---	---	+++	---	+++	---
A. junii	1335	+++	+++	+++	+++	---	+++	+++	---	---	+++	+++	---	+++	-- +
A. lwoffii	1338	+++	+++	+++	- -+	---	+++	---	---	---	---	---	---	+++	---
A. schindleri	1339	+++	+++	---	---	---	+++	---	---	---	---	---	---	---	---
A. ursingii	1334	+++	+++	+++	+++	+++	+++	+++	---	---	+++	+++	---	+++	---

To examine the antibiotic sensitivity of a number of *Acinetobacter* strains, the agar dilution method was used. TSA and DDA media which did not contain the antibiotics were included as controls. *A. baumannii,* including the type strain 1102 (12156^{T}), showed greater antibiotic resistance compared to other species. Resistance ranging between 91.7% (11/12) and 100.0 % was shown by *A. baumannii* strains. The only sensitivity shown was at 356mg/l vancomycin with 29.2% (7/24) of the *A. baumanii* strains being inhibited. *A. haemolyticus*, *A. lwoffii*, and *A. schindleri* showed particular sensitivity and were inhibited by 58.3% (7/12), 75.0% (9/12), and 91.6 % (11/12) of the antibiotics respectively. *A. schindleri* was the only strain whose recovery was affected by the presence of aztreonam (4mg/l). The food (418) and the clinical (1097) isolates of *A. calcoaceticus* exhibited similar susceptibilities. Aztreonam, ceftazidime, ciprofloxacin, norfloxacin, and temocillin had the least inhibitory effect on *Acinetobacter* (≤ 3 strains inhibited). They were, therefore, considered as potential selective agents for the DDA medium. Azt Aztreonam, Cefo Cefotaxime, Cefs Cefsulodin, Ceft Ceftazidime, Cipr Ciprofloxacin, Eno Enoxacin, Lom Lomefloxacin, Net Netilmicin, Norfloxacin, Nor Norfloxacin, Pip Pipemidic acid, Tem Temocillin, Van Vancomycin, +++ triplicate positive recovery, --- triplicate negative recovery, ++- duplicate positive recovery with the relevant strain counted as resistant, --- duplicate negative recovery.

Table 3.7 Sensitivity of Gram-positive organisms to aztreonam, ceftazidime, ciprofloxacin, norfloxacin, and temocillin on the DDA medium.

Species	Strain number	Control medium		Aztr.	Ceft.	Cipr.	Nor.	Tem.
		TSA	DDA	4mg/l	1mg/l	0.05mg/l	0.5mg/l	100mg/l
Bacillus cereus	1170	+++	+++	+++	+++	+++	---	---
Enterococcus faecalis	1172	+++	+++	+++	+++	+++	---	---
Lactococcus lactis	1171	+++	+++	+++	+++	+++	+++	---
Lactobacillus plantarum	574	+++	+++	+++	+++	---	---	+++
Staphylococcus aureus	1167	+++	+++	+++	+++	+++	+++	---
Staphylococcus aureus	1168	+++	+++	+++	+++	+++	+++	---

Due to the minimised sensitivity of *Acinetobacter* to these five antibiotics (as shown earlier), their inhibitory effect was further tested against a set of Gram-positive bacteria. Two *S. aureus* strains were used, due to availability. Temocillin proved to be the most selective against the tested Gram-positive organisms compared to the other antibiotics at the specified concentrations. Azt Aztreonam, Ceft Ceftazidime, Cipr Ciprofloxacin, Nor Norfloxacin, Tem Temocillin, +++ triplicate positive recovery, --- triplicate negative recovery.

Table 3.8 Sensitivity of *Acinetobacter* and other Gram-negative and positive organisms to a range of lincomycin concentrations included in the DDA medium.

Species	Strain number	Control medium		Lincomycin													
		TSA	DDA	10mg/l	20mg/l	40mg/l	60mg/l	80mg/l	100mg/l	120mg/l	140mg/l	160mg/l	180mg/l	200mg/l	220mg/l	240mg/l	260mg/l
A. baumannii	1095	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1096	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1098	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1099	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1102	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1109	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1110	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1111	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1112	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1113	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1114	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1115	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1116	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1117	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1118	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1119	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1120	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1121	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1122	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1123	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1124	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1125	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1126	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1127	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. calcoaceticus	418	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. calcoaceticus	1097	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. calcoaceticus	1103	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Acinetobacter gensp. 3	415	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. haemolyticus	1337	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. johnsonii	1336	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. junii	1335	++	++	++	++	+-	++	++	++	++	++	++	++	++	++	++	++
A. lwoffii	1338	++	++	++	++	++	--	--	--	--	--	--	--	--	--	--	--
A. ursingii	1334	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
A. radioresistens	1340	++	++	++	--	--	--	--	--	--	--	--	--	--	--	--	--
B. cereus	1170	++	++	--	--	--	--	--	--	--	--	--	--	--	--	--	--
B. subtilis	1248	++	++	--	--	--	--	--	--	--	--	--	--	--	--	--	--
E.faecalis	1172	++	++	--	--	--	--	--	--	--	--	--	--	--	--	--	--
L.lactis	1171	++	++	--	--	--	--	--	--	--	--	--	--	--	--	--	--
L. plantarum	574	+++	+++	--	--	--	--	--	--	--	--	--	--	--	--	--	--
S. aureus	1167	++	++	--	--	--	--	--	--	--	--	--	--	--	--	--	--
S. aureus	1168	++	++	--	--	--	--	--	--	--	--	--	--	--	--	--	--

At 10mg/l lincomycin, there was 100% recovery of the *Acinetobacter* strains and 100% inhibition of the Gram-positive strains. The increase of the antibiotic concentration to 20mg/l (MIC) prevented the growth of *A. radioresistence*. At 60mg/l, *A. lwofii* was also unable to grow. ++ duplicate positive recovery, -- duplicate negative recovery.

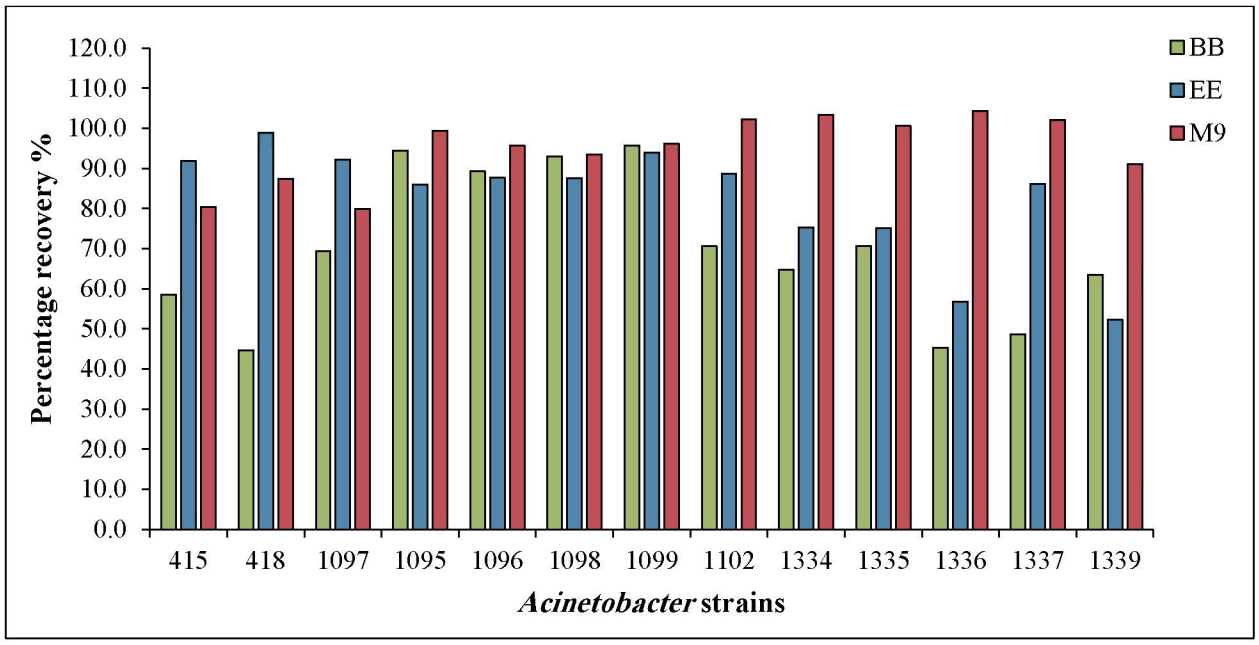
3.3.3 Optimization of the enrichment stage

The main purpose of the survey was to screen PIF products for the presence of *Acinetobacter*. Each PIF sample was analysed by reconstituting 25g of the powder in 225ml BPW, followed by the enhancement of bacterial growth in an appropriate enrichment broth medium. Enriched cultures were then inoculated onto DDA and LAM medium for a comparative assessment. Morphological and biochemical tests were applied for presumptive identification of the isolates.

To select a suitable broth for the enrichment stage of the survey, the recovery of *Acinetobacter* in EE, modified M9, and BB was evaluated. The results are presented in **Figure 3.1**. Percentage recoveries (PRs) were higher in M9, with 10 strains, and generally significant ($p < 0.05$). The lowest PRs exhibited in BB and EE broth were 44.6 and 52.3 % respectively, while that recorded with M9 was 79.9%. Ten of the 13 tested strains (76.9%) showed higher recovery when grown in M9 compared to EE broth. Furthermore, higher numbers were also detected with all of the strains in M9 in contrast to BB. Viable counts increased from the original inocula size with 5 of the strains (*A. calcoaceticus* 418, *A. baumannii* 1102, *A. ursingii* 1334, *A. johnsonii* 1336, and *A. haemolyticus* 1337) cultivated in M9 (PR > 100.0%). This growth was not recorded in BB or in EE broth.

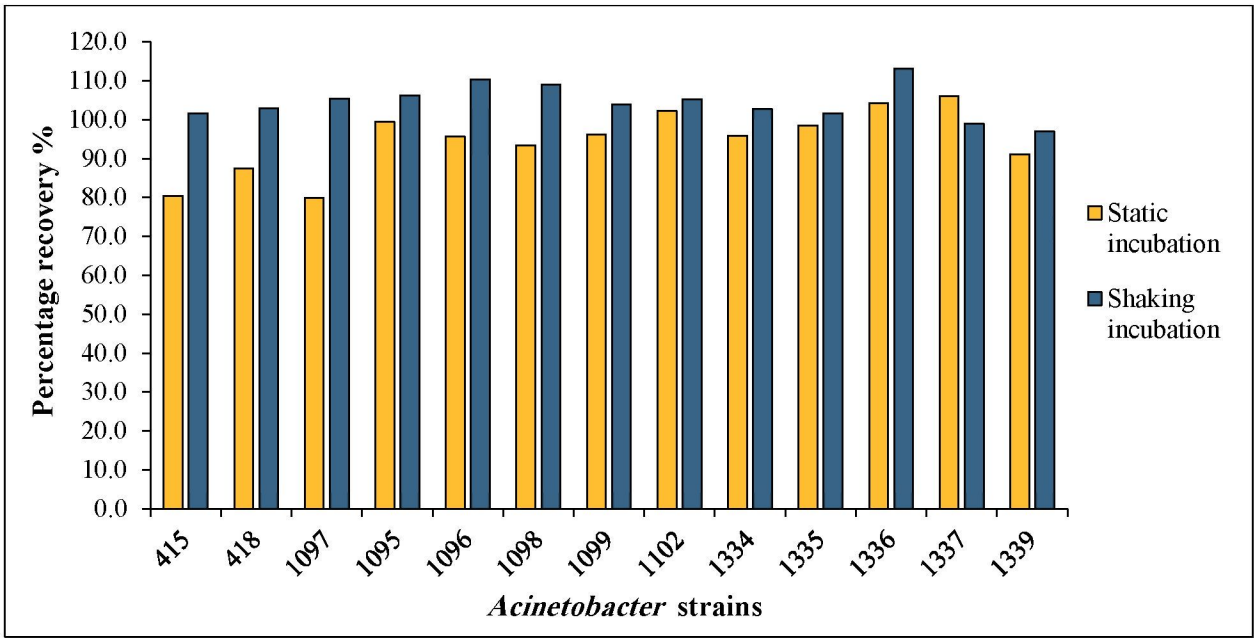
Since the majority of strains showed enhanced recovery in M9 in comparison to the other two broths, it was selected as the enrichment broth for the survey of PIF. The next step was optimizing the incubation conditions during the enrichment stage. *Acinetobacter* cultures in M9 were incubated statically and on a rotary shaker at 30°C for 18h. The PRs were determined relatively to BPW and compared **(Figure 3.2)**. The vigorous shaking (280 rpm) proved a significant ($p < 0.05$) enrichment factor for the strains tested. Eleven strains (84.6%) had a PR > 100.0% when the air circulation increased during incubation. The only reduction was shown with *A. haemolyticus* 1337. However, it was not significantly different ($p \geq 0.05$). Therefore, the shaking method was appropriate for the incubation of overnight cultures during the enrichment in M9 broth.

Figure 3.1 Percentage recovery of *Acinetobacter* strains in Baumann (BB), Enterobacteriaceae enrichment (EE), and modified M9 broths.



To select an appropriate broth for the enrichment stage of the PIF survey, Baumann (BB), Enterobacteriaceae enrichment (EE), and modified M9 broths were compared based on the number of bacterial cells recovered. Viable counts were determined in each medium using the Miles & Misra method and percentage recoveries were calculated in relation to the viable count in the pre-enrichment medium (BPW). The percentages in BB and EE were both significantly lower than in M9. The lowest PRs exhibited in BB and EE broths were 44.6 and 52.3 % respectively, while that recorded with M9 was 79.9%. Viable counts increased from the original inocula size with 5 of the strains cultivated in M9 (PR>100.0%). This growth was not recorded in BB or in EE broth. Strain 415 is *Acinetobacter* gensp. 3; 418 and 1097 are *A. calcoaceticus*; 1095 to 1127 are *A. baumannii*; 1334 is *A. ursingii*; 1335 is *A. junii*; 1336 is *A. johnsonii*; 1337 is *A. haemolyticus* and 1339 is *A. schindleri*.

Figure 3.2 Recovery of *Acinetobacter* strains in M9 broth when incubated statically and on a rotary shaker.



To optimize the incubation condition so that the recovery of *Acinetobacter* isolates, from PIF, would be enhanced, bacterial cultures in M9 were incubated statically and on a rotary shaker. Viable counts of overnight cultures were determined using the Miles & Misra method and the percentage of bacterial cells recovered was calculated in relation to the original inoculum. The percentage recoveries under each condition were then compared. Strain 415 is *Acinetobacter* gensp. 3; 418 and 1097 are *A. calcoaceticus*; 1095 to 1127 are *A. baumannii*; 1334 is *A. ursingii*; 1335 is *A. junii*; 1336 is *A. johnsonii*; 1337 is *A. haemolyticus* and 1339 is *A. schindleri*.

3.3.4 Evaluation of the DDA medium

The developed medium was assessed in terms of selectivity and sensitivity during the survey of PIF. The results were used to optimize the DDA medium during the course of the study. The number of isolates (acinetobacters and non-acinetobacters) recovered, and their percentages were calculated and compared between the DDA and LAM **(Table 3.9)**.

The DDA medium at this stage was designed to prevent the growth of Gram-positive bacteria. This is while allowing the differentiation mostly between fermentative and non-fermentative Gram-negative organisms based on the latter's pigmented appearance on the medium. Presumptive Enterobacteriaceae were also able to grow on VRBGA medium and ferment Glucose. Non-fermentative Gram-negative/variable coccobacilli that were oxidase-negative were proposed as *Acinetobacter*. The identity of the Gram-positives, on the other hand, was beyond the scope of this study and therefore was not investigated further.

Although 10mg/l lincomycin was incorporated into the DDA medium, 69.5% (57/82) of the total number of isolates detected on DDA was Gram positive organisms **(Table 3.9)** which indicated the low levels of selectivity of the medium. In contrast, it was apparent that LAM was highly selective by showing no recovery of the Gram-positives detected on the DDA medium. These presumptively included *Bacillus* spp., *Staphylococcus* spp., and *Enterococcus* spp. based on their appearance under the microscope. From the four isolates that grew on LAM, one was a Gram-variable rod (presumptively *Bacillus* spp. according to the microscopic examination).

With respect to sensitivity, from 63 PIF products, only 1 was positive for *Acinetobacter* and was identified as *A. haemolyticus* using API 20NE. One *A. haemolyticus* (as identified using API 20NE), was detected on DDA but not LAM. This isolate was a Gram-variable coccobacillus and also colourless on DDA.

Despite the absence of a selective agent against Gram-positive bacteria (non-acinetobacters), only one Gram-negative cocobacillus/short rod isolate was found in PIF. It produced

pigmented colonies on DDA medium and was identified as *Enterobacter amnigenus* group2 using API 20E. Another twenty one Gram-negative rod isolates did not produce acceptable profiles by API 20NE, but were designated as *Lysinibacillus* spp. by partial 16S rDNA sequencing.

The most isolated Gram-negative organism was *Pseudomonas luteola* (8 isolates from 8 different PIF products). All of which grew on DDA medium only. Isolates which were Gram-negative rods and positive for glucose fermentation on VRBGA were regarded as presumptive Enterobacteriaceae. The only Enterobacteriaceae recovered on DDA was identified as *Enterobacter amnigenus* group2 using API 20E. The recovery of this organism was also detected on LAM. *Enterobacter cloacae* and *Klebsiella pneumoniae* were each isolated from LAM and not DDA. Only 43.5% (10/23) of the Gram-negative isolates and 75.0% (3/4) of the Gram-variable organisms, recovered on DDA and LAM respectively, were acceptably identified by API biochemical profiling (**Table 3.10)**. The remaining isolates had either unacceptable or low discrimination profiles when the same test was applied. It is of note that 6 isolates which also appeared as Gram-negative rods were identified as 99.9% *Burkholderia cepacia* by API20NE but were in fact *Lysinibacillus fusiformis*, *Bacillus amyloliquefaciens/atrophaeus/mojavensis/subtilis* and *Enterococcus casseliflavus* based on 16S rDNA sequence-based identification. A *Lysinibacillus boronitolerans* was also misidentified as *Myroides* spp. with an ID percentage of 93.1% by the same API biochemical test.

Table 3.9 Morphological and biochemical analysis of PIF isolates recovered on DDA (containing 10mg/l lincomycin) and LAM.

Gram reaction	Morphology	Oxidase (-/+)	Isolation medium		Total
			DDA	LAM	
Gram positive	cocci	-	10	0	10
	rods	-	47	0	47
Gram negative	rods	22/0	21	1	22
	coccobacilli/short rods*	3/0	1	2	3
Gram variable	cocci	-	0	0	0
	rods	3/0	2	1	3
	coccobacilli/short rods*	1/0	1	0	1
Total numbers of isolates (%)			**82 (95.3)**	**4 (4.7)**	**86**

Despite the incorporation of 10mg/l lincomycin, 69.5% (57/82) of the total number of isolates on DDA was Gram-positive organisms. These organisms were completely inhibited on LAM. Only 40.0% (10/25) and 75.0% (3/4) of the total Gram-negative and Gram-variable isolates recovered on DDA and LAM respectively were acceptably identified by API biochemical profiling as indicated in Table 2.10. The remaining isolates had either unacceptable or low discrimination profiles when the same test was applied. * Presumptive acinetobacters, (-/+) Negative and positive oxidase reactions, DDA Druggan-Dimmer agar, LAM Leeds *Acinetobacter* medium.

Table 3.10 Gram-negative organisms recovered on selective media and biochemically identified during PIF screening.

PIF product	Bacterial species (ID %)	API profile	Isolation media		
			DDA	LAM	VRBGA
ULKER HERO BABY 1	*Acinetobacter haemolyticus* (92.7%) *	0010141	+		
NESTLE NAN H.A. 1	*Enterobacter amnigenus* group 2 (84.6%) **	3205553	+	+	+ [a]
MILUPA CONFORMIL 1	*Enterobacter cloacae* (99.4%) **	3305563		+	+ [a]
SUPER PREMIUM	*Klebsiella pneumonia* spp. *pneumonia* (97.6%) **	5215773		+	+ [a]
BEBELAC 1	*Pseudomonas luteola* (99.0, 99.1%) *	1577740	+		
GOAT MILK BASED ORGANIC INFANT FORMULA	*P. luteola* (97.2%) *	1475741	+		
NESTLE NAN H.A. 1	*P. luteola (99.9%)* *	0777541	+		
NESTLE NIDINA 1 H.A.	*P. luteola* (99.7%) *	1577771	+		
NESTLE NIDINA CONFORT 1	*P. luteola* (99.9%) *	1777741	+		
MOJUNG	*P. luteola* (99.1%) *	0477541	+		
SCIENCE 2	*P. luteola* (99.7%) *	1577771	+		
SNOW BRAND, STEP 1	*P. luteola* (99.9%) *	0777541	+		

Organisms listed in this table are the only Gram-negative isolates that gave valid profiles using API tests. VRBGA medium was used to presumptively identify organisms from the Enterobacteriaceae before using the appropriate API kit (API 20E). API 20NE was used for the non-Enteric bacteria. * API 20NE identification, ** API 20E identification, + positive recovery, [a] red/pink colonies.

3.3.5 Modification of the DDA medium (Second antibiotic sensitivity testing)

The low levels of selectivity of the DDA medium against the Gram-positive isolates found in PIF showed the need for further optimization of this medium. Therefore, the growth of some of these randomly selected PIF isolates was tested at different lincomycin concentrations **(Table 3.11).** A 100.0% inhibition was observed at all concentrations including the 10mg/l originally incorporated into the medium prior to the survey. Nevertheless, the lincomycin concentration was increased from 10mg/l to 20mg/l for a possibly more effective dose against Gram-positives.

To enhance the specificity of the medium, another set of antibiotics (ceftazidime and aztreonam) was tested against Gram-negative bacteria other than *Acinetobacter* spp., and the minimum inhibitory concentration (MIC) was determined **(Tables 3.12 & 3.13)**. These antibiotics caused the least inhibition of *Acinetobacter* (0.0% and 3.0% (1/33) at 4mg/l and 1mg/l respectively) as previously shown in **Table 3.6**, and therefore were selected for further testing.

When ceftazidime was used, the only *Acinetobacter* undetected on DDA medium was *A. radioresistens* (1340) **(Table 3.12)**. Of the 6 non-*Acinetobacter* strains tested, only 2 (*Klebsiella pneumoniae* 1013, and *Escherichia coli* 1247) were inhibited. Therefore, it was not considered as the best option with regard to increasing the selectivity of the medium. However, in the presence of aztreonam (1mg/l), 4 non-*Acinetobacter* strains showed inability to grow **(Table 3.13)**. These strains were 1 *Enterbacter cloacae*, 2 *Escherichia coli*, and 1 *Klebsiella pneumoniae*. At this MIC, 2 *Acinetobacter* type strains (*A. calcoaceticus* 1103 and *A. radioresistens* 1340) were not also recovered. The increase of the concentration to 4mg/l caused *A. schindleri* to also be inhibited. *Pseudomonas aeruginosa*, *Pseudomonas luteola*, and *Serratia liquefaciens* remained resistant. The 2 latter strains did not appear to be affected by any of the aztreonam concentrations. Conversely, *Pseudomonas aeruginosa* became sensitive at a higher concentration of this antibiotic (8mg/l). However, this was also accompanied with an inhibition of another *Acinetobacter* strain (*A. haemolyticus*) indicating an overall negative recovery of 25.0% (4/16). Zero inhibition of *Acinetobacter* could not be achieved. It was hence decided to select the maximum concentration (2mg/l) of aztreonam that would inhibit the least possible number of *Acinetobacter* strains on DDA medium. This is

while maintaining the highest levels of selectivity against non-*Acinetobacter* Gram-negatives. In summary, 2mg/l aztreonam and 20mg/l lincomycin were both used in the final formula of the DDA medium, which were to be characterised in the following experiments.

Table 3.11 Activity of lincomycin against Gram-positive organisms recovered from PIF.

Species	Strain number	Control medium		Lincomycin						
		TSA	DDA	10mg/l	20mg/l	40mg/l	60mg/l	80mg/l	100mg/l	120mg/l
Bacillus sp.	33	++	++	--	--	--	--	--	--	--
Bacillus sp.	94	++	++	--	--	--	--	--	--	--
Staphylococcus sp.	50	++	++	--	--	--	--	--	--	--
Staphylococcus sp.	76	++	++	--	--	--	--	--	--	--

The number of Gram-positive bacteria detected on the DDA medium during the PIF survey indicated the low levels of selectivity of this medium. Therefore, the sensitivity of some of these randomly selected PIF isolates was tested to choose an effective lincomycin concentration. Complete inhibition (100.0%) of all the isolates was observed at all concentrations including that originally used in the medium prior to the survey.

Table 3.12 Activity of ceftazidime against *Acinetobacter* spp. and other Gram-negative species at different concentrations.

Species	Strain number	Control medium		Ceftazidime							
		TSA	DDA	1mg/l	2mg/l	4mg/l	6mg/l	8mg/l	10mg/l	12mg/l	16mg/l
A. baumannii	1095	++	++	++	++	++	++	++	++	++	++
A. baumannii	1096	++	++	++	++	++	++	++	++	++	++
A. baumannii	1098	++	++	++	++	++	++	++	++	++	++
A. baumannii	1099	++	++	++	++	++	++	++	++	++	++
A. baumannii	1102	++	++	++	++	++	++	++	++	++	++
A. baumannii	1109	++	++	++	++	++	++	++	++	++	++
A. baumannii	1110	++	++	++	++	++	++	++	++	++	++
A. baumannii	1111	++	++	++	++	++	++	++	++	++	++
A. baumannii	1112	++	++	++	++	++	++	++	++	++	++
A. baumannii	1113	++	++	++	++	++	++	++	++	++	++
A. baumannii	1114	++	++	++	++	++	++	++	++	++	++
A. baumannii	1115	++	++	++	++	++	++	++	++	++	++
A. baumannii	1116	++	++	++	++	++	++	++	++	++	++
A. baumannii	1117	++	++	++	++	++	++	++	++	++	++
A. baumannii	1118	++	++	++	++	++	++	++	++	++	++
A. baumannii	1119	++	++	++	++	++	++	++	++	++	++
A. baumannii	1120	++	++	++	++	++	++	++	++	++	++
A. baumannii	1121	++	++	++	++	++	++	++	++	++	++
A. baumannii	1122	++	++	++	++	++	++	++	++	++	++
A. baumannii	1123	++	++	++	++	++	++	++	++	++	++
A. baumannii	1124	++	++	++	++	++	++	++	++	++	++
A. baumannii	1125	++	++	++	++	++	++	++	++	++	++
A. baumannii	1126	++	++	++	++	++	++	++	++	++	++
A. baumannii	1127	++	++	++	++	++	++	++	++	++	++
A. calcoaceticus	418	++	++	++	++	++	++	++	++	++	++
A. calcoaceticus	1097	++	++	++	++	++	++	++	++	++	++
A. calcoaceticus	1103	++	++	++	++	++	++	++	++	++	++
Acinetobacter gensp. 3	415	++	++	++	++	++	++	++	++	++	++
A. haemolyticus	1337	++	++	++	++	++	++	++	++	++	++
A. johnsonii	1336	++	++	++	++	++	++	++	++	++	++
A. junii	1335	++	++	++	++	++	++	++	++	++	++
A. lwoffii	1338	++	++	++	++	++	++	++	++	++	++
A. radioresistens	1340	++	++	++	++	--	--	--	--	--	--
A. schindleri	1339	++	++	++	++	++	++	++	++	++	++
A. ursingii	1334	++	++	++	++	++	++	++	++	++	++
Enterobacter cloacae	49	++	++	++	++	++	++	++	++	++	++
Escherichia coli	1247	++	++	++	++	--	--	--	--	--	--
Klebsiell pneumoniae	1013	++	++	++	++	--	--	--	--	--	--
Pseudomonas aeruginosa	1169	++	++	++	++	++	++	++	++	++	++
P. luteola	133	++	++	++	++	++	++	++	++	++	++
Serratia liquefaciens	579	++	++	++	++	++	++	++	++	++	++

To enhance the specificity of the medium, susceptibilities of *Acinetobacter* and non-*Acinetobacter* strains to aztreonam were tested and the minimum inhibitory concentrations were determined on the DDA medium. Results showed that 2 *Acinetobacter* strains and 4 non-acinetobacters were unable to recover at the lowest tested concentration of aztreonam (1mg/l). The increase of the concentration to 8mg/l inhibited 5 of the 7 non-*Acinetobacter* strains while the total number of unrecoverable acinetobacters rose to 4. ++ duplicate positive recovery, -- duplicate negative recovery.

Table 3.13 Activity of aztreonam against *Acinetobacter* spp. and other Gram-negative species at different concentrations.

Species	Strain number	Control medium		Aztreonam								
		TSA	DDA	1mg/l	2mg/l	4mg/l	6mg/l	8mg/l	10mg/l	12mg/l	14mg/l	16mg/l
A. baumannii	1095	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1096	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1098	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1099	++	++	++	++	++	++	++	++	++	++	++
A. baumannii	1102	++	++	++	++	++	++	++	++	++	++	++
A. calcoaceticus	418	++	++	++	++	++	++	++	++	++	++	++
A. calcoaceticus	1097	++	++	++	++	++	++	++	++	++	++	++
A. calcoaceticus	1103	++	++	--	--	--	--	--	--	--	--	--
Acinetobacter gensp. 3	415	++	++	++	++	++	++	++	++	++	++	++
A. haemolyticus	1337	++	++	++	++	++	++	--	--	--	--	--
A. johnsonii	1336	++	++	++	++	++	++	++	++	++	++	++
A. junii	1335	++	++	++	++	++	++	++	++	++	++	++
A. lwoffii	1338	++	++	++	++	++	++	++	++	++	++	++
A. radioresistens	1340	++	++	--	--	--	--	--	--	--	--	--
A. schindleri	1339	++	++	++	++	--	--	--	--	--	--	--
A. ursingii	1334	++	++	++	++	++	++	++	++	++	++	++
Enterobacter cloacae	49	++	++	--	--	--	--	--	--	--	--	--
Escherichia coli	605	++	++	--	--	--	--	--	--	--	--	--
Escherichia coli	1247	++	++	--	--	--	--	--	--	--	--	--
Klebsiella pneumoniae	1013	++	++	--	--	--	--	--	--	--	--	--
Pseudomonas aeruginosa	1169	++	++	++	++	++	++	--	--	--	--	--
P. luteola	133	++	++	++	++	++	++	++	++	++	++	++
Serratia liquefaciens	579	++	++	++	++	++	++	++	++	++	++	++

Ceftazidime was tested against *Acinetobacter* and non-*Acinetobacter* strains and the minimum inhibitory concentration was determined. *A. radioresistens* (ATCC 43998^T) was the only *Acinetobacter* strain to be undetected in the presence of 4mg/l ceftazidime. However, this concentration did not prove effective against 4 of the 6 non-*Acinetobacter* strains. ++ duplicate positive recovery, -- duplicate negative recovery.

3.3.6 Characterization of DDA and other selective *Acinetobacter* media

Following the optimization of DDA medium in light of the survey results, a second comparative investigation of the selective *Acinetobacter* media was conducted and involved DDA, LAM, as well as CHROMagar *Acinetobacter*. The latter was newly introduced to the market as the first chromogenic agar for *Acinetobacter*. Thus it was of particular interest to examine its effectiveness in relation to the other above-mentioned media. A total number of 67 strains including *Acinetobacter* and non-*Acinetobacter* species were used to examine the media in terms of the percentage recovery (PR), selectivity, sensitivity, and detection limit.

Based on presence and absence, the selectivity of the media was expressed as the percentage of the negative recovery of non-*Acinetobacter* strains. CHROMagar was the most selective medium inhibiting 96.9% (n = 31) of the total 32 tested strains **(Table 3.14)**. The only strain recovered on this medium was *Burkholderia cepacia* which was also detected on DDA and LAM. The latter, on the other hand, showed the least selectivity at 65.6% (n=21). Amongst the bacteria that grew on LAM but not on CHROMagar or DDA were *Serratia marcescens*, *Escherichia coli*, and *Klebsiella pneumoniae*. In contrast to CHROMagar and LAM, *Pseudomonas aeruginosa* was not inhibited on DDA. DDA was 12.5% lower on selectivity than CHROMagar but was more efficient than LAM with 84.4% (n = 27) growth inhibition of non-*Acinetobacter* spp.

In terms of sensitivity, which was determined by the percentage of the positive growth of *Acinetobacter*, DDA was the best (94.3%) with 33 out of the 35 strains recovered, followed by CHROMagar inhibiting four strains (88.6%) **(Table 3.15)**. LAM showed the least sensitivity performance as eight strains failed to grow (77.1%). *A. calcoaceticus* and *A. radioresistens* were distinctively the only strains to be inhibited on the three media mentioned above.

PRs were analysed by performing the Miles & Misra plate count method on all media plus TSA. TSA was used as a control given that a 100.0% of *Acinetobacter* recovers on this all-purpose medium. Applying the One way-analysis of variance (ANOVA), the P-value (0.09) showed no significant difference between the PR on the three selective media. The minimum

PR on CHROMagar, DDA, and LAM was 80.3, 88.1, and 90.4% respectively **(Figure 3.3)**. Twelve of thirty-three recovered strains (36.4%) were counted in higher numbers (PR > 100.0%) on DDA relative to the control TSA. The increase in viable counts of these strains was averaged at 4.1% (0.3 $\log_{10}$ cfu/ml). On CHROMagar, 29.0% (9/31) of the strains showed a total average of 2.8% growth increase (0.2 $\log_{10}$ cfu/ml) in comparison to TSA. LAM encouraged the bacterial growth of 14.8% (4/27) of the strains by 2.7% in average (0.2 $\log_{10}$ cfu/ml).

The detection limit was assessed by the lowest number of *Acinetobacter* colonies each medium can detect. The culture media of interest were inoculated with 0.1, 1, 10, 100, 1000 cfu/ml of overnight cultures of *Acinetobacter*. The viable counts were determined by the spread plate method. TSA had a detection limit of 1-10 cfu/ml. That of LAM was 10 cfu/ml, while CHROMagar and DDA media were detecting bacterial cells at 10-100 cfu/ml.

Table 3.14 Comparison of the selectivity of *Acinetobacter* agar media.

Species	Strain number	Selectivity			
		TSA	CHROMagar	DDA	LAM
Bacillus subtilis	702	+++	---	---	---
B. cereus	1170	+++	---	---	---
Burkholderia cepacia	627	+++	+++	+++	+++
Citrobacter freundii	624	+++	---	---	---
Citr. koseri	224	+++	---	---	---
Citr. koseri	225	+++	---	---	---
Citr. koseri	226	+++	---	---	---
Citr. koseri	826	+++	---	---	---
Cronobacter sakazakii	1	+++	---	---	---
C. sakazakii	2	+++	---	---	---
Enterococcus cloacae	49	+++	---	+++	+++
E. cloacae	218	+++	---	---	---
Enterococcus faecalis	1172	+++	---	---	---
Escherichia coli	100	+++	---	---	+++
E. coli	540	+++	---	---	+++
E. coli	571	+++	---	---	+++
E. coli	800	+++	---	---	+++
Klebsiella pneumoniae	68	+++	---	---	+++
K. pneumoniae	541	+++	---	---	+++
K. pneumoniae	1013	+++	---	---	---
Lactococcus lactis	1171	+++	---	---	---
Pseudomonas aeruginosa	117	+++	---	+++	---
P. aeruginosa	401	+++	---	+++	---
P. aeruginosa	570	+++	---	+++	---
Serratia marcescens	763	+++	---	---	+++
S. marcescens	834	+++	---	---	+++
S. marcescens	835	+++	---	---	+++
Staphylococcus aureus	152	+++	---	---	---
S. aureus	616	+++	---	---	---
S. aureus	1167	+++	---	---	---
S. aureus	1168	+++	---	---	---
S. epidermidis	671	+++	---	---	---

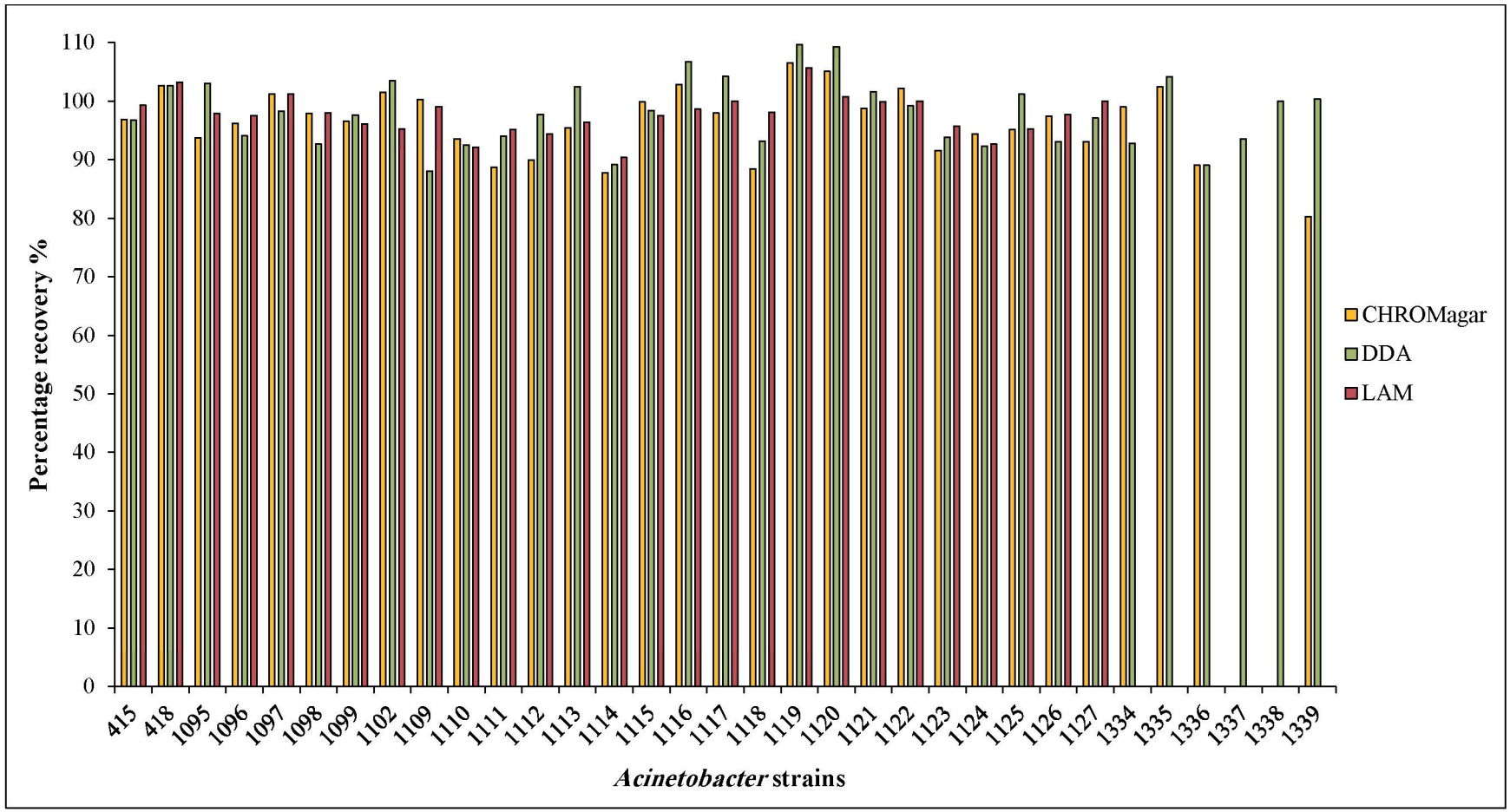
Based on presence and absence, the selectivity of the media was expressed as the percentage of the negative recovery of non-*Acinetobacter* strains. CHROMagar was the most selective medium inhibiting 96.9% (n = 31) of the total 32 tested strains. The only strain recovered on this medium was *Burkholderia cepacia* which was also detected on DDA and LAM. LAM showed the least selectivity at 65.6% (n = 21). DDA was 12.5% lower on selectivity than CHROMagar but was more efficient than LAM with 84.4% growth inhibition of non-*Acinetobacter* spp. +++ triplicate positive recovery, --- triplicate negative recovery.

Table 3.15 Comparison of the sensitivity of *Acinetobacter* agar media.

Species	Strain number	Sensitivity			
		TSA	CHROMagar	DDA	LAM
A. baumannii	1095	+++	+++	+++	+++
A. baumannii	1096	+++	+++	+++	+++
A. baumannii	1098	+++	+++	+++	+++
A. baumannii	1099	+++	+++	+++	+++
A. baumannii	1102	+++	+++	+++	+++
A. baumannii	1109	+++	+++	+++	+++
A. baumannii	1110	+++	+++	+++	+++
A. baumannii	1111	+++	+++	+++	+++
A. baumannii	1112	+++	+++	+++	+++
A. baumannii	1113	+++	+++	+++	+++
A. baumannii	1114	+++	+++	+++	+++
A. baumannii	1115	+++	+++	+++	+++
A. baumannii	1116	+++	+++	+++	+++
A. baumannii	1117	+++	+++	+++	+++
A. baumannii	1118	+++	+++	+++	+++
A. baumannii	1119	+++	+++	+++	+++
A. baumannii	1120	+++	+++	+++	+++
A. baumannii	1121	+++	+++	+++	+++
A. baumannii	1122	+++	+++	+++	+++
A. baumannii	1123	+++	+++	+++	+++
A. baumannii	1124	+++	+++	+++	+++
A. baumannii	1125	+++	+++	+++	+++
A. baumannii	1126	+++	+++	+++	+++
A. baumannii	1127	+++	+++	+++	+++
A. calcoaceticus	418	+++	+++	+++	+++
A. calcoaceticus	1097	+++	+++	+++	+++
A. calcoaceticus	1103	+++	---	---	---
Acinetobacter gensp. 3	415	+++	+++	+++	+++
A. haemolyticus	1337	+++	---	+++	---
A. johnsonii	1336	+++	+++	+++	---
A. junii	1335	+++	+++	+++	---
A. lwoffii	1338	+++	---	+++	---
A. radioresistens	1340	+++	---	---	---
A. schindleri	1339	+++	+++	+++	---
A. ursingii	1334	+++	+++	+++	---

Sensitivity of the media was defined as the percentage of the positive growth of *Acinetobacter*. DDA came first (94.3%) with 33 out of the 35 strains recovered, followed by CHROMagar inhibiting 4 strains (88.6%). LAM showed the least sensitivity performance as 8 strains failed to grow (77.1%). *A. calacoaceticus* and *A. radioresistens* were the only strains to be inhibited on the 3 above-mentioned media. +++ triplicate positive recovery, --- triplicate negative recovery.

Figure 3.3 Comparison of the percentage recovery on *Acinetobacter* agar media.



Percentage recovery (PR) was analysed by performing the Miles and Misra plate count method on all media plus TSA. TSA was used as a non-selective control medium with a 100.0% recovery of *Acinetobacter*. The proportion of the viable counts on each medium relative to that of TSA was calculated and expressed as a percentage. These percentages were then compared. The minimum PR on CHROMagar, DDA, and LAM was 80.3, 88.1, and 90.4% respectively. Twelve of 33 recovered strains (36.4%) were counted in higher numbers (PR>100.0%) relative to the control TSA. The increase was averaged at 4.1%. On CHROMagar, 29.0% (9/31) of the strains showed a total average of 2.8% increase in recovery in comparison to TSA. LAM encouraged the bacterial growth of 14.8% (4/27) of the strains by 2.7% in average. 415 *Acinetobacter* gensp. 3; 418 and 1097 *A. calcoaceticus*; 1095 to 1127 *A. baumannii*; 1334 *A. ursingii*, 1335 *A. junii*, 1336 *A. johnsonii*, 1337 *A. haemolyticus*, 1338 *A. lwoffii*, 1339 *A. schindleri*.

3.4 DISCUSSION

The use of chromogenic agars for the differentiation of *Acinetobacter* has not been widely applied. The differentiation of the organism on LAM is based on the utilization of complex nitrogenous resources and the consequent release of ammonium ions. The base of the medium becomes alkaline (pH 8-9) and changes from dark orange to mauve as a result (Jawad *et al.*, 1994). DDA, on the other hand, exploits the presence of DHF-riboside to distinguish between Gram-negative bacteria according to their positive or negative hydrolysis of this substrate. DDA medium made use of the incapability of *Acinetobacter* to produce this enzyme for the purpose of presumptively differentiating and identifying the organism. This is particularly efficient if other β-ribosidase negative organisms are inhibited. According to Butterworth *et al.*, (2004), other Gram-negative bacteria showing no activity with DHF-riboside included *Yersinia enterocolitica*, *Yersinia pseudotuberculosis, Yersinia kristensenii, Proteus mirabilis, Proteus penneri, Vibrio parahaemolyticus, Vibrio cholerae*, *Ralstonia* spp., and *Pandoraea* spp. whose colonies also appeared non-pigmented. Without any selective agents, the Gram-negative organisms mentioned above would all produce non-pigmented colonies on the medium along with Gram-positive bacteria. Therefore, it was important to test different selective agents to achieve the maximum inhibition possible of non-*Acinetobacter* spp. One of the agents tested against Gram-positives was sodium deoxycholate. Generally, Gram-positive organisms are more sensitive to bile salts than the Gram-negatives due to the absence of the outer membrane as a protective barrier (Gunn, 2000). However, when sodium deoxycholate was incorporated into the medium at 0.15g/l, *A. calcoaceticus* NCTC 12983^T and *A. radioresistens* ATCC 43998^T were unable to grow. This might be due to some membrane alteration as designated type strains usually undergo intensive studies and repeated subculturing, which can generate mutations and lead to some changes in their characteristics.

Susceptibility data of this study demonstrated the resistance, amongst all of the tested *A. baumannii* strains, to at least 11 of the 12 antibiotics used. These strains were clinical in origin. In contrast, *A.* genomospecies 3, and *A. calcoaceticus* (418, 1097, and 1103) as well as the *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. lwoffii*, *A. radioresistens, A. schindleri,* and *A. ursingii* varied in their sensitivity. The levels of resistance may explain why *A. baumannii* is

able to maintain its existence and be frequently isolated from the clinical setting compared to other species of *Acinetobacter*. Of note is that the food/cheese isolates, *Acinetobacter* gensp. 3 (415) and *A. calcoaceticus* (418), were resistant to 83.3 and 58.3% of the antibiotics respectively. Food isolates are generally less exposed to antibiotics as opposed to the clinical ones which are, consequently, more prone to developing antibiotic resistance due to the extensive use of suboptimal antimicrobial agents. However, as stated by the World Health Organization (World Health Organization WHO, 2001) the emergence of increased levels of antibiotic resistance of micro-organisms found in food produced from animal sources is anticipated to rise as a result of inappropriate use of antibiotics. Giving antibiotics to livestock or adding them to animal-feed may give rise to this resistance. Moreover, it has been suggested that probiotics may serve as likely reservoirs for antibiotic resistance genes (von Wright, 2005; European Food Safety Authority-The panel on additives and products or substances used in animal, 2008).

As well as its potential use for diagnostic or screening purposes in laboratories, industry, and hospitals, the DDA medium was initially aimed for the analysis of PIF. Therefore, the medium was formulated for the general detection of *Acinetobacter* species and not merely for the commonly isolated MDR-*A. baumannii* isolates. Achieving both high selectivity against non-acinetobacters and 100% recovery of *Acinetobacter* spp. was not possible. In this case, the species and their frequent isolation and importance as nosocomial pathogens needed to be considered before choosing the final selective agents for the medium. The final formulation of the medium included 20mg/l lincomycin and 2mg/l aztreonam. The type strains *A. calcoaceticus* NCTC 12983^T and *A. radioresistens* ATCC 43998^T were both inhibited as a result. Nevertheless, this was not of particular concern since type strains do not necessary represent their species owing to their atypical behaviour as previously observed by Caubilla-Barron and Forsythe (2007). This was also indicated by the positive recovery of both the food (418) and the clinical (1097) isolates of *A. calcoaceticus* on DDA in contrast to their type strain (NCTC 12983^T).

For a comprehensive and robust validation of selective/differential culture media in general, it is necessary not only to use culture collection strains but also natural samples containing competitive microflora that are relevant to the application of the medium. The usefulness of

DDA medium was assessed by analysing 63 PIF samples. The number of Gram-positive bacteria recovered on DDA showed its reduced selectivity compared to LAM. However, this lower level of selectivity is caused by the low selectivity of the broth used for the enrichment stage and not an indication of the effectiveness of the medium. Gram-positives including *Bacillus* spp., and *Staphylococcus* spp. were sensitive to 10mg/l lincomycin when tested prior to the survey of PIF. However, these organisms, as a part of the natural inhabitants of PIF, were able to grow on DDA medium containing the same concentration of lincomycin as mentioned above. Pure cultures of some of these PIF isolates were examined in the presence of lincomycin and they were in fact sensitive. The resistance of these isolates, inoculated directly from the enrichment broth, can be due to the large number of bacterial cells that might exist in clumps and hence be protected or at least less affected by the antibiotics in the medium. The bacterial concentration used for lincomycin susceptibility testing was ~10^5cfu/ml and so it can be assumed that the amount of these Gram-positive cells may be much greater than the number above.

Despite the ability of some non-*Acinetobacter* strains cultured on DDA medium to grow, they were not mistaken with the target organism. *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* were easily distinguished from *Acinetobacter* spp. by their coloured appearance. The positive oxidase reaction would then differ the latter organisms (*P. aeruginosa* and *B. cepacia*) from the Enterobacteriacae in general. However, the differentiation between these two bacteria would require further characterization. Biochemical tests such as benzyl-arginine arylamidase (BAA) can be applied for this purpose since *P. aeruginosa* possesses the BAA activity which is otherwise lacking in *Burkholderia cepacia* (Laffineur *et al.*, 2002). *P. aeruginosa* can be a significant contaminant of wet environmental sources but as far as the detection of desiccated organisms in PIF, the proportion of *Pseudomonas* spp. is unlikely to be significant (Druggan and Iversen, 2009). With regard to *Burkholderia cepacia*, its presence in PIF has not been reported. While the *P. aeruginosa* strains obtained from culture collections were negative for their recovery on CHROMagar in this study, scant growth of red colonies was observed with 2 clinical isolates (1imipenem sensitive and 1 resistant) examined by Akers *et al.* (2010). Four out of 6 *Staphylococcus aureus*, 2 *Enterococcus faecalis*, and 1 *Enterobacter cloacae* were also able to grow on the same medium and produced a red to red-orange colour (Akers *et al.*, 2010).

The growth of organisms with similar colony color as *Acinetobacter* means that it is more likely to have a misidentification and report a false positive result if additional tests were not performed. This can be of particular concern with respect to infection control, appropriateness of treatment, and unnecessary exposure to antibiotics. Akers *et al.* (2010) also inspected the growth of clinical isolates of *Citrobacter freundii*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Serratia marcescens*. Apart from one *Escherichia coli* ATCC 25922 and *Citrobacter freundii*, all of these strains were recovered on CHROMagar. In comparison, strains from the same species were examined in our study and showed negative recovery on the same medium. This observation of strain variations highlighted the need to use larger numbers of clinical isolates of each species, in order to properly validate whether the current formula of the DDA medium is suitable for future applications in the hospital setting.

Using 10^5 cfu/ml inocula, CHROMagar proved to be 12.5% and 31.3% more selective for *Acinetobacter* than DDA, and LAM respectively. The latter was too selective in that the recovery of eight *Acinetobacter* species (*A. calcoaceticus*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. lwoffii*, *A. radioresistens*, and *A. schindleri, A. ursingii*) was affected. Despite its lower selectivity compared to CHROMagar, DDA medium was the most sensitive medium with 94.3% of the *Acinetobacter* strains showing positive recovery. CHROMagar came second at 88.6%, followed by LAM (77.1%). Seventy-five percent sensitivity of the prior medium was previously reported by Akers *et al.* (2010) when eight mixed cultures containing the *calcoaceticus-A. baumannii* complex were inoculated into CHROMagar at 10^4 cfu/ml. Earlier during the PIF screening, better sensitivity of DDA over LAM was also demonstrated whereby one *A. haemolyticus* was only isolated on DDA but not LAM. When the medium was inoculated with sterile pre-made infant formula artificially spiked with 0.1, 10, 100, and 1000 cfu/ml, the lowest number of bacterial cells detected was 10 cfu/ml on CHROMagar, DDA, and LAM. The lower the detection limit the better it is for the isolation of *Acinetobacter* from a sample specifically if the organism is present in low numbers, otherwise, a false negative might be obtained. The opposite correlation applies to the low percentage recovery on a medium and the sensitivity. In other words, the lower the percentage recovery of an organism on an isolation medium the lower the sensitivity of that medium will be. For this reason, PRs on the media were determined and compared and

recoveries below 50.0% were regarded as a failure for the evaluated media. However, all strains showed PRs greater than 80.0% on CHROMagar, DDA, and LAM. DDA presented the highest percentage (36.4%) of strains whose PR was greater than 100.0%. However, generally there was no significant difference between the levels of recovery on these three media.

The DDA medium described here showed promising results for its applicability as a selective and a differential medium in laboratories, industry, hospitals where the presence of *Acinetobacter* is investigated. All acinetobacters tested were abundantly recovered (PR>80.0%) on this medium, which was also highly sensitive and showed good selectivity. The latter can be further improved with the incorporation of an antimicrobial agent against *Pseudomonas* spp. Moreover, DDA is simple and quick to prepare as it is composed of few, uncomplicated, and inexpensive ingredients. The only costly part is the chromogenic substrate. However, this could be justified by the pronounced advantages of better specificity offered by chromogenic media. A 100% accuracy of isolation and presumptive identification is seldom achieved as false negatives and false positives may occur in any selective medium (Greenhalgh, 1997). Incorporating a differential agent into a selective medium can increase the accuracy and reliability of the presumptive identification. Consequently, it would reduce the need for complementary testing (biochemical or morphological), culture media, and time spent on the analysis of samples containing mixed populations of bacteria, that are generally insignificant to the case scenario or the screening purpose.

With regards to the species specificity of the DDA, the medium was not designed for the selective recovery of MDR-*A. baumannii* and therefore cannot be applied for clinical diagnosis or screening for this particular strain in the hospital without conducting confirmatory identification and susceptibility testing. As mentioned before, the medium as it stands was designed for PIF sampling where the prevalence of all species of *Acinetobacter* is of interest. Future practical research applications of the medium may include clinical, environmental, and food screening of general *Acinetobacter* species.

This research study had another goal, which was investigating the PIF for *Acinetobacter* contamination. The procedure required the inoculation of recovered cells into an enrichment broth to enhance the growth of the target organism in relation to competitive microflora

present in the same sample. Although M9 broth increased the growth of *Acinetobacter* as shown in the preliminary experiments, it was apparent that the broth was not effective in terms of inhibiting the recovery of Gram-positive organisms. These organisms were consequently isolated abundantly on the DDA medium despite the presence of 20mg/l lincomycin. The idea of the modified M9 broth was to enhance the recovery of *Acinetobacter* spp. and at the same time avoid the reduction in sensitivity, or in other words avoid the inhibition of some *Acinetobacter* strains. This was believed to be achieved by making the broth more suitable for the target organism and less so for others without incorporating a selective agent. Therefore, the glucose was omitted from the original formula of the medium so that *Acinetobacter* have an advantage over organisms that require this nutrient for their growth.

The pH of the medium was also reduced to 6.0 to selectively enhance the recovery of *Acinetobacter* as observed by Baumann (1968). However, PIF samples contain mixed bacterial populations. Consequently, predominant bacteria, mainly Gram-positives, can outgrow the organisms that are present in smaller proportions, if they are not inhibited in the enrichment stage. In the survey here, *Bacillus* spp. presented the majority of the isolates detected from PIF. Their frequent isolation from PIF is not uncommon (Anderton, 1986; Crielly *et al.*, 1994; Reyes *et al.*, 2007; Ronimus *et al.*, 2006; Rueckert *et al.*, 2005). Endospore-formation enables them to withstand extreme conditions during manufacturing which would otherwise limit the growth of many Gram-negative organisms in particular (Atrih and Foster, 2001; Setlow, 2006). Genera from the Bacillaceae family can also produce antibiotics such as colistin and polymyxin which are active against both Gram-negative and positive organisms (He *et al.*, 2007; Katz and Demain, 1977). Such antibiotics are used as selective agents in isolation media for Gram-positive bacteria found in water and food (Bisson and Cabelli, 1979; Handford, 1974; Holbrook and Anderson, 1980). The above-mentioned factors may have lowerd the sensitivity of the enrichment broth (M9) and therefore it is possible that some *Acinetobacter* in the samples were missed.

Overall, *Pseudomonas luteola* was the most prevalent Gram-negative organism isolated in this study and was found in 12.7% (8/63 products) of PIF products. In contrast to *Pseudomonas aeruginosa*, this species is oxidase negative. Although the organism was

colourless on DDA medium, it had a distinctive morphological appearance that differentiates it from *Acinetobacter*. *P. luteola* has been associated with nosocomial infections such as endocarditis, peritonitis, cellulitis, septicaemia, and bacteraemia (Casalta *et al.*, 2005; Connor *et al.*, 1987; Dalamaga *et al.*, 2004; Freney *et al.*, 1988; Rastogi and Sperber, 1998). However, PIF contamination with this organism has not been previously reported.

From the Enterobacteriaceae, *K. pneumoniae, E. cloacae,* and *E. amnigenus* were also detected in different PIF products. Infections caused by *E. amnigenus* have been occasionally reported (Capdevila *et al.*, 1998; Korah *et al.*, 2007) but were not associated with neonates or PIF. On the other hand, epidemiological link between contaminated feeds and new-borns colonized with multi-resistant *K. pneumoniae* has been found (Vieira *et al.*, 1999). Overgrowth of this organism in the gut has been observed following treatment with broad-spectrum antibiotics. The organism has the potency to acquire and/or develop antibiotic resistance which can serve as a reservoir for antibiotic resistance genes (Sirot *et al.*, 1991). The presence of *E. cloacae* in PIF has also been previously reported by Chap *et al.* (2009), and Iversen and Forsythe (2004). Similar to *Acinetobacter* spp., this species along with *K. pneumoniae* were classified by FAO-WHO (2004) (2006), according to their potential risks to neonates, as category B whereby causality is plausible but not yet demonstrated.

Only one *Acinetobacter* isolate was isolated. It was identified as *A. haemolyticus* using API 20NE. According to the surveillance programme of bacteraemia resistance, *A. haemolyticus* was implicated in 24 and 21 of the total *Acinetobacter* 1246 and 984 bacteraemia cases in the UK and Ireland between 2007 and 2008 respectively. However, there was no specific data available regarding the rates of neonatal infections caused by this species.

The detection of one *Acinetobacter* isolate may or may not accurately reflect the organism's actual occurrence in the PIF products included in this study. No conclusion can be drawn in this respect unless an efficient screening method, which is both highly sensitive and selective, is established. Nevertheless, the occasional isolation of *Acinetobacter* by no means minimises the importance of this organism as a potential agent of neonatal infections. This should be evaluated in terms of clinical impact of such infections on feeding rather than frequent occurrences in PIF.

CHAPTER 4. LONG-TERM PERSISTENCE OF *ACINETOBACTER* IN DESICCATED INFANT FORMULA

4.1 INTRODUCTION

Infant formulas are designed to provide the nutritional requirements of growing infants. To ensure they are suitable for their consumption, the Codex Alimentarius Commission (CAC), established by the WHO and FAO, developed a global safety, quality, and compositional standard for infant formula which was first adopted in 1981 and revised in 2007 (CAC, 2007). However, despite the 'good manufacturing practices' regulations, bacterial contamination of PIF remains an issue since sterilization cannot be achieved during production. Consequently a variety of Gram-positive and Gram-negative micro-organisms (Edelson-Mammel *et al.*, 2005; Iversen and Forsythe, 2004) including *Acinetobacter* spp. may contaminate the product (Cawthorn *et al.*, 2008; Marino *et al.*, 2007; Miled *et al.*, 2010). Although no epidemiological link between *Acinetobacter* infections in neonates and contaminated PIF has been established, the organism was included in the FAO-WHO (2006) list of organisms of plausible concern for neonatal health due to powdered formula. *A. baumannii/calcoaceticus* were classified under category B representing 'plausible causality, but not yet demonstrated' according to their potential risk of causing neonatal illnesses.

In addition to the isolation of *Acinetobacter* from the inanimate surfaces in hospitals, *Acinetobacter* has been experimentally shown to survive desiccation for up to ten months (Getchell-White *et al.*, 1989; Jawad *et al.*, 1996; Jawad *et al.*, 1998b; Wagenvoort and Joosten, 2002; Wendt *et al.*, 1997). These studies were conducted using suspensions of *Acinetobacter* in distilled water and/or bovine serum albumin, or phosphate-buffered saline. Infant formula has been used to study the desiccation persistence of various *Enterobacteriaceae* including *Cronobacter sakazakii* (a major neonatal pathogen found in PIF) (Bar-Oz *et al.*, 2001; Caubilla-Barron and Forsythe, 2007; Lai, 2001). However, the persistence during desiccated storage in this carrier medium has not been previously investigated for *Acinetobacter*. The documented presence in PIF may be indicative of the risk of neonatal exposure to *Acinetobacter* infections through the consumption of a reconstituted PIF intrinsically contaminated with this organism. This study, therefore, quantitively examines the ability of *Acinetobcter* strains to maintain their viability in dehydrated infant

formula over a storage period consistent with the shelf-life of the commercial PIF products (2 years).

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains

A total number of nine *Acinetobacter* strains were evaluated for their desiccation survival in infant formula. *Acinetobacter* strains were obtained from clinical (n = 7) and environmental (n = 2) sources. For comparison, species of such as *Enterobacter cloacae* (n = 2) and *Enterobacter hormaechei* (n = 1) from the Enterobacteriacaeae family were also included. Bacterial species, their sources and culture collection designation are presented in **Chapter 1**.

4.2.2 Capsule formation

All strains included in this study were examined by microscopy for the presence of the capsule, using the India ink stain, prior to the desiccation experiment. A loopful of sterile distilled water was placed on a clean and dry slide. A small amount of one colony from TSA plates was emulsified in the water, and was then mixed with a drop of India ink and covered with a clean coverslip. A small drop of immersion oil was added before viewing under the microscope at the magnification of 1000x.

4.2.3 Preparation of cultures prior to the desiccation assay

Cultivation of *Acinetobacter* for desiccation studies required some method development. *Acinetobacter* strains were grown on milk agar consisting of 3.0g of bacteriological agar No. 3, and 0.4g of ammonium sulphate. Both were dissolved in 40ml of distilled water. After that, the agar was autoclaved at 121^{o}C for 15 min. Meanwhile, 200ml ready to use sterile liquid infant formula (Cow & Gate Premium 1) was kept in the 55^{o}C incubator to be added aseptically to the agar and mixed. Milk agar was then poured and allowed to set before inoculation. The medium, however, based on plate counts did not support sufficient bacterial growth required for the desiccation assay. An alternative method evaluated included growing the strains in 25ml sterile liquid infant formula, centrifugation at 500xg for 5 min and re-suspension of the pellet in 2ml of infant formula. This method also proved to be an

inadequate method for obtaining ~10^{10} *Acinetobacter* cfu/ml. In the final procedure, sufficient growth was obtained when skimmed milk-tryptic soy agar (SM-TSA) was used. It was prepared by adding 200ml TSA to 50ml of sterilised 10% skimmed milk (w/v) (LAB M, MC027). The skimmed milk was autoclaved separately from the TSA for 5 min at 121°C, while the TSA was autoclaved for 15 min at 121°C. They were then mixed together aseptically, poured into Petri dishes, allowed to set, and then inoculated. Each strain was streaked onto five SM-TSA plates. The plates were incubated for 48h at 37°C.

4.2.4 Desiccation procedure

The desiccation assay applied followed the same procedure as previously described by Caubilla-Barron and Forsythe (2007). Bacterial cultures (48h incubation period) were harvested from the five plates using a sterilized spatula. The cells were dispensed in 7ml of liquid infant formula (Cow & Gate premium 1) and vortexed. An aliquot (0.5ml) of each bacterial suspension (cell density 10^8-10^{10} cfu/ml) was then transferred into 4.5ml of sterile liquid infant formula and serially diluted up to 10^{-8} cfu/ml. Next, 12.5µl of the undiluted bacterial suspension, and the multiple decimal dilutions, as well as un-inoculated liquid infant formula (Cow and Gate premium 1) were transferred into 40 of 96-microtitre trays giving a total of 480 trays for the 12 desiccated strains. Two plates containing 16 replicates per dilution were assigned for each sampling point. Plate counts were performed using the Miles and Misra method on TSA and incubated at 30°C for 24h to determine the initial concentration of the bacterial suspension before desiccation. To air-dry these suspensions, the 96-well plates were left open overnight (18h) in a class two cabinet. The plates were then sealed and stored away from light at room temperature (21°C).

4.2.5 Reconstitution of the desiccated strains

At 20 timed intervals, bacterial cells desiccated in two 96-microtitre trays were rehydrated with 200µl of sterile liquid infant formula (Cow and Gate Premium 1), and then incubated for 48h at 37°C. Reconstitution time points were 18h, 3, 7, 14, and 20 days, then every two months for up 24 month.

4.2.6 Determination of cell viability

Following the 48-hour incubation of the reconstituted cells, a replicator was used to aseptically inoculate two TSA plates with aliquots from each rehydrated well. The TSA plates were incubated overnight at 37°C. Next, the plates were checked for the presence or absence of growth of the sixteen replicates per dilution. The number of positive wells were entered into the BAM-MPN Excel software (Food and Drug Administration, 2006) to determine the viability of each strain at each time point based on the estimation of the most probable number (MPN) that caused these positive results per millilitre of the original sample. Survival curves of bacterial cells were generated by plotting the viable count ($\log_{10}$ cfu/ml) against the time points in order to compare desiccation survival trends of all the strains tested. The recovery of the strains tested was followed for a maximum period of 24 months or until the bacteria became undetectable.

4.3 RESULTS

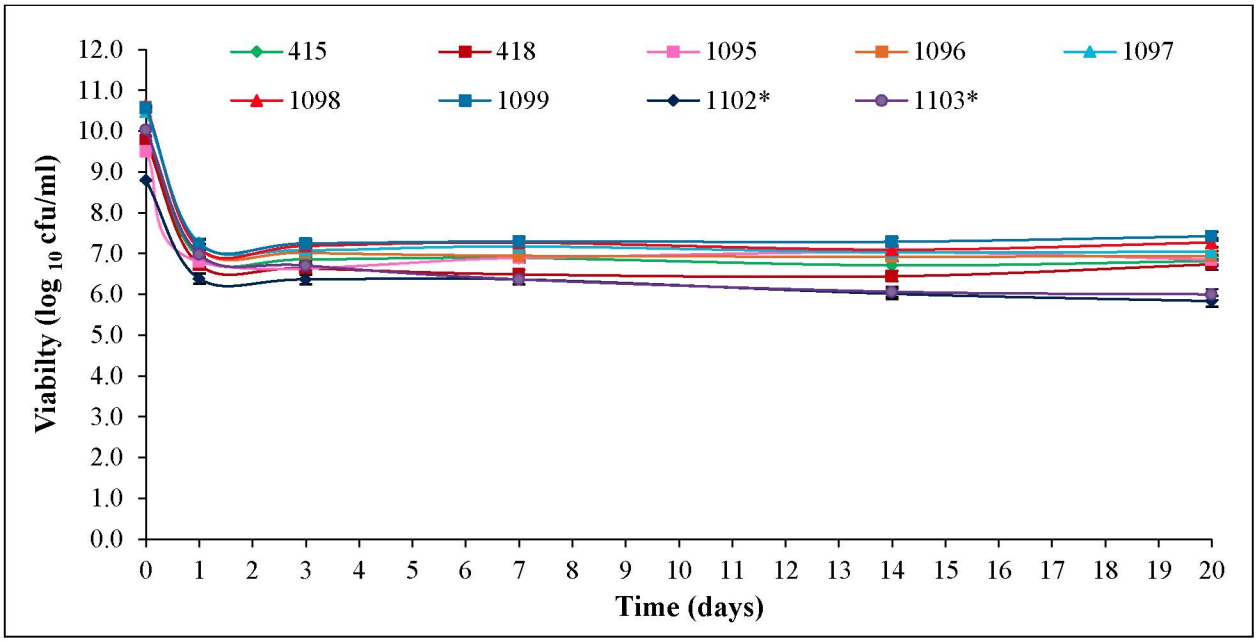
4.3.1 Capsule formation

India ink staining showed that all the *Acinetobacter* strains were capsulated. These capsules were negative stained and appeared as clear areas surrounding bacterial cells.

4.3.2 Long-term desiccation survival of *Acinetobacter* strains

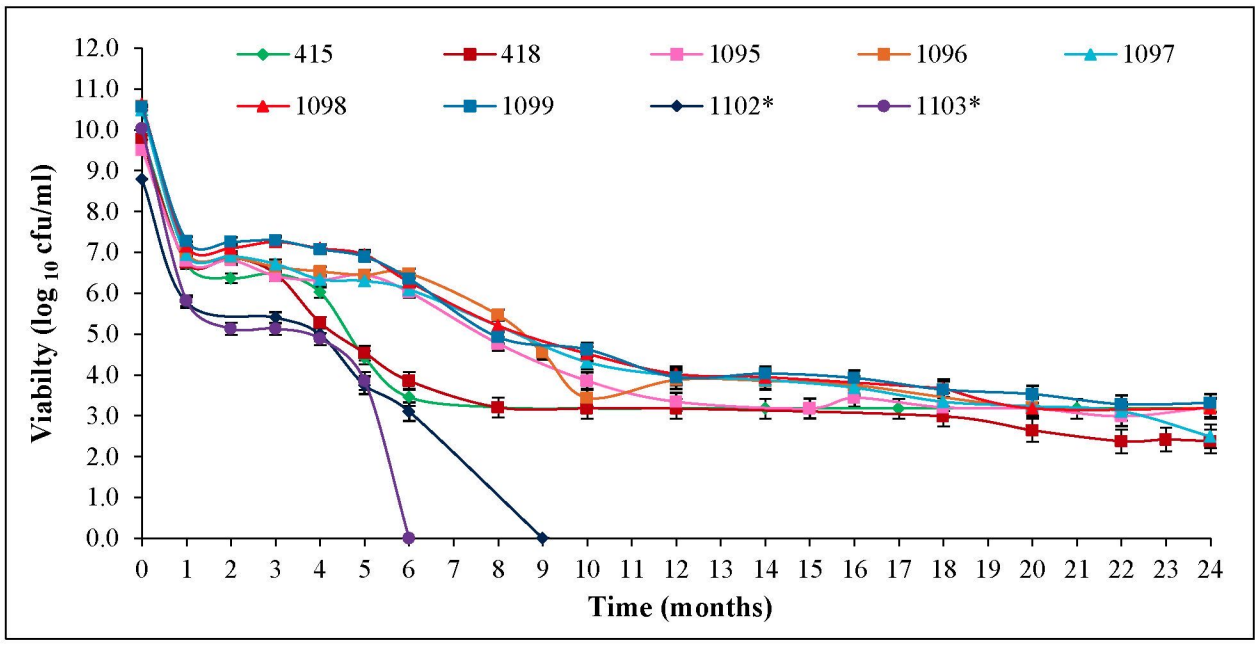
The first 18h of desiccation in infant formula caused the viability of all *Acinetobacter* strains to decrease by 2.4-3.5 $\log_{10}$ cfu/ml **(Figure 4.1)**. However, the reduction observed over the next 19 days ranged between 0.0 and 0.98 $\log_{10}$ cfu/ml **(Figure 4.1)**. Thereafter, the viable cells of strains 1095, 1096, 1098, and 1099 (*A. baumannii*) as well as 1097 (*A. calcoaceticus*) did not appear to be reduced for 5 months before starting to consistently decline **(Figure 4.2)**. Bacterial counts were at the order of 10^3-10^4 cfu/ml (3.1-3.7 $\log_{10}$ cfu/ml decrease) when a plateau was reached by 12 months of desiccation. The food isolates (415, and 418) exhibited similar desiccation survival trends to those of the clinical strains **(Figure 4.2)**. However, the decrease in viability was apparent after 3 months of desiccation. Unlike that of the clinical strains (12 months), the decline in the recovery of strain 415 and 418 continued only for up to 8 months until the persistence phase was reached. At this time point (8 months), the number of detected cells was 1.6×10^3 cfu/ml (total log decrease = 3.5 and 3.7 for strain 418 and 415 respectively). Overall, 7 out of the 9 desiccated strains were resistant to long-term desiccation in infant formula and remained detectable for the whole duration of the study (24 months) **(Figure 4.2)**. On the other hand, the type strains *A. baumannii* 1102 (NCTC 12156^T) and *A. calcoaceticus* 1103 (NCTC 12983^T) both exhibited a reduced desiccation tolerance compared to the other strains. The initial drop in viable count (2.4 and 3.1 $\log_{10}$ cfu/ml respectively) was followed by a lag phase which lasted 3 months at the order of 10^5 cfu/ml. After which, strains 1103 and 1102 continued to lose their viability until they were no longer detectable by 6, and 9 months respectively (total reduction = 3.1 and 3.3 $\log_{10}$ cfu/ml).

Figure 4.1 Survival of *Acinetobacter* strains during the first twenty days of desiccation.



The figure shows an intial decrease in viability (2.4 and 3.5 $\log_{10}$ cfu/ml) as a result of overnight desiccation stress. The reduction observed by 20 days ranged between 0.0 and 0.98 $\log_{10}$ cfu/ml**.** The number of viable cells recovering after reconstitution was calculated based on the MPN estimation of the number of cells showing growth in each well. Visible growth was seen when at least one bacterial cell was present in the sample. Strain 415 is *Acinetobacter* gensp. 3; 418 and 1097 are *A. calcoaceticus*; 1095, 1096, 1098, and 1099 are *A. baumannii*. Strains 1102 and 1103 are *A. baumannii* NCTC 12156^T and *A.calcoaceticus* NCTC 12983^T respectively.* Type strain. Error bars represent 95% confidence intervals.

Figure 4.2 Survival of *Acinetobacter* strains during 24 months of desiccation.

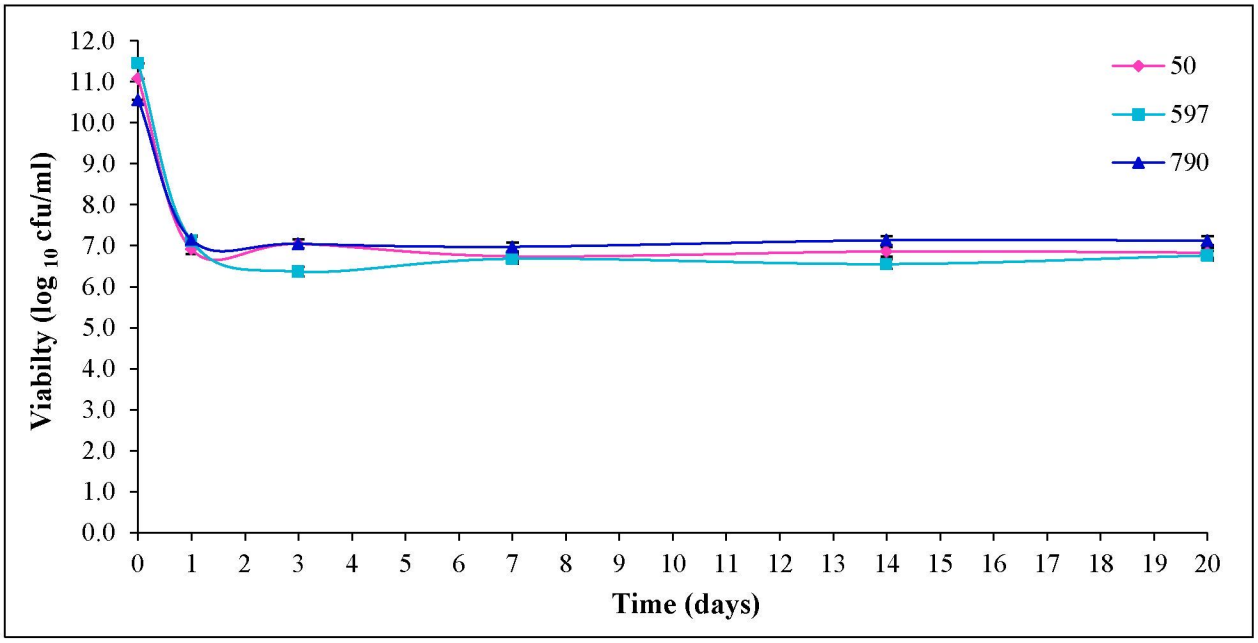


The figure shows three distinctive survival trends that separated the test strains into three groups. The food isolates (415 and 418), and the clinical isolates (1095, 1096, 1097, 1098, and 1099) both maintained their viability for 24 months in desiccated infant formula. However, the clinical isolates incurred lower viability loss than the food isolates particularly after 3 months of desiccation stress. The type strains 1103 and 1102 proved to be the most sensitive and were undetectable by 6 and 9 months respectively. Strain 415 is *Acinetobacter* gensp. 3; 418 and 1097 are *A. calcoaceticus*; 1095, 1096, 1098, and 1099 are *A. baumannii*. Strains 1102 and 1103 are *A. baumannii* NCTC 12156^{T} and *A.calcoaceticus* NCTC 12983^{T} respectively.* Type strain. Error bars represent 95% confidence intervals.

4.3.3 Desiccation persistence of bacterial species belonging to the Enterobacteriaceae family

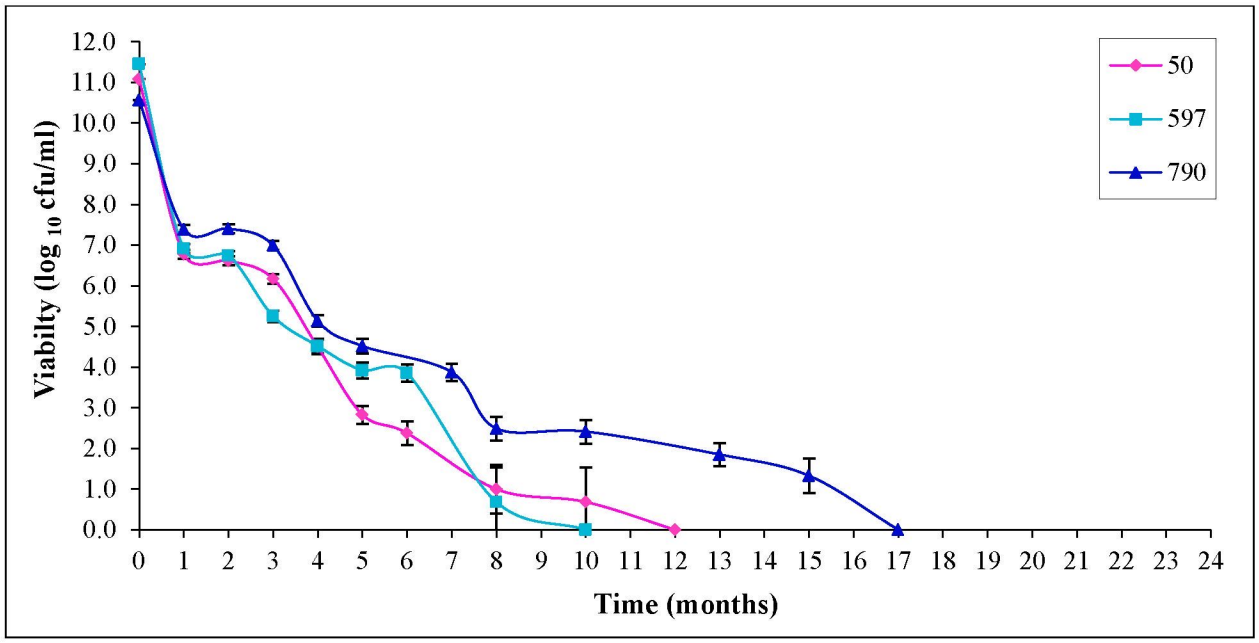
Exposure of *E. cloacae* 50 and 597, and *E. hormaechei* 790 to desiccation generated an initial 4.2, 4.3, and 3.4 $\log_{10}$ cfu/ml decrease in viability, while that monitored during the next 19 days was 0.1, 0.4, and 0.0 $\log_{10}$ cfu/ml respectively **(Figure 4.3)**. Afterwards, the cell count continued to decrease until no detection was achieved **(Figure 4.4)**. Strain 790 was the longest to persist under desiccated stress and remained recoverable until the last sampling point (17 months) (5.8 $\log_{10}$ cfu/ml decrease). On the other hand, strain 597 was the first of the Enterobacteriaceae to become undetectable by 10 months of desiccation, followed by strain 50 which was no longer recoverable at 12 months. The total loss of these strains was determined at 6.4 and 6.2 $\log_{10}$ cfu/ml respectively.

Figure 4.3 Survival of *E. cloaceae* and *E. hormaechei* strains during the first twenty days of desiccation.



Overnight desiccation in infant formula caused the viability of strains 50, 597, and 790 to initially decrease by 4.2, 4.3, and 3.4 $\log_{10}$ cfu/ml, while the loss of 0.1, 0.4, and 0.0 $\log_{10}$ cfu/ml was detected during the next 19 days respectively. Strains 50 and 597 are *E. cloacae*; 790 is *E. hormaechei*. Error bars represent 95% confidence intervals.

Figure 4.4 Survival of *E. cloaceae* and *E. hormaechei* strains during 24 months of desiccation.



All the strains presented in the figure showed consistent reduction in viability after two months of desiccated storage in infant formula. *E. hormaechei* 790 appeared to persist longer than *E. cloacae* 50 and 597. The total loss of these strains was determined at 5.8, 6.2 and 6.4 $\log_{10}$ cfu/ml respectively. Error bars represent 95% confidence intervals.

4.4 DISCUSSION

This desiccation survival study was designed to resemble the conditions present in PIF. The proposed period for monitoring the persistence was 2 years corresponding to the shelf-life of commercially available PIF products. Bacterial cultures were suspended in ready-to-use sterile infant formula so that the bacteria of interest were exposed to similar nutritional components to those in PIF. Commercially available PIF could not be used due to non-sterility and skimmed milk powder does not have similar nutritious content of PIF. Hence, both were not deemed suitable for the purpose of the study.

This study aimed to also optimise the recovery of injured bacterial cells after reconstitution. The incubation temperature and period, as well as the temperature of the water used for reconstitution were not intended to replicate the feed preparation and storage conditions in the hospital. The reason for this was to start the study with a high bacterial concentration in order to avoid a quick possible loss of detection of the persistent cells that could have otherwise been monitored over a longer period.

To produce powdered infant formula, the liquid in the milk suspension is evaporated by exposing it to enough heat for a very short period of time that the material dries almost instantaneously. The equipment commonly employed for this procedure in the food industry is the spray dryer. For research purposes, laboratory scale spray dryers can be employed. However, this method could not be feasibly applied to this study due to its high cost, and the impracticality when multiple organisms are involved. In contrast, the procedure used in this desiccation study is more practical and reproducible in any laboratory.

The biphasic survival curves observed in **Figures 4.2** could be due to the presence of mixed populations in which a small proportion is more resistant. Desiccation stress imposed on the microbial cells may have generated desiccation-resistant mutants in part of the population that survived the non-lethal selective pressure, which was illustrated by the decline in viable cells followed by the stationary period. An example of these survival changes was described elsewhere (Ramos *et al.*, 2001) in which a large proportion of the *E. coli* population exposed to carbon starvation died after a few days, and survivors were nourished on what was released

from the dead cells. Eventually, the resistant mutants outnumbered the sensitive cells and remained viable for years (Ramos *et al.*, 2001).

Based on the comparison of the survival curves **(Figure 4.2)**, the desiccated *Acinetobacter* strains were divided into 3 groups. Strains in the first category were considered the most sensitive to desiccation, and included type strains *A. calcoaceticus* NCTC 12983^T and *A. baumannii* NCTC 12156^T. The food isolates *A. calcoaceticus* (415), and *Acinetobacter* gensp. 3 (418) formed the second cluster. The final group consisted of the clinical isolates; *A. baumannii* 1095, 1096, 1097, 1098, and 1099. Although the food and the clinical isolates all recovered after 2 years of storage, the latter group seemed to have a smaller proportion of the more sensitive cells in its populations, while the food isolates appeared to contain a larger proportion of these cells. This was suggested by the sharper declining of the second group. However, overall the organism is clearly able to successfully adapt to the prolonged desiccation stress, regardless of the source of isolation. The higher sensitivity of the type strains of *A. baumannii* and *A. calcoaceticus* compared to the remaining strains is not unusual and is in agreement with previous studies that monitored the desiccation persistence of species of the Enterobacteriaceae family (Caubilla-Barron and Forsythe, 2007; Jawad *et al.*, 1998a; Wendt *et al.*, 1997). Although the *A. baumannii* type strain did not survive as long as the other strains, it persisted for longer than reported in other studies when desiccated in distilled water (Jawad *et al.*, 1998b; Wendt *et al.*, 1997), which may indicate the protective role of the nutritional content of infant formula. Milk-based infant formula products contain milk fat, proteins, and lactose. These compositions are believed to provide some degree of protection to desiccated *Acinetobacter* strains through their protective osmotic effects (Gardiner *et al.*, 2000; Lian *et al.*, 2002). A previous observation by (Wang *et al.*, 2004b) showed that the percentage recovery of spray-dried bifidobacteria increased from 16% and 82.6% when dried with 10% skimmed milk compared to gum arabic, gelatin, and soluble starch. Another factor contributing to the protection of bacterial cells against desiccation is believed to be the presence of the capsule by retaining water molecules, and hence prolonging the time taken by these cells to dry (Ophir and Gutnick, 1994; Roberson and Firestone, 1992). All the *Acinetobacter* strains tested in this study appeared to be capsulated and these capsules may have consequently enhanced their persistence under desiccated conditions.

It is apparent in this study that Enterobacteriacae strains (*E. cloacae* and *E. hormaechei*) reported to cause neonatal infections were more sensitive to desiccation than the *Acinetobacter* strains. *E. cloacae*, which is commonly found in PIF, was no longer recoverable by 10 and 12 months. Caubilla-Barron and Forsythe (2007) reported an 8 months survival period for *E. cloacae* when the same desiccation procedure was applied. The survival variation between strains within the same species is not exceptional. For instance, desiccation persistence of 10 *Cronobacter sakazakii* strains was described to be ranging from 12 to 30 months (Caubilla-Barron and Forsythe, 2007). Different repair mechanisms adopted by different strains within the same species may attribute to these strain-dependent responses to dehydration. Of interest is that *Acinetobacter* strains studied here survived for longer than some *Cronobacter sakazakii* strains that were no longer detectable after 20 months (Caubilla-Barron and Forsythe, 2007). *C. sakazakii* has been well known for its implication in life-threatening neonatal infections such as meningitis and necrotizing enterocolitis in association with contaminated PIF (Bar-Oz *et al.*, 2001; Lai, 2001). This observation highlights the virulence potential of *Acinetobacter* and also the possible relevance between the consumption of a feed containing *Acinetobacter* and the risk of neonatal infections.

The ingested dose of *Acinetobacter* cells required for the initiation of a neonatal infection is not known. However, it is reasonable to propose that it could be relatively low given the state of the immune system and the deficiency in the competing gut flora of the neonate. High recovery of *Acinetobacter* from reconstituted PIF given to infants at a hospital in Cape Town has been reported (Marino *et al.*, 2007). Eighty-two samples were analysed pre-incubation at different temperatures, while ninety-two samples were examined post-incubation. Pre-incubated samples were found to contain 31 *Acinetobacter* isolates, 12 *Bacillus* isolates, and 6 mixed cultures of *Bacillus* and *Acinetobacter*. When PIF was left to stand for 6h at 30°C, the detectable bacterial contamination in PIF increased from 8/34 to 18/34 samples. Overall, 20/82 and 25/92 samples showed heavy contamination (> 10^4 cfu/ml) pre- and post-incubation, respectively (Marino *et al.*, 2007). The ingestion of this number of bacterial cells in a contaminated feed could potentially expose the neonates to a high risk of consequent infections. According to the Codex Alimentarius Commission (2008), pathogenic organisms in PIF should not be considered safe at any level since illnesses resulting from the

administration of contaminated PIF do not require a large number of pathogens, as even small numbers can colonize the neonate.

In summary, this work is the longest reported desiccation persistence study to involve *Acinetobacter* and the first to be conducted in infant formula. The findings demonstrated the considerable ability of this organism to maintain its viability during long-term desiccation in infant formula and to efficiently recover after reconstitution. This recovery and subsequent proliferation, if allowed to happen, can be considered as a risk factor to the health of the neonate receiving the contaminated feed in the ICU. This risk would be greater if bacterial cells were provided with an environment where they could establish themselves and increase in numbers. Such environment could be the enteral tubes, which are placed inside the nasogastric tract of the neonate and used for feeding. The next study investigated the biofilm formation inside enteral feeding tubes and the potential hazard associated with it for the neonate.

CHAPTER 5. BIOFILM FORMATION OF *ACINETOBACTER* INSIDE ENTERAL FEEDING TUBES

5.1 INTRODUCTION

One of the predisposing factors for *Acinetobacter* infections is the use of invasive devices (Bergogne-Berezin and Towner, 1996; Dima *et al.*, 2007; Jerassy *et al.*, 2006; Thongpiyapoom *et al.*, 2004; Wang *et al.*, 2004a). On one hand, these devices are an essential part of medical care but on the other hand they serve as a source of acquisition and transmission of micro-organisms. *Acinetobacter* spp. is known for its ability to attach and form biofilms on abiotic surfaces such as glass and plastic (Loehfelm *et al.*, 2008; Tomaras *et al.*, 2003; Vidal *et al.*, 1997; Vidal *et al.*, 1996). Such a trait facilitates the colonization and survival of pathogens not only in the hospital environment but also in medical devices (Donlan and Costerton, 2002) where biofilms have been associated with severe clinical manifestations including bacteraemia, meningitis, and lower respiratory infections (Chen *et al.*, 2007a; Rodriguez-Bano *et al.*, 2008).

Biofilms are likely to develop at the point where the medical device is inserted or on its surface as a result of local colonization, hematogenous spread from a distant site, or infusion of contaminated material (Crnich and Maki, 2002; Raad and Bodey, 1992). Enteral feeding is a standard practice used for nutritional provision to patients who have a functional gastrointestinal system but cannot ingest food orally (Matlow *et al.*, 2003; Roberts, 2007). In contrast to parenteral feeding, enteral nutrition is generally associated with fewer complications (Kirby *et al.*, 1995), as well as being cost effective (Tucker and Miguel, 1996), and helping to maintain the structure and the function of the gastrointestinal system (Abou-Assi *et al.*, 2002; Kalfarentzos *et al.*, 1997; Gupta *et al.*, 2003). However, bacterial contamination of the enteral formula is one of the recognized disadvantages of this method of nutritional support. Studies have previously identified enteral tubes as a reservoir for microbial contamination (Matlow *et al.*, 2003; Mehall *et al.*, 2002a; Roy *et al.*, 2005). The acquisition of antimicrobial-resistant organisms such as vancomycin-resistant *Enterococcus* (Matlow *et al.*, 2003; Mehall *et al.*, 2002a; Weinstein *et al.*, 1996), methicillin-resistant *Staphylococcus aureus* (MRSA) (Mehall *et al.*, 2002a), and carriers of extended-spectrum beta lactamases (*Escherichia coli* and *Klebsiella* spp.) (Wiener *et al.*, 1999) is not unusual via this route. Epidemiological and microbiological links between enteral feeding and feeding

intolerance, abdominal distention, aspiration pneumonia, diarrhoea, necrotizing enterocolitis, and even systemic infections such as sepsis caused by bacterial contamination have been found (Anderson *et al.*, 1984; Anderton, 1993; Freedland *et al.*, 1989; Wagner *et al.*, 1994).

Ingestion of enteral feeds contaminated with *Acinetobacter* spp. can also potentially serve as a risk factor for neonatal infections (FAO-WHO, 2006). Powdered infant formula can be intrinsically contaminated (Cawthorn *et al.*, 2008; Marino *et al.*, 2007; Miled *et al.*, 2010) where this organism is likely to survive owing to its desiccation resistance capacity (as shown in the previous chapter). Alternatively, the prepared feed can be contaminated during preparation by the carer, environment, or the equipment. The presence of the organism in nasogastric feeding tubes collected from hospitalized patients has been previously reported. Desport *et al.* (2004) described that *Acinetobacter* was one of the most common contaminants of the enteral feeding lines administered to 14-89 years patients. In addition, Okuma *et al.*(2000) determined high contamination levels of *A. baumannii* (10^7 cfu/ml) from a nasogastric feeding formula sample given to a 62 year-old female patient. Contact with the abiotic surface of an enteral feeding tube could subsequently lead to attachment and biofilm formation.

The World Health Organization (WHO, 2007) have recommended using water at 70 °C or above to reconstitute PIF followed by the consumption of the feed within 2 hours of preparation. Avoiding the ambient temperature storage of the reconstituted feed in order to minimize the risks of neonatal infections was also recommended. This recommendation is not followed in all countries, such as USA, and is impractical when preparing small volumes of feed and fortifying breast milk. This proposition also did not take into account the time for which the enteral feeding tubes are at body temperature inside the nasogastric tract of the neonate, which can be for more than 48h (Hurrell *et al.*, 2009a; Hurrell *et al.*, 2009b; Mehall *et al.*, 2002b). During this time, bacterial biofilm formation occurs and can act as loci for repeated microbial contamination of subsequent feeds entering the neonate stomach and intestines (Hurrell *et al.*, 2009b; Mehall *et al.*, 2002b). As the biofilm ages the cells begin to disperse in the form of clumps which may protect the cells from the stomach acidity owing to the presence of bacterial capsular material. Consequently, bacterial biofilm formation may pose a risk to neonatal health once the bacterial cells could evade the detrimental effects of

the acidity and transit into the intestinal tract (Kim *et al.*, 2006). The complications resulting from this are significant in premature neonates due to the increased permeability of mucosa, immaturity of the immune system, and the undeveloped microflora of the gut (Greenough, 1996; Mehall *et al.*, 2002b). In addition, neonates in ICUs often require antibiotic treatment (Westerbeek *et al.*, 2006) which, combined with the intrinsic susceptibility of the neonate, may render them highly vulnerable for potential infections when exposed to organisms such as *Acinetobacter*. These infections may not only be local gastrointestinal infections, but also systematic infections following translocation from the immature gastrointestinal system. This could be particularly serious considering that common antibiotics may not be effective against multidrug-resistant *Acinetobacter* strains.

Although the isolation of *Acinetobacter* spp. from enteral feeding samples has been documented, the potential risk of exposure to neonatal *Acinetobacter* infections through enteral feeding required further elucidation. This was achieved by investigating the contamination of both the fresh enteral feeds administered to neonates and the lumen of the tubes due to biofilm formation. These investigations were conducted using practices similar to those applied in the healthcare setting, and based on applying the indirect Rapid Automated Bacterial Impedance Technique (RABIT) (Owens *et al.*, 1989; Silley and Forsythe, 1996). Quantification of biofilm cells through this technique relied on measuring the decrease in the conductivity of the growth medium consequently to the production of carbon dioxide, which resulted from the metabolic activities of the organism present.

5.2 MATERIALS & METHODS

5.2.1 Bacterial cultures and growth media

Whey (Cow & Gate1), casein (SMA1), and soy-based (Cow & Gate infasoy) infant formula were used to grow eleven *Acinetobacter* cultures for 18-20h (stationary phase) at 37°C. The test strains, their sources and designated culture collection numbers are presented in **Chapter 1**. All the strains were part of the preceding study **(Chapter 4)** except for three strains (1440, 1182, and1183) which were newly included here due to their later availability in the research culture collection.

5.2.2 Determination of attachment and biofilm formation using the indirect impedance technique

5.2.2.1 Preparation of potassium hydroxide agar

The KOH agar was prepared as indicated by Dézenclos *et al.* (1994). Ten grams per litre of KOH (Sigma-Aldrich, 221473) was dissolved in distilled water. Twenty grams per litre of bacteriological agar No. 2 (LAB M, MC006) was also prepared in distilled water and autoclaved for 15min at 121°C. Equal volumes of the KOH solution and the agar were then mixed and kept at 60°C for 3h before used for the indirect impedance method described next.

5.2.2.2 Construction of calibration curves

Acinetobacter cultures grown in casein, whey, and soy-based infant formula for 18-20h at 37°C were serially diluted to 10^{-7} cfu/ml. Sterile two-by-half tubes containing 2ml of the required infant formula type were inoculated, in duplicate, with 0.1ml of the dilutions. Two tubes containing un-inoculated infant formula were also included as negative controls. These test tubes were then placed inside specific electrode assembly tubes containing 0.75ml KOH agar, which was previously prepared and allowed to set across the electrodes. Neoprene rubber bungs were used to seal the tubes before loading into the Rapid Automated Bacterial

Impedance equipment (Don Whitley Scientific Ltd., UK). This equipment is composed of heating blocks into which the electrode assembly tubes are inserted. It also has a heating system for different incubation temperatures (± 0.001) in addition to a computer control system. Specific manufacturer's software designed to detect the conductivity was used. The metabolism of bacterial cells, present in the sample, would result in the production of CO_2. The KOH agar would in turn absorb the released CO_2 and lead to a reduction in conductivity. The detection criterion was set at -11μS change monitored at 6 min intervals. The detection time was defined as the time at which three repeated decreases in conductivity, which were equal or larger than -11μS, occurred. The values of time to detection (TTD) for all the bacterial dilutions in infant formula, incubated in the RABIT instrument at 37°C for 24h, were plotted against the viability ($\log_{10}$ cfu/ml) of the inoculum. The regression equations derived from these plots were used to calculate the doubling time of the test organisms.

5.2.2.3 Biofilm setup

Acinetobacter cultures grown in infant formula for 24 hours at 37°C were diluted to 10^4-10^5 cfu/ml in sterile casein, whey, and soy-based infant formula before inoculation into 40 cm sterile polyvinylchloride enteral feeding tubes (Unomedical Ltd., UK). These tubes were placed aseptically in sterile bottles and then incubated at 37°C. Two hours later, air was pushed gently through the tubes using a sterile syringe. The lumen liquid was retained for a viable count of the detached cells using the Miles and Misra plate count method. Four 2 cm lengths were cut from the feeding tubes, resulting in two duplicate samples. Within 10 min of preparation, the four pieces were transferred to two sterile two-by-half tubes each containing 2ml sterile infant formulas. The tubes were then placed inside the impedance electrode assembly tubes containing 0.75ml KOH agar across the electrodes, and sealed with rubber bungs. These were then connected to the RABIT equipment. The remaining portion of the tubes had fresh sterile infant formula flushed into them before placing back in the 37°C incubator until the next time point. The procedure was repeated at 2h intervals for up to 10, 24, and 48h. Each strain was tested for biofilm formation in duplicate. The same procedure was repeated three times with different types of infant formula and the results were compared.

5.2.2.4 Quantification of biofilm

The tubes containing the test samples were connected to the RABIT equipment for 48h at 37°C. The reduction in conductivity was detected and reported by the relevant computer software. Bacterial cells in the biofilm were calculated using the following formula:

$$\text{Bacterial concentration } (\log_{10} \text{cfu/2cm}) = c + (m \times \text{TTD})$$

Where c is the intercept, m is the slpoe, and TTD is the time to detection. These c and m values were derived from the calibration curves established in the same media for each test strain.

5.2.3 Statistical analysis

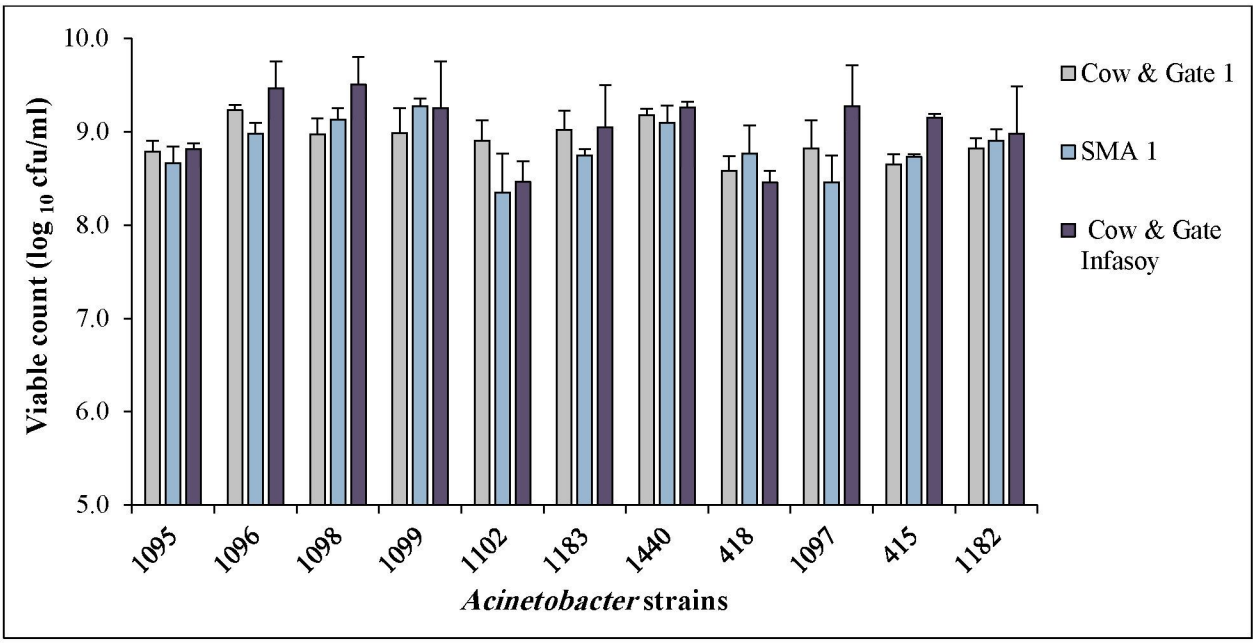
The data were statistically analysed in terms significant differences using the Student t-test. *P*-values lower than 0.05 indicated significantly different results. In addition, those lower than 0.01 and 0.001 were considered highly significant, and very highly significant respectively. To examine the relationship between two datasets, the Standard correlation test (Pearson product-moment correlation) was applied. The measure of correlation (r) ranging from -1 to 1 shows the strength of the negative or positive correlation. The closer the r value to the zero the weaker the correlation is.

5.3 RESULTS

Eleven *Acinetobacter* strains were evaluated for their time-course biofilm formation on enteral feeding tubes. Seven of which were clinical, and the remaining four were from food (2/4) and PIF samples (2/4). Viable counts of overnight cultures grown in the casein- (Cow & Gate 1), whey- (SMA 1), and soy-based infant formulas (Cow & Gate Infasoy) were determined before the start of the biofilm experiment in order to see if there was any preference towards a particular growth medium. The highest counts were found with eight of the eleven strains in Infasoy. However, they were not consistently significant. The average overnight cell count was 8.8, 8.9, and 9.1 $\log_{10}$ cfu/ml in SMA, Cow & Gate, and Infasoy respectively. Results are shown in **Figure 5.1**

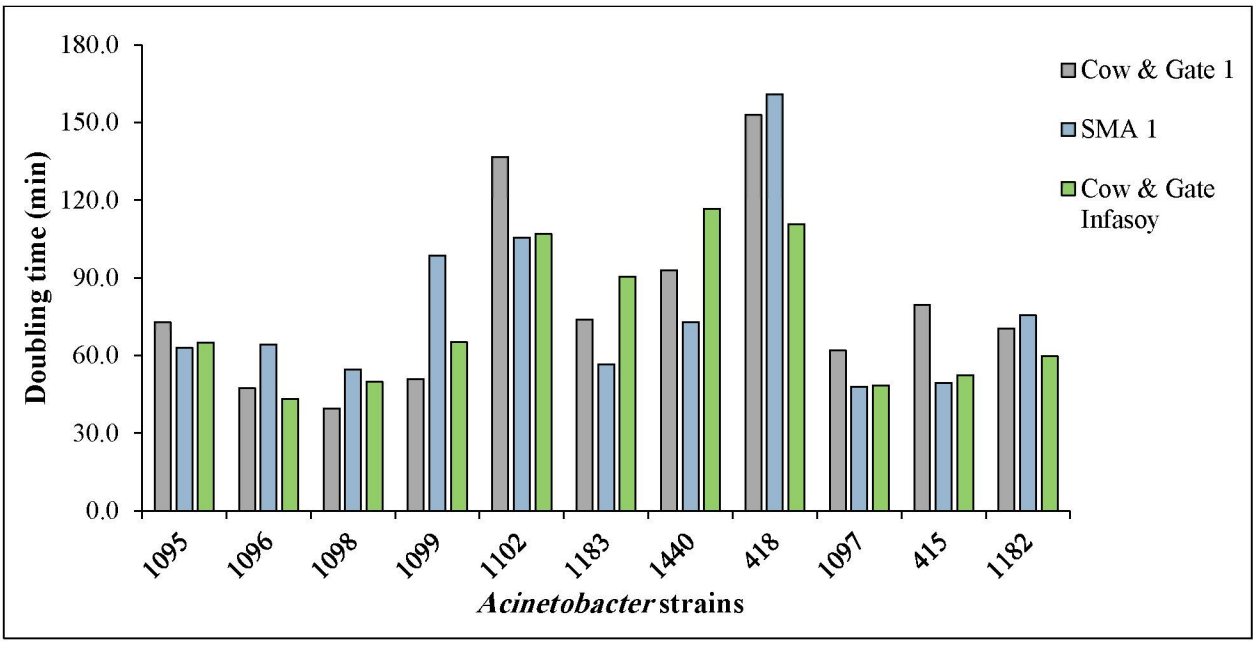
The doubling time of *Acinetobacter* strains varied between the infant formulas used, though no apparent tendencies was found among different species or isolates from different sources **(Figure 5.2)**. The mean time taken for the populations of test strains to double in numbers was 73.5 min in Infasoy formula, while that in Cow & Gate and SMA was 79.9 and 77.2 min respectively. When the strains were compared against each other, both *A. baumannii* 1102 (NCTC 12156^T) and *A. calcoaceticus* 418 appeared to be distinctly slower to double in all infant formulas (> 100 min).

Figure 5.1 Viable count of overnight cultures grown in casein- (Cow & Gate 1), whey- (SMA 1), and soy-based (Cow & Gate Infasoy) infant formulas.



Viable counts of overnight cultures in different types of infant formula were determined before the start of the biofilm experiment in order to see if there was any preference towards a particular growth medium. However, no significant difference was found. Strains 1095, 1096, 1098, 1099, 1102, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3. Error bars represent the standard deviation of at least two independent results.

Figure 5.2 Doubling times of *Acinetobacter* cultures grown in casein- (Cow & Gate 1), whey- (SMA 1), and soy-based (Cow & Gate Infasoy) infant formulas.



Doubling times of the test organisms in different types of infant formula were determined by plotting the viable counts of each bacterial dilution (from 10^{-2} to 10^{-8}) against the values of time to detection (TTD) monitored by the RABIT instrument. The regression equations derived from these plots were then used to calculate the doubling time, which was performed once. The mean time taken for the populations of test strains to double in numbers was 73.5 min in Infasoy formula, while that in Cow & Gate and SMA was 79.9 and 77.2 min respectively. Strains 418 and 1102 were distinctly slower to double in all infant formulas (>100 min). Strains 1095, 1096, 1098, 1099, 1102, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3.

According to the Pearson correlation test, the biofilm density determined at 2h and the doubling time for all strains were very weakly correlated (positive correlation; $r = 0.16$). Only 2.42% ($r^2 = 0.024$) of the variation in biofilm cell counts can be accounted for by the doubling time. When the same statistical analysis was applied, a positive correlation between the incubation duration and biofilm densities was found ($r = 0.70$, $P < 0.001$). Squared-r was 0.49 meaning that 49.0% of the variation in biofilm cell counts can be explained by the length of time the feeding tube was placed at body temperature (37ºC). The impact of the length of incubation time on the biofilm density was apparent. The numbers of cells forming biofilms progressively increased as the feeding tubes were left longer at the body temperature **(Figure 5.3-5.5)**. The results can be interpreted as a best-fit line of biofilm formation= 0.0556 x Incubation time + 4.45. In comparison with data collected at 2h, cell counts at 4h were not regarded as statistically significant ($P > 0.05$). In contrast, numbers of biofilm cells determined at 6 to 48h were generally significantly to highly significantly greater ($P < 0.05$ and $P < 0.01$). However, some occasional drop in the biofilm density values was recorded during the time-course across all the test strains. These decreases did not exhibit a consistent trend that could be associated with an incubation time of the infant formula inside the enteral feeding tubes.

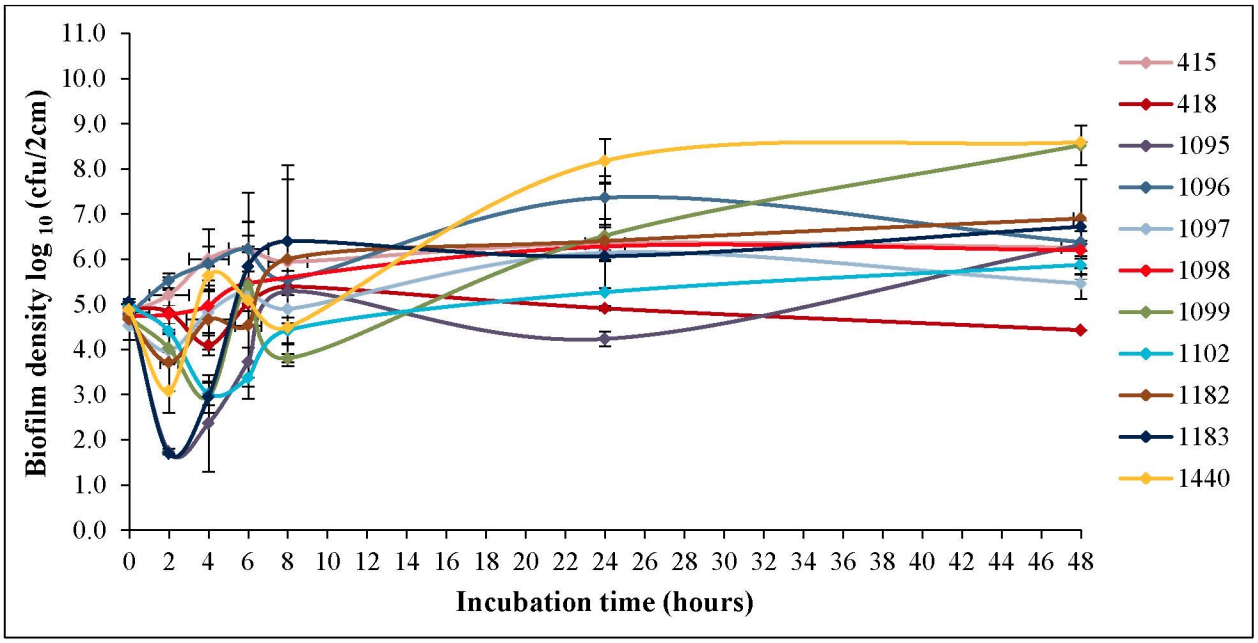
Overall values of biofilms formed on enteral feeding tubes varied between strains in each formula **(Figure 5.3-5.5)**. In contrast, the quantities of these biofilms did not differ significantly ($P > 0.05$) between the types of infant formulas over the time-course. The ability to attach to these tubes, within 2h of incubation at body temperature (37°C), was evident with all the test strains. Generally, 0.3-1.2, 0.6-1.2, and 0.6-1.1 $\log_{10}$ cfu/2cm of the inocula in Cow & Gate, SMA, and Infasoy were found adhering, respectively **(Figure 5.3-5.5)**. Cell counts of 1098, 415, and 1096 were 0.8, 7.9, and 15.5% higher than the initial inocula, after 2h, when grown in Cow & Gate. This equated to an increase of 0.04, 0.4, and 0.7 $\log_{10}$ cfu/2cm respectively **(Figure 5.3)**. The remaining strains were counted at between 1.7 and 4.8 $\log_{10}$ cfu/2cm, decreasing by 3.4 to 0.1 $\log_{10}$ cfu/2cm. In SMA, four strains (1099, 418, 1098, and 1440) showed adherence percentages > 100.0%, with 0.2, 0.3, 0.4, and 1.2 $\log_{10}$ cfu/2cm cell growth respectively **(Figure 5.4)**. That of the other seven strains was 56.8-99.3% (2.2-0.03 $\log_{10}$ cfu/2cm lower than the inocula). In Infasoy, nine of the eleven strains

exhibited 56.6-99.6% attachment, while 1099 adhered at 108.5% and 1097 at 111.1% corresponding to increases of 0.4 and 0.5 $\log_{10}$ cfu/2cm respectively **(Figure 5.5)**.

At 24h, biofilm cell counts reached 4.2 to 8.2 $\log_{10}$ cfu/2cm (mean = 6.2) in Cow & Gate and 5.0 to 8.0 $\log_{10}$ cfu/2cm in SMA (mean = 6.9) **(Figure 5.3 & 5.4)**. Those in Infasoy were averaged at 6.1 $\log_{10}$ cfu/2cm, and ranged between 3.0 and 7.7 $\log_{10}$ cfu/2cm **(Figure 5.5)**. It was evident that biofilm formation was significantly high in Infasoy ($p < 0.01$), and also very significantly high in Cow & Gate and SMA ($p < 0.001$) in comparison to cell counts obtained at 2h post-infection of the enteral feeding tubes. The mean increase in the biofilms formed was 55.0, 60.6, and 78.6% in Infasoy, SMA, and Cow & Gate respectively (2.1, 2.6, and 2.2 $\log_{10}$ cfu/2cm). By 48h, biofilm counts ranged from 4.4 to 8.6 $\log_{10}$ cfu/2cm (mean = 6.5) in Cow & Gate **(Figure 5.3)**, and from 5.7 to 9.2 $\log_{10}$ cfu/2cm (mean = 7.6) in SMA **(Figure 5.4)**. In Infasoy, biofilm densities were between 2.9 and 9.1 $\log_{10}$ cfu/2cm (mean = 6.3) **(Figure 5.5)**. Bacterial cells adhered inside the enteral feeding tubes continued to grow by an average of 1.0 $\log_{10}$ cfu/2cm in Cow & Gate, 1.1 $\log_{10}$ cfu/2cm in SMA, and 1.4 $\log_{10}$ cfu/2cm in Infasoy, relatively to values at 24h.

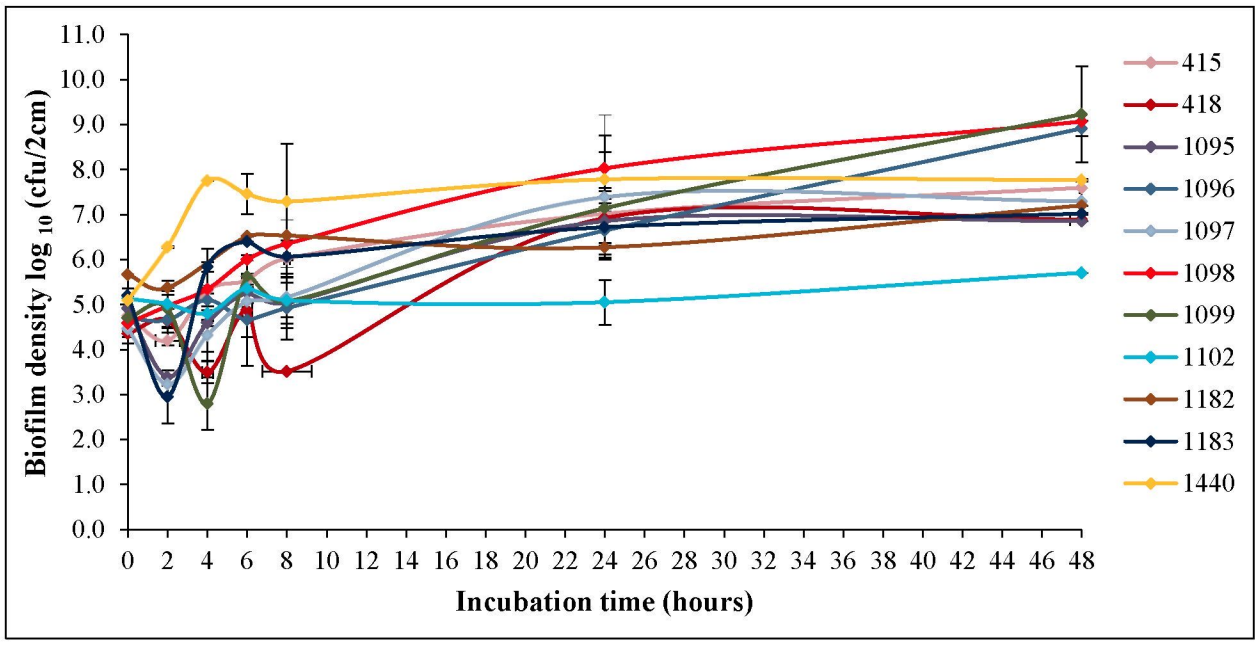
The number of bacterial cells in the lumen, which would be flushed out with each feed and replaced with sterile infant formula, was determined by viable plate count **(Figure 5.6-5.8)**. No significant variation was, generally, found between the different types of formulas apart from the cell counts in Infasoy at 48h, which were highly ($p < 0.01$) to very highly greater ($p < 0.001$) than those in Cow & Gate, and SMA respectively. When the data for all strains at each time point were pooled and compared, the number of bacterial cells in the fresh feeds, administered two hours after inoculation with 10^4-10^5 cfu/ml, was found to vary between 5.1 and 8.9 $\log_{10}$ cfu/ml. The average viable count that could have gained access to the neonatal stomach was 6.7 $\log_{10}$ cfu/ml. Following incubation for 6 and 8 hours, the bacterial count had respectively increased by 0.1-2.9 $\log_{10}$ cfu/ml and 0.3-3.3 $\log_{10}$ cfu/ml compared to the 2h samples. At 24h, much higher ($P < 0.001$) levels of bacteria were detected in the lumen, reaching between 7.8 and 10.0 $\log_{10}$ cfu/ml. After 48h, the lowest level of contamination detected was 6.9 $\log_{10}$ cfu/ml, while the highest was 10.9 $\log_{10}$ cfu/ml ($P < 0.001$).

Figure 5.3 Biofilm densities of *Acinetobacter* strains in casein-based infant formula (Cow & Gate 1).



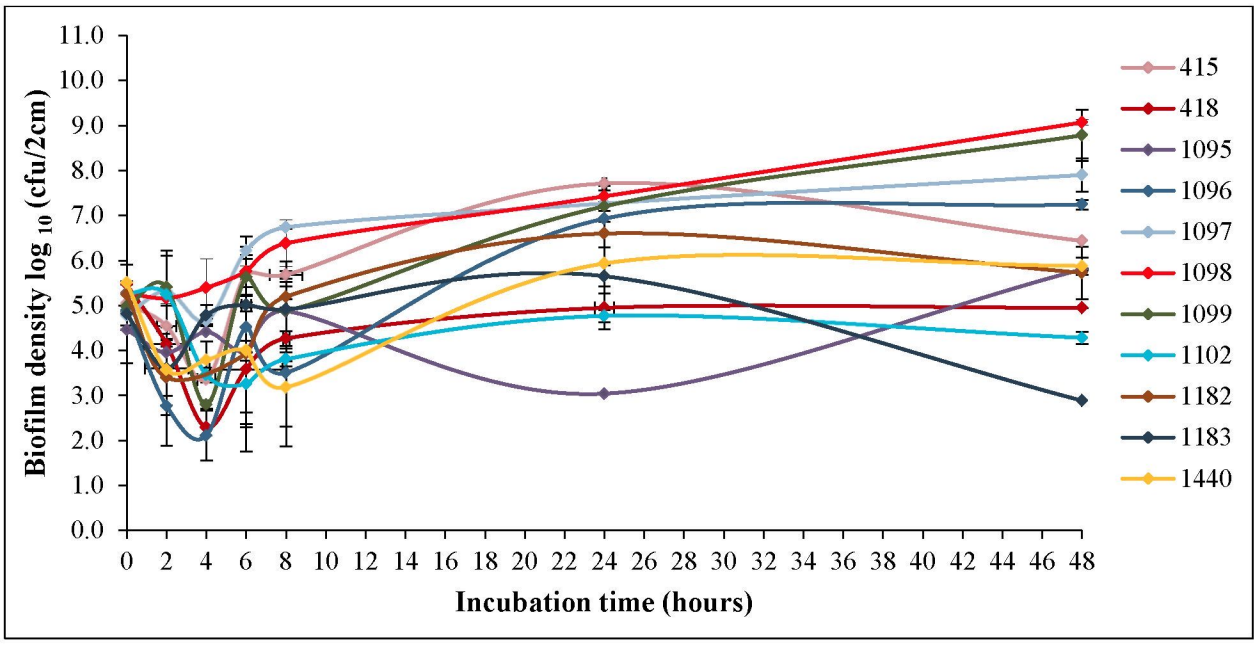
Cow & Gate 1 infant formula containing the test strains were inoculated into the enteral feeding tubes and incubated for 2h at 37°C. Fresh sterile infant formula was then flushed into these tubes at two hourly intervals for 48h. Quantification of biofilm cells attached the tubes was achieved using the direct rapid automated bacterial impedance technique at the sampling times specified above. The reduction in conductivity, due to the activity of the organism in the sample, was detected. The detection time was compared to that of the calibration curve to determine the number of bacterial cells forming the biofilm. Attachment to the feeding tubes was evident with all strains within 2h of incubation, and the majority of the biofilms formed by different strains reached their highest densities after 48h. Some of the biofilm values displayed above were widely spread. These variations were assumed to be due to the loss of weakly adhered cells. Strains 1095, 1096, 1098, 1099, 1102, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3. Error bars represent the standard deviation of two values per sampling point.

Figure 5.4 Biofilm densities of *Acinetobacter* strains in whey-based infant formula (SMA 1).



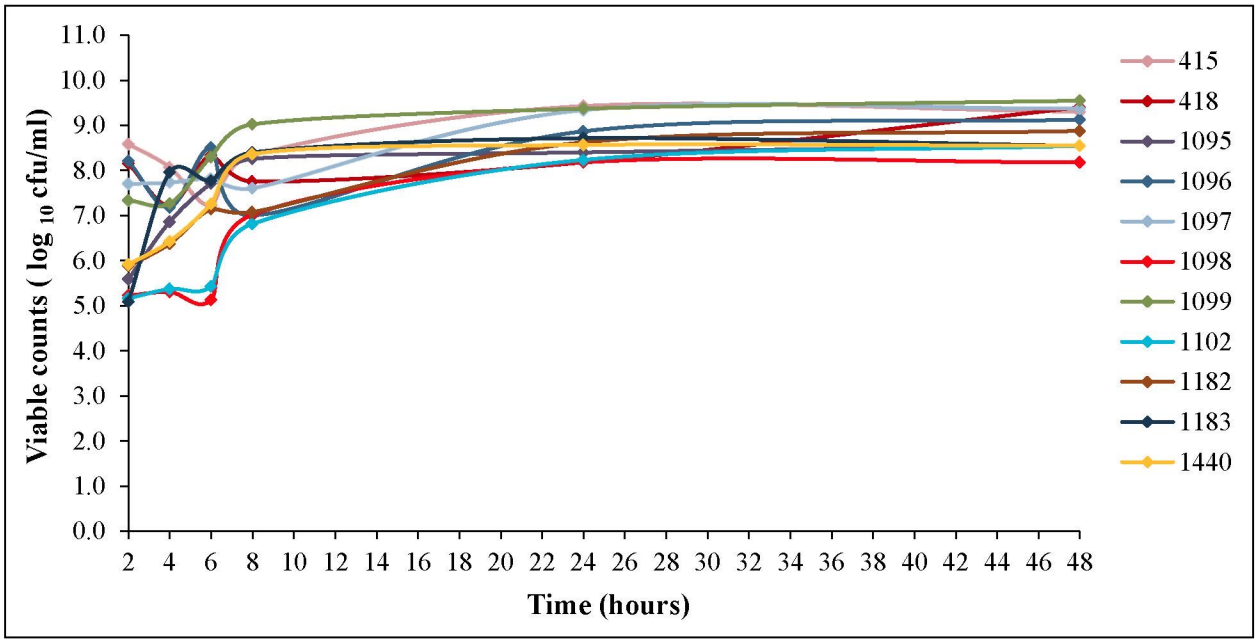
Biofilm formation tendencies exhibited in the whey-based formula (SMA 1) were similar to those in the casein-based formula under the same experiment conditions. Within 2 h of incubation at body temperature (37°C), 0.6-1.2 log10 cfu/2cm of the inocula ($T_0 = 10^4$-10^5 cfu/ml) were found adhering. At 24h, biofilm cell counts reached 5.0 to 8.0 $\log_{10}$ cfu/2cm (mean = 6.9) and were very highly significant ($p < 0.001$) compared to those obtained at 2h. Cells adhered to the enteral feeding tubes continued to grow by an average of 1.1 $\log_{10}$ cfu/2cm relatively to values at 48h. By 48h, biofilm counts ranged from 5.7 to 9.2 $\log_{10}$ cfu/2cm (mean = 7.6). Bacterial Strains 1095, 1096, 1098, 1099, 1102, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3. Error bars represent the standard deviation of two values per sampling point.

Figure 5.5 Biofilm densities of *Acinetobacter* strains in soy-based infant formula (Cow & Gate Infasoy).



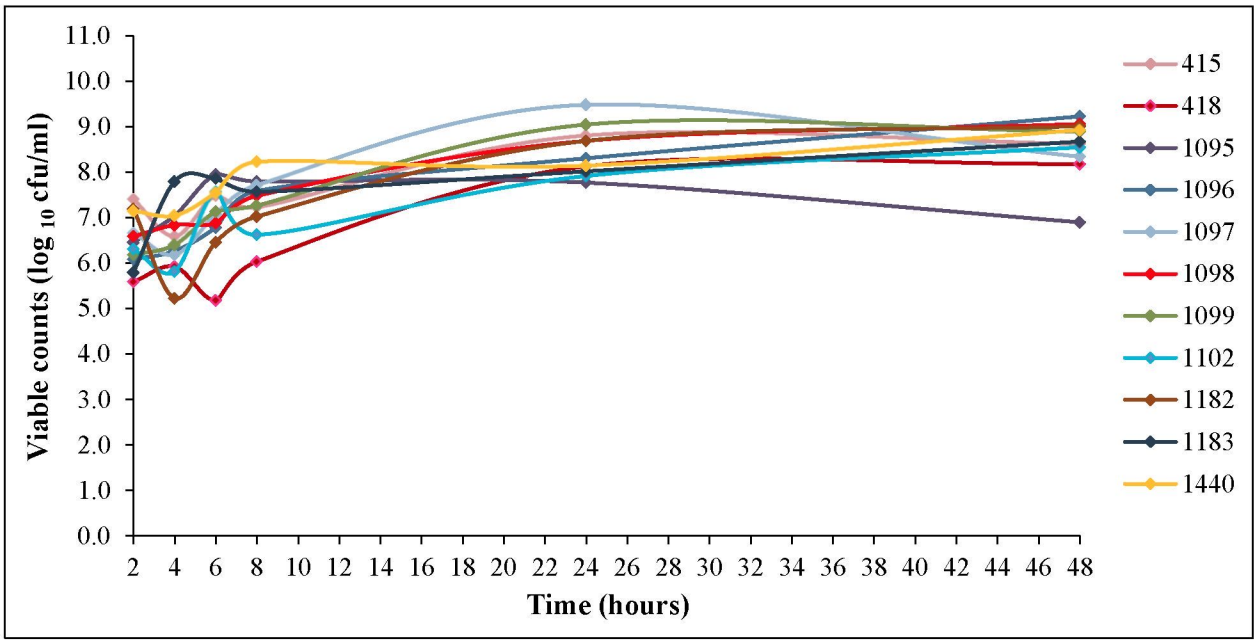
By 2 hours of incubation, strains inoculated into the Infasoy formula exhibited 0.6-1.1 $\log_{10}$ cfu/2cm attachment of the inocula. Biofilm cell counts ranged between 3.0 and 7.7 $\log_{10}$ cfu/2cm and were highly significant ($p < 0.01$) after 24h in comparison to cell counts obtained 2h post-inoculation of the enteral feeding tubes. The mean increase in the biofilms formed was 2.1 $\log_{10}$ cfu/2cm. Bacterial cells continued to grow by an average of 1.4 $\log_{10}$ cfu/2cm relatively to the values at 48h. By 48h, biofilm counts ranged from 2.9 to 9.1 $\log_{10}$ cfu/2cm (mean = 6.3 $\log_{10}$ cfu/2cm). Strains 1095, 1096, 1098, 1099, 1102, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3. Error bars represent the standard deviation of two values per sampling point.

Figure 5.6 Cell numbers of *Acinetobacter* strains in the residual casein-based infant formula (Cow & Gate 1) inside feeding tubes.



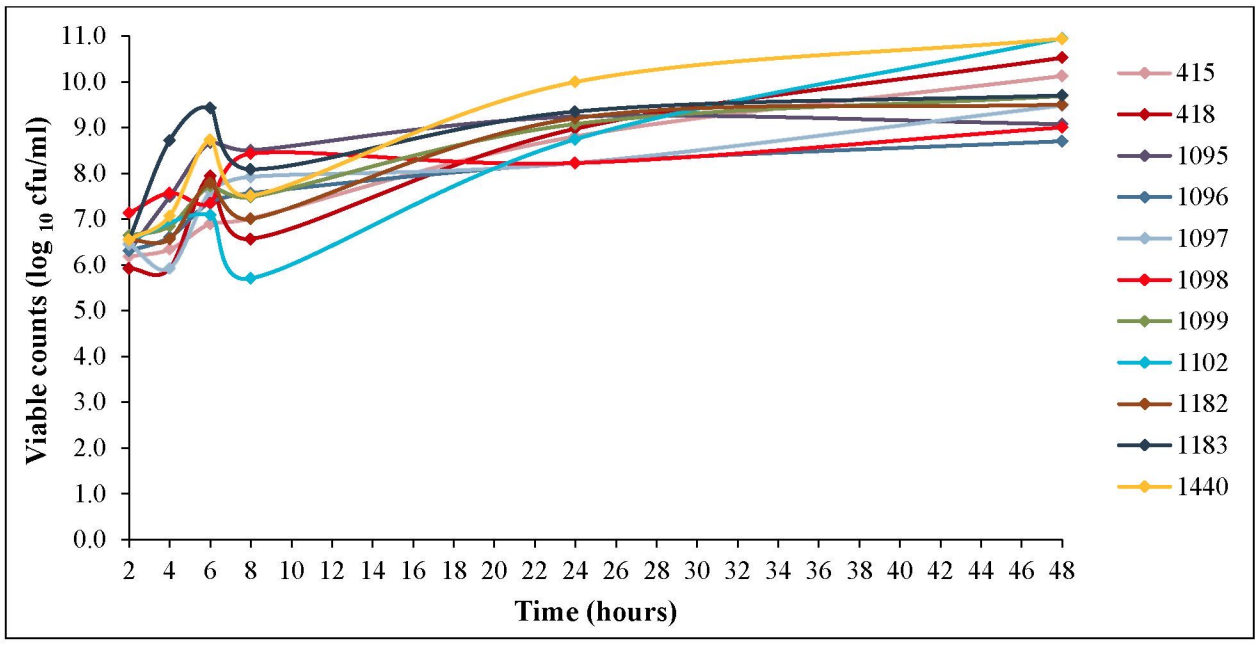
The number of bacterial cells in the lumen, which would be flushed out with each feed and replaced with sterile infant formula, was determined by viable plate count. Two hours after inoculation with 10^4-10^5 cfu/ml, bacterial cells in the fresh feed were found to vary between 5.1 and 8.9 $\log_{10}$ cfu/ml. The average viable count that could have gained access to the neonatal stomach was 6.8 $\log_{10}$ cfu/ml. Following incubation for 6 and 8 hours, the bacterial count had respectively increased by 0.1-2.7 $\log_{10}$ cfu/ml and 1.2-3.3 $\log_{10}$ cfu/ml compared to the 2h samples. At 24h, much higher ($P < 0.001$) levels of bacteria were detected in the lumen, reaching between 8.2 and 9.8 $\log_{10}$ cfu/ml. After 48h, the lowest level of contamination detected was 8.5 $\log_{10}$ cfu/ml, while the highest was 9.9 $\log_{10}$ cfu/ml ($P < 0.001$). Strains 1095, 1096, 1098, 1099, 1102, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3.

Figure 5.7 Cell numbers of *Acinetobacter* strains in the residual whey-based infant formula (SMA 1) inside feeding tubes.



Two hours after inoculation, bacterial cells in the fresh feed were found to vary between 5.8 and 7.8 $\log_{10}$ cfu/ml. The average viable count that could have gained access to the neonatal stomach was 6.7 $\log_{10}$ cfu/ml. Following incubation for 6 and 8 hours, the bacterial count had respectively increased by 0.4-2.1 $\log_{10}$ cfu/ml and 0.3-1.8 $\log_{10}$ cfu/ml compared to the 2h samples. At 24h, much higher ($P < 0.001$) levels of bacteria were detected in the lumen, reaching between 7.8 and 9.9 $\log_{10}$ cfu/ml. After 48h, the lowest level of contamination detected was 6.9 $\log_{10}$ cfu/ml, while the highest was 9.6 $\log_{10}$ cfu/ml ($P < 0.001$). Strains 1095, 1096, 1098, 1099, 1102, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3.

Figure 5.8 Cell numbers of *Acinetobacter* strains in the residual soy-based infant formula (Cow & Gate Infasoy) inside feeding tubes.



Two hours after inoculation, bacterial cells in the fresh feed were found to vary between 6.2 and 7.5 $\log_{10}$ cfu/ml. The average viable count that could have gained access to the neonatal stomach was 6.7 $\log_{10}$ cfu/ml. Following incubation for 6 and 8 hours, the bacterial count had respectively increased by 0.3-2.9 $\log_{10}$ cfu/ml and 0.5-2.2 $\log_{10}$ cfu/ml compared to the 2h samples. At 24h, much higher ($P < 0.001$) levels of bacteria were detected in the lumen, reaching between 8.7 and 10.0 $\log_{10}$ cfu/ml. After 48h, the lowest level of contamination detected was 9.1 $\log_{10}$ cfu/ml, while the highest was 10.9 $\log_{10}$ cfu/ml ($P < 0.001$). Strains 1095, 1096, 1098, 1099, 1102, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3.

5.4 DISCUSSION

The exposure of neonates to *Acinetobacter* in the healthcare setting was simulated using enteral feeding tubes as an inert support, and sterile ready to feed infant formula (suitable for the needs of babies from birth to 3 months) as a nutrient source. The formula was spiked with the test organisms grown to the early stationary phase. This was standardized so that variations in the attachment and the formation of biofilm, owing to different physiological states of bacterial cells, were avoided. Of note is that bacterial cells were not exposed to desiccation stress, as would be the case with those present in PIF.

This study showed no significant variation between strains of different *Acinetobacter* species in terms of the density of the biofilms formed. The biofilm cell density was not dependent upon strain source; follow up infant formula, food, or clinical. All tested strains persisted in the feeding tubes where cells adhered within 2h of inoculation and multiplied thereafter. The biofilm formation capacity of *A. baumannii* strain 1183 and *Acinetobacter* genosp. 3 strain1182, both isolated from follow up formula, was comparable to that of the other clinical strains giving support to the clinical significance of *Acinetobacter* contaminated feed. The ability of *A. baumannii* 1102 (ATCC 19606^T) to form biofilm in the enteral feeding tube was consistent with Tomaras *et al.* (2003) who demonstrated the strain's capacity to colonise polypropylene, polystyrene, and Teflon.

In a previous work by Hurrell *et al.* (2009a), the average cell density of biofilms formed by *A. calcoaceticus* strain 418 were 0.5, and 1.3 $\log_{10}$ cfu/cm in different types of feeding tubes (polyurethane and polyvinylchloride respectively). The same strain in this current study showed greater biofilm densities (2.5, 3.5, and 2.5 $\log_{10}$ cfu/cm in casein-, whey-, and soy-based infant formula respectively). Differences could be attributed to the different impedance technique applied in this study (indirect impedance) which appears to be more suited to the non-fermentative nature of *Acinetobacter* as opposed to the direct method previously used by Hurrell *et al.* (2009a).

Biofilm formation is influenced by the components of the medium in which the biofilm develops (Gerstel and Romling, 2001; Hood and Zottola, 1997; Kim *et al.*, 2006; Stepanovic *et al.*, 2003). This is due to changes in the characteristics of the bacterial cell surface (Kim *et al.*, 2006). The biofilm production by organisms such as the *Cronobacter* spp. is higher under nitrogen-rich conditions (whey-casein) than under carbohydrate-rich conditions (Dancer *et al.*, 2009). The infant formulas used were rich in milk protein and contained various nitrogen sources (casein, whey, and soy). The other components in these media were similar. *Acinetobacter* strains did not show significant differences in terms of the rates of cell growth or the levels of biofilm formation. The results are in agreement with Dancer's observation (2009) that the biofilm density of *Cronobacter* was similar in skimmed milk containing casein and that with added whey. To know whether various carbon sources would make any difference in terms of the biofilm formation, Tomaras *et al.* (2003) also examined *Acinetobacter* cells cultivated in a chemically defined medium (Simmons salts) supplemented with different carbon sources such as acetate, citrate, ethanol, lactate, pyruvate, or succinate. No significant effect was found in this respect.

Adhesion characteristics of *Acinetobacter* cells cultivated on innate surfaces can change as a response to the nutrient state (James *et al.*, 1995). Infant formula is rich in nutrients and in such environments the attachment of *Acinetobacter* cells may become unstable and continuously migrate across the surface in what is called the spreading maneuver (Lawrence and Caldwell, 1987). A similar type of spreading behavior was observed during surface growth of *Pseudomonas* (Marshall, 1988). Although this dispersive surface colonization allows expansion of the population over a surface, these weakly attached cells may detach more easily due to the liquid flow of subsequent enteral feeds (James *et al.*, 1995). The non-uniform disruption of adhered cells due to the sheer forces of the liquid stream was apparent in the fluctuation in the biofilm viable count during the 48h time-course. An additional reason could be irregular colonization of the tube surface. This inconsistency of the biofilm density is of clinical importance as it underlines the fragile nature of the biofilm inside the enteral feeding tube and the frequent dispersion of bacterial clumps in fresh feeds as a consequence. Colonization of enteral feeding tubes by *Acinetobacter* may occur not only during placement but also as a result of the administration of contaminated feeds. Therefore, to reduce this risk, the microbiological safety of neonatal feeds should be ensured during the general preparation

and handling of formula and feeding systems. The time the feed is left at room temperature, after opening and before administration, should also be minimized. Reconsideration of the prolonged nasogastric positioning of the feeding tube is also advised.

According to Hurrell *et al.* (2009b), the enteral nutritional regime given to hospitalized low birth weight infants in the NICUs involves regular fresh feeding every 2 to 3h via enteral feeding tubes. It is also reported that 13% of the feeding tubes are in place for more than 48h (Hurrell *et al.*, 2009b). This study demonstrates the consequential contamination of feeds due to lumen bacteria. The time during which the tubes are in place at body temperature in the nasogastric tract may pose a greater risk for allowing bacterial proliferation, than during the actual feeding that may last less than 30 min (Hurrell *et al.*, 2009b). The prolonged use of the same feeding tubes for enteral feeding allowed these tubes to serve as a reservoir for continuous exposure of the neonate to rapidly growing opportunistic pathogens. The average viable counts of *Acinetobacter* isolated from the lumen liquid increased to 9.3 $\log_{10}$ cfu/ml in 48h and that reflects the number of bacterial cells that could be flushed, with the each regular feed, into the stomach of the neonate. The colonized tubes may also result in clumps of bacteria entering the stomach, protected from the low acidity due capsule formation.

The bioburden of bacterial cells shed from the biofilm that could overcome the host defenses is unknown. The risk is not only the competency of the developing immune system and the lack of a competitive intestinal microflora, but also resistance to host defenses and antibiotics (Fux *et al.*, 2004; Lee *et al.*, 2008). Biofilm cells can grow and thrive in the presence of large concentrations of antibodies directed against epitopes on their surfaces, and can withstand the attack of activated phagocytes (Jensen *et al.*, 1990). These cells can also be less sensitive to acidic conditions (Hurrell *et al.*, 2009b), which is rarely sustained in the neonate stomach as the pH tends to be above 4.0 for prolonged periods of time due to frequent feeding (Hurrell *et al.*, 2009b; Mitchell *et al.*, 2001; Washington *et al.*, 1999). Frequent and short intervals in feeding do not allow enough time for the pH to go down to levels similar to that in the adult stomach. The long maintenance of the high pH levels with the highest reached at feeding times (Sondheimer *et al.*, 1985), when the organism in the contaminated feed is ingested, may offer a greater chance of bacterial survival, colonization, and potentially subsequent infections in the neonate. Although some pathogens lose their viability to a certain extent,

they are not completely killed or eradicated at such pHs (Edelson-Mammel *et al.*, 2006; Koutsoumanis *et al.*, 2004; Koutsoumanis and Sofos, 2004).

Cells growing in biofilms are generally highly resistant to antimicrobial agents (Lee *et al.*, 2008). The enhanced rates of horizontal gene transfer and recombination between bacterial cells within biofilm communities facilitate the spread of antibiotic resistance (Bergogne-Berezin and Towner, 1996; Donlan and Costerton, 2002), and provide biofilms a very high level of genomic diversity where it is expected that at least a small proportion of cells will withstand the impact of any antibiotic (Boles *et al.*, 2004). Antibiotic pressure might also select for antibiotic resistant strains, which are capable of forming biofilm. The problem with the high levels of antibiotic resistance is that it may not only be challenging in terms of the choice of treatment and the difficulty to sometimes clear the infection but also lies in the possibility of severe consequences to vulnerable neonates. This could be caused as a result of the delay in recognising that the bacterium is actually resistant to the antibiotic of choice and hence the delay in using the effective treatment which takes between 24-48h for culturing and susceptibility testing (Hurrell *et al.*, 2009b).

In summary, this research study investigated the extent to which *Acinetobacter* strains form biofilms over 48h at the neonate's body temperature using an enteral feeding routine that mimics the healthcare practices in NICUs. Biofilm densities were also compared in three infant formula made by different manufacturers for our target age (0-3 months) to meet the different nutritional needs of neonates. This work also provided detailed evidence of the role that enteral feeding plays in prolonging the exposure to *Acinetobacter* commonly associated with multiple antibiotic resistances. It also highlighted that the enteral feeding tube serves as a risk factor by providing bacterial cells with an environment where they can establish themselves and increase in numbers, which may hence increase the risk of infections to infection-prone neonates.

Although *Acinetobacter* strains have proven their ability to form biofilms on enteral feeding tubes, success in causing an infection through the ingestion route would also require them to survive during gastrointestinal passage. Therefore, the aim of the next chapter was to study the survival of this organism under conditions similar to those present in the gastrointestinal tract.

CHAPTER 6. GASTROINTESTINAL SURVIVAL OF *ACINETOBACTER*

6.1 INTRODUCTION

Acidity in the stomach acts as a major line of defence in protecting against infections through ingestion (Martinsen *et al.*, 2005; Smith, 2003). The average stomach's pH value of neonates given ready-to-feed and powdered infant formulas ranges from 3.5 to 4.3 (Hurrell *et al.*, 2009b). According to Zhu *et al.* (2006), the majority of bacteria are killed at a pH lower than 2.0. The bacterial killing in the stomach is also facilitated by the proteolytic activity of the gastric enzymes as they cause the release of peptides from, for example, lactoferrin which in turn act as antimicrobials (Nibbering *et al.*, 2001; Ryley, 2001). Other studies showed that adding lactate can overcome the acid tolerance of *E. coli* O157:H7 at a pH 3.0 and lead to cell death as a result of the drop in the pH of the bacterial cytoplasm (Jordan *et al.*, 1999).

Survivors of the acid barrier in the stomach will pass through the duodenum and jejunum in the small intestines where they would be subjected to the stress of the flushing forces of peristalsis, digestive enzymes, and bile secretions which all hinder the bacterial colonisation of these regions (Wilson *et al.*, 2002). Digestive enzymes (e.g. lipase, nuclease, peptidase, and trypsin) aid the process of degrading food macromolecules in order to produce products which have low molecular mass and can be absorbed by the intestinal cells (Wilson *et al.*, 2002). This is not their sole activity as they also exert an antibacterial effect (Rubinstein *et al.*, 1985; Wilson *et al.*, 2002). Beside their main role in emulsifying and solubilizing lipids (Wilson *et al.*, 2002), bile acids form another part of the intestinal defence system due to their ability to dissolve the proteins of cell membranes, phospholipids, and cholesterol, resulting in cell lysis (Flahaut *et al.*, 1996; Hofmann, 1994; Kristoffersen *et al.*, 2007; Leverrier *et al.*, 2003; Schmidt and Zink, 2000). Their antimicrobial effect is also caused by the oxidative stress and DNA damage (Kandell and Bernstein, 1991; Sokol *et al.*, 1995). Bile secretion is a mixture of primarily bile acids found physiologically in the form of sodium salts. These bile acids (deoxycholic, cholic, and lithocholic acids) are synthesized and conjugated with either taurine or glycine in the liver. They are then stored and concentrated in the gallbladder during the fasting state (Hofmann and Mysels, 1992). The effects of bile salts have rendered them useful as selective agents, specifically against Gram-positive bacteria, in various growth and enrichment media. Considering the substantial challenge represented by the exposure to bile,

it has been argued that bile tolerance may be a prerequisite for organisms to survive and colonize the intestinal region (Begley *et al.*, 2002). Intestinal pathogens as well as the commensal microbiota all possess such ability (Pace *et al.*, 1997; Pope *et al.*, 1995; Prouty *et al.*, 2002; Thanassi *et al.*, 1997; van Velkinburgh and Gunn, 1999).

The effects of stress factors present in the gastrointestinal tract have not been explored with respect to *Acinetobacter*. The role of the gastrointestinal juices in preventing the colonization by this organism is yet to be elucidated. The available information is mainly about the clinical isolation of the organism from neonates and adult patients admitted to ICUs. Isolation from the stomach was reported by Garvey *et al.* (1989), Garrouste-Orgeas *et al.* (1997), and Ma *et al.* (1999). Colonization by *Acinetobacter* spp. (Ofori-Darko *et al.*, 2000) and *A. lwoffii* (Zavros *et al.*, 2002) was also shown in the mouse stomach. A clinical isolate of *A. lwoffii* was later found in a gastric tissue culture (Kim *et al.*, 2011a). Parm *et al.* (2011a, b) reported the rectal colonization of neonates with *Acinetobacter* spp. during the first five weeks of their lives, while Thom *et al.* (2010) identified 6 critically ill adult patients with *A. baumannii* from perirectal cultures. *A. baumannii* was also found in the gut of 11% (25/228) of the neonates in NICUs (Roy *et al.*, 2010). In addition, 23 cases of neonatal gut colonization with *Acinetobacter* spp. were described in another study (Parm *et al.*, 2010).

Limited information is available with respect to the capacity of *Acinetobacter* to colonize the gastrointestinal system. Hence, scientific evidence shedding light on this relatively unexplored topic would be of great importance. The aim of this study, therefore, was to assess the survival of *Acinetobacter* under different physiological elements, which can have implications on the establishment of this organism in the gastrointestinal environment. The effects of acidic pH, gastric and pancreatic enzymes, and the bile secretion were investigated for this purpose.

6.2 MATERIALS & METHODS

6.2.1 Bacterial strains

The same strains that were previously studied for their desiccation survival in infant formula **(Chapter 4.)** were also examined in this study in order to mimic their behaviour through the ingestion route.

6.2.2 Inocula preparation

Acinetobacter cultures were grown in °ml sterile ready-to-feed infant formula (Cow & Gate 1) and incubated for 18h at 37°C. The cell density of these overnight cultures on the day of the experiment was ~10^6-10^8 cfu/ml.

6.2.3 Preparation of simulated gastrointestinal fluids and acidified infant formula

6.2.3.1 Gastric fluid

The gastric fluid was prepared as described by Charteris *et al.* (1998), as well as Huang and Adams (2004) with slight modifications. The gastric juice was simulated by dissolving 3g/l pepsin in 0.5% (w/v) saline before it was filter sterilised using a 0.45µm pore diameter cellulose filter. However, the pH (7.0) for the pepsin solution was kept neutral and was not adjusted. This was on purpose in order to separately study the effect of the pepsin enzyme as a single stress condition in this experiment.

6.2.3.2 Small intestinal fluid

The small intestinal fluid was prepared according to Koo *et al.* (2001). Porcine pancreatin (Sigma-Aldrich, P8096) (3.5g/l) and 0.1g/l porcine trypsin type 1X-S (Sigma-Aldrich, T0303) were added together and dissolved in distilled water at pH 7.0. A 0.45µm-pore diameter cellulose filter was used to sterilise this solution.

6.2.3.3 Biliary fluid

Simulated biliary secretions (pH 7.0), which were described by Koo *et al.* (2001), consisted of 40g/l porcine bile (Sigma-Aldrich, B8631) dissolved in distilled water and filtered by a 0.45µm pore diameter cellulose filter.

6.2.3.4 Acidified infant formula

Sterile infant formula was acidified to pH 2.5, 3.5, and 4.5 using 1M HCl (Sigma-Aldrich, H1758). The pH was determined aseptically by transferring 3ml of the acidified infant formula into a small bijou. The pH reading of the transferred volume was determined on a pH meter. The pH was adjusted to the desired value by the addition of small aliquots of 1M HCl whereas the 1M NaOH (Sigma-Aldrich, 72082) was added where the pH for the infant formula was lower than required.

6.2.4 Acid, pepsin, small intestinal fluid, and bile salts tolerance assays

To examine the effect of simulated acidic conditions in a neonatal stomach on the viability of *Acinetobacter* ingested through a contaminated feed, 15µl of the overnight (18h) cultures in infant formula were inoculated into 135µl of the pH adjusted formula, which was pre-aliquoted into 96-well plates. The temperature of the formula at the time of the inoculation was 37°C. The inoculated plates were then kept at 37°C for 2h. At specific time points (0, 15, 30, 45, 60, 90, 120 min), aliquots (15µl) were transferred into fresh 96 well plates containing sterile saline and then serially diluted. The viable count of these samples were enumerated on nutrient agar (NA) using the Miles and Misra method.

Similarly to the acid survival assay, the viability of *Acinetobacter* during the exposure to the enzyme pepsin and the simulated small intestinal fluid containing porcine pancreatin and porcine trypsin was assessed using the Miles and Misra method. Overnight (18h) cultures in infant formula were inoculated (15µl) into 135µl of the test solution (37°C), which was pre-dispensed into 96-well plates. Serial dilutions of the inoculated samples were prepared directly after inoculation (T_0) and plated onto NA applying the Miles and Misra method. The

same plate count procedure was repeated after 15, 30, 60, 90, 120 min of incubation at 37°C. The plates remained at this temperature for the whole duration of the experiment and were only taken out of the incubator during the rapid transfer of the 15μl of all the samples at once, using a multi-channel pipette. The rest of the viable count steps (serial dilutions and plating) were carried out while the original inoculated solutions were kept at the required temperature (37°C).

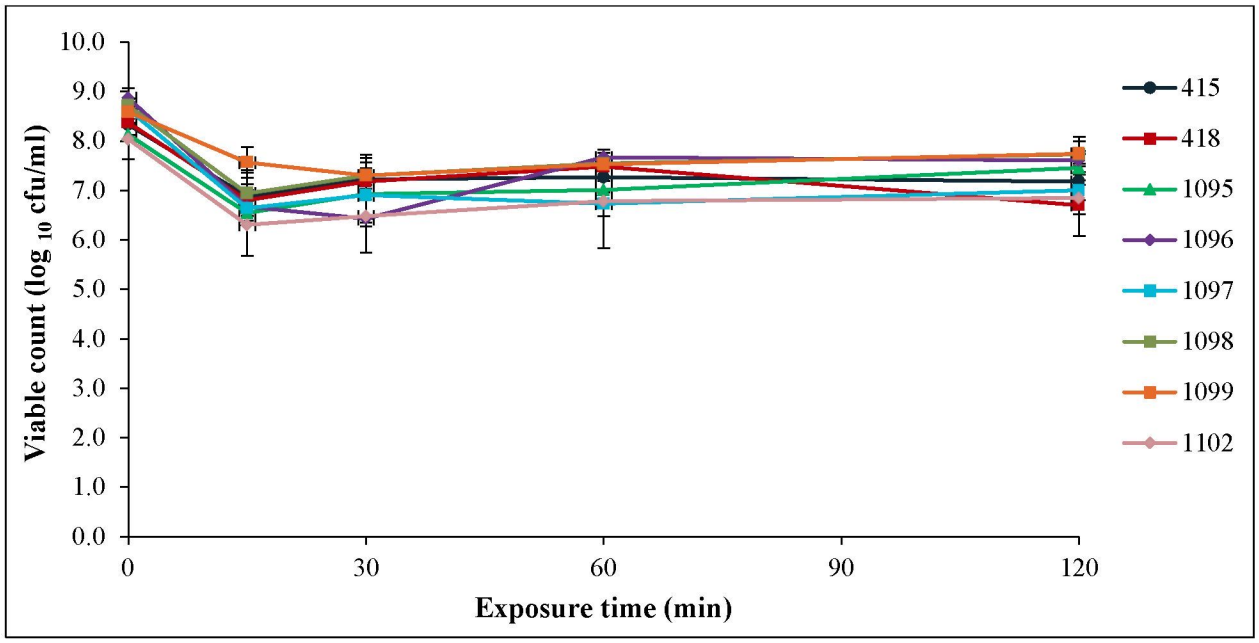
The presence or absence of *Acinetobacter* recovery was also examined in 0.125, 0.25, 0.5, 1, and 2% porcine bile solutions. Approximately 10μl aliquots of the overnight cultures, diluted ten-fold in the specified bile solutions, were transferred onto NA plates after 5, 10, 15, 30, 45, 60, 90, and 120 min of incubation at 37°C. The inoculated plates were incubated at 37°C and were checked 24h later. The initial inocula sizes were determined by carrying out the Miles and Miles plate count directly after inoculation (T_0). To evaluate the viability of *Acinetobacter* strains in 4% porcine bile, the same procedure as those of the pepsin and intestinal survival assays was followed at 0, 30, 60, 90, and 120 min. All the experiments, described above, were conducted twice and the results were expressed as the average of the $\log_{10}$ cfu/ml values.

6.3 RESULTS

6.3.1 Acid tolerance

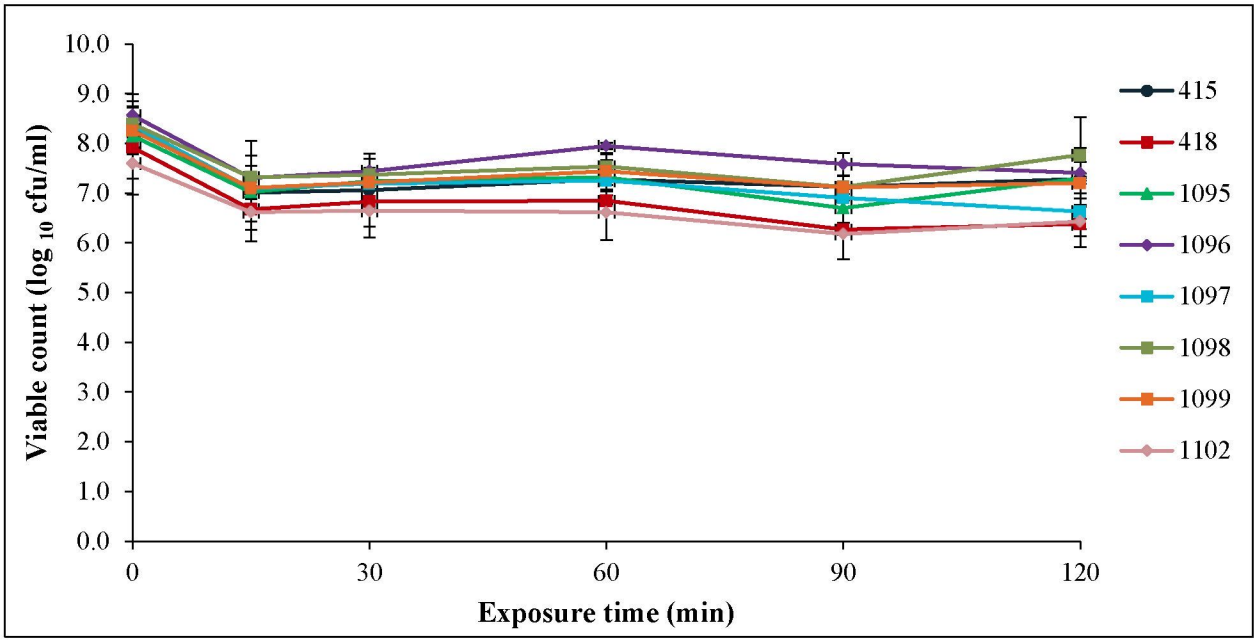
Acinetobacter strains were challenged for 120 min under three acidic levels (pH 2.5, 3.5, and 4.5). The upper value (pH 4.5) exhibited an initial effect on the viability of all 8 strains where there was an average decrease between 1.0 and 2.2 $\log_{10}$ cfu/ml (strain 1099 and 1096 respectively) during the first 15 min of acid exposure **(Figure 6.1)**. The following sampling points did not show significant reduction in cell counts of seven of the test strains ($P > 0.05$). The exception was strain 418 after 60 min. Overall, survival tendencies of the different strains at this pH (4.5) were statistically similar ($P \geq 0.05$). When the pH was lowered to 3.5, acidity caused the number of viable cells to drop by 1.0 to 1.3 $\log_{10}$ cfu/ml within 15 min of exposure **(Figure 6.2)**. Persistence of the *Acinetobacter* strains at consistent levels ($P > 0.05$) was apparent afterwards for the remaining duration of the assay (120 min). The acidified pH 2.5 solution caused a very highly significant ($P < 0.001$) decline of the bacterial populations which was evident in all test strains **(Figure 6.3)**. The complete cell loss by the last detection times ranged between 2.9 and 5.8 $\log_{10}$ cfu/ml. Different strains showed different acidity endurances and according to the comparison of their survival times they were categorised into acid sensitive, moderately acid tolerant, and acid tolerant at pH 2.5. *A. baumannii* (1096, 1099), *A. calcoaceticus* (1097), and *Acinetobacter* gensp. 3 (415) all remained viable for 60 min and therefore were regarded as acid tolerant. The, moderately, acid tolerant *A. baumannii* (1098) was last detected at 45 min of exposure to low acidity. *A. baumannii* ATCC 19606^T (1102) and strain 1095, as well as *A. calcoaceticus* (418) all exhibited the shortest survival time at 30 min.

Figure 6.1 Survival curves of *Acinetobacter* strains in infant formula at pH 4.5.



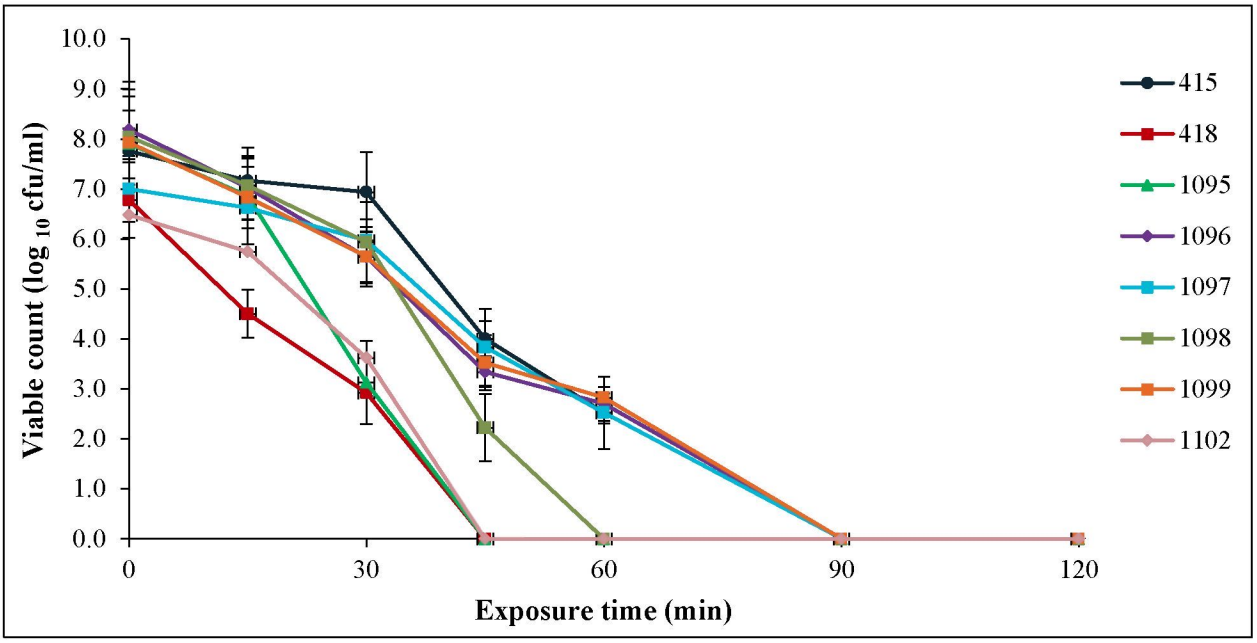
Acinetobacter strains were challenged for 120 min under pH 4.5 and the viable counts were determined using the Miles & Misra method. This pH value exhibited an initial effect on the viability of all 8 strains where there was a decrease between 1.0 and 2.2 $\log_{10}$ cfu/ml (strain 1099 and 1096 respectively) during the first 15 min of acid exposure. The following sampling points did not show significant reduction in cell counts of seven of the test strains ($P > 0.05$). The exception was strain 418 after 60 min. Overall, survival tendencies of the different strains at this pH were statistically similar ($P \geq 0.05$). Strain 415 is *Acinetobacter* gensp. 3.; 418 and 1097 are *A. calcoaceticus;* 1095, 1096, 1098, 1099, and 1102 are *A. baumannii*. Error bars represent the standard deviation of two independently repeated experiments.

Figure 6.2 Survival curves of *Acinetobacter* strains in infant formula at pH 3.5.



When the pH was lowered to 3.5, acidity caused the number of viable cells to drop by 1.0 to 1.3 $\log_{10}$ cfu/ml within 15 min of exposure. Persistence of the *Acinetobacter* strains at consistent levels was apparent afterwards for the remaining duration of the assay (120 min). Strain 415 is *Acinetobacter* gensp. 3.; 418 and 1097 are *A. calcoaceticus;* 1095, 1096, 1098, 1099, and 1102 are *A. baumannii*. Error bars represent the standard deviation of two independently repeated experiments.

Figure 6.3 Survival curves of *Acinetobacter* strains in infant formula at pH 2.5.

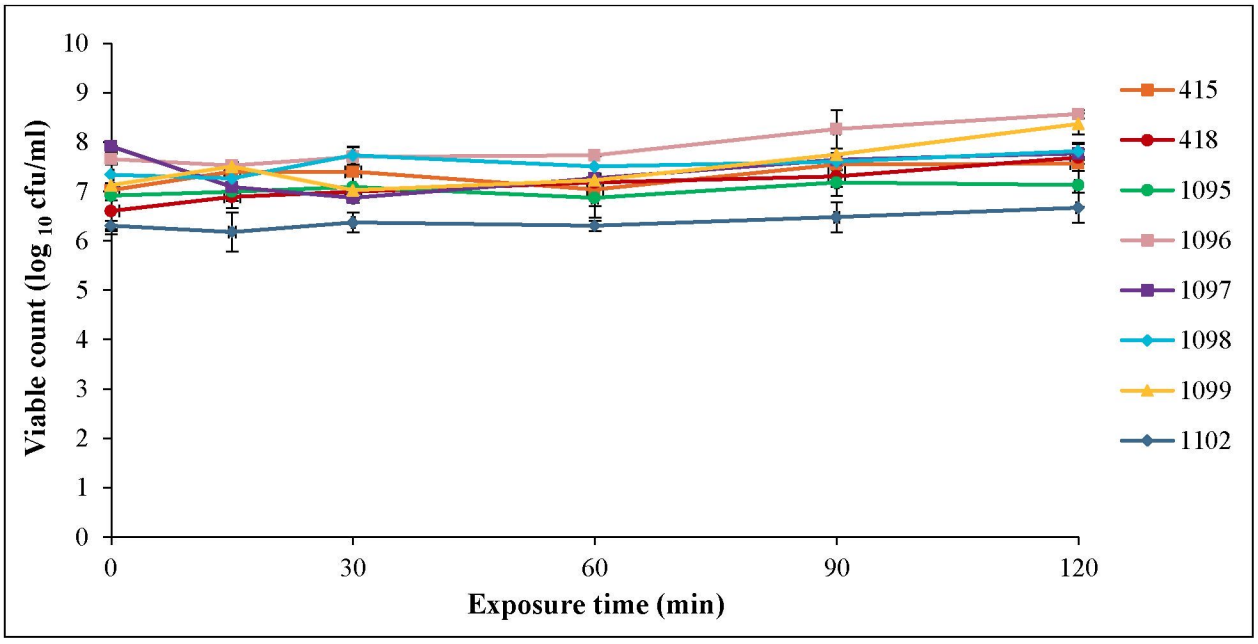


The acidified pH 2.5 infant formula caused a very highly significant ($P < 0.001$) decline of the bacterial populations which was evident in all test strains. The complete cell loss by the last detection time ranged between 2.9 and 5.8 $\log_{10}$ cfu/ml. Different strains showed different acidity endurances. *A. baumannii* (1096, 1099), *A. calcoaceticus* (1097), and *Acinetobacter* gensp. 3 (415) all remained viable for 60 min and therefore were regarded as acid tolerant. The moderately acid tolerant *A. baumannii* (1098) was last detected at 45 min. *A. baumannii* ATCC 19606^T (1102) and strain 1095 and *A. calcoaceticus* (418) all exhibited the shortest survival time at 30 min. Error bars represent the standard deviation of two independently repeated experiments.

6.3.2 Pepsin tolerance

The effect of the pepsin enzyme on *Acinetobacter* was determined by the enumeration of viable cells at 0, 15, 30, 60, 90, 120 min **(Figure 6.4)**. The presence of *Acinetobacter* cultures in the pepsin solution neither caused an early decrease in the numbers of viable cells nor a gradual decline over the period of 120 min. The only significant reduction was observed at 15 min for strain 1097 ($P < 0.05$). In contrast, increases in survival rates were generally recognised by the end of the experiment in comparison to the initial cell counts (T_0). Strain 1095 increased by 0.2 $\log_{10}$ cfu/ml over the test period. *A. baumannii* ATCC 19606^T (1102) was 0.4 $\log_{10}$ cfu/ml higher than the inoculum. Strain 1098 and 415 both showed a 0.5 $\log_{10}$ cfu/ml growth. A notable recovery was exhibited by strains 1096 (0.9 $\log_{10}$ cfu/ml), 418 (1.1 $\log_{10}$ cfu/ml), and 1099 (1.2 $\log_{10}$ cfu/ml) **(Figure 6.4)**.

Figure 6.4 Survival curves of *Acinetobacter* strains in pepsin solution.

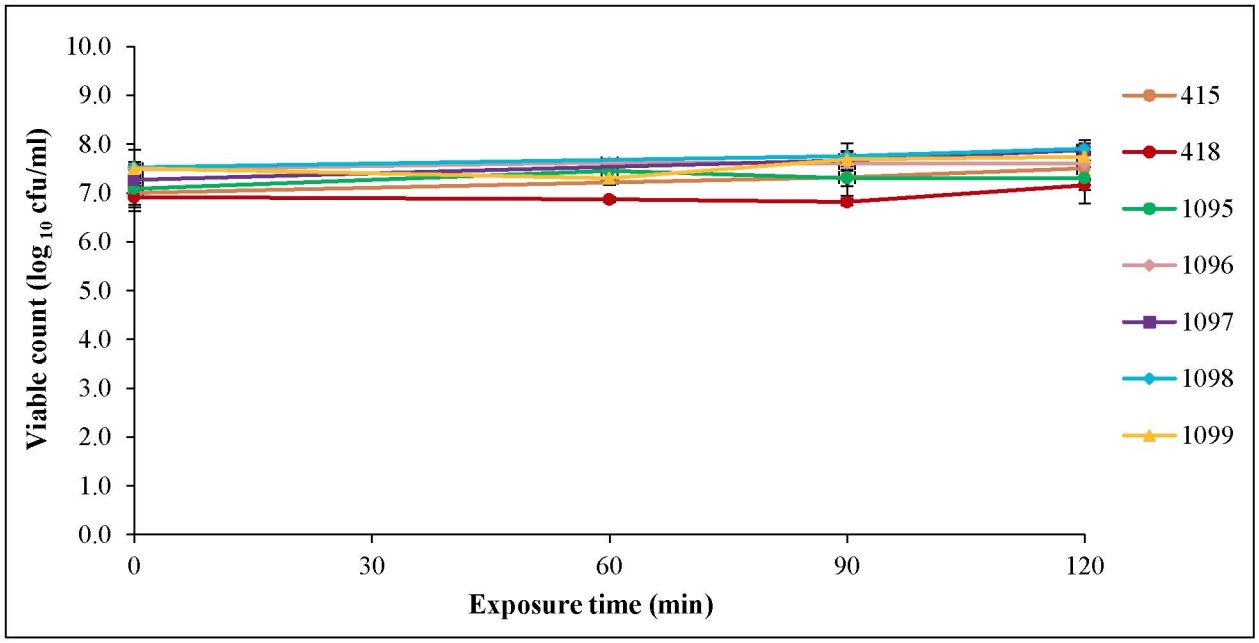


The pepsin enzyme did not appear to cause an early decrease in viable counts nor a gradual decline over the period of 120 min. The only significant decrease was observed at 15 min for strain 1097. In contrast, increases in survival rates were generally recognised by the end of the experiment in comparison to the initial cell counts (T_0). Strain 415 is *Acinetobacter* gensp. 3.; 418 and 1097 are *A. calcoaceticus;* 1095, 1096, 1098, 1099, and 1102 are *A. baumannii*. Error bars represent the standard deviation of two independently repeated experiments.

6.3.3 Tolerance to the small intestinal fluid

The simulated small intestinal juice was inoculated with *Acinetobacter* cultures and incubated for 120 min at 37ºC. The viable counts of the bacterial suspensions, determined at 0, 60, 90, and 120 min, demonstrated the ability of all strains to sustain their viability during the complete time-course. Survival trends are illustrated in **Figure 6.5**. The recovered cells at the end of the assay were between 0.1 to 0.6 $\log_{10}$ cfu/ml higher than the inoculum (T_0) with the largest increase shown by strain 1097 ($P > 0.05$).

Figure 6.5 Survival curves of *Acinetobacter* strains in small intestinal fluid.

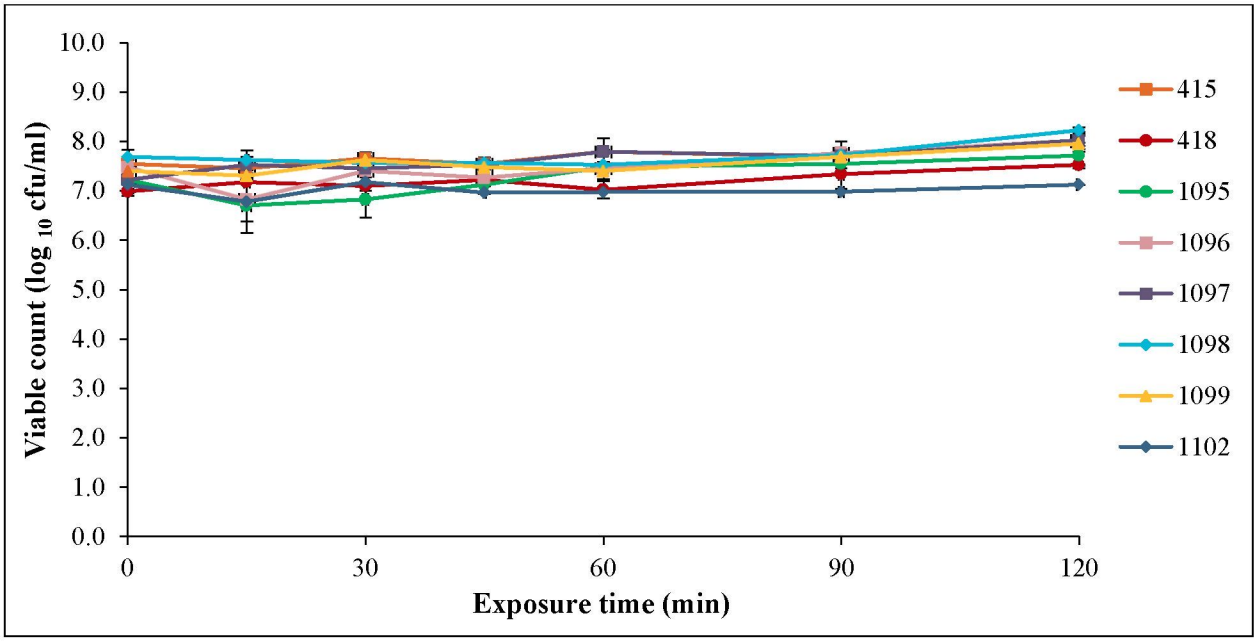


The simulated small intestinal juice, containing porcine pancreatin and porcine trypsin, was inoculated with *Acinetobacter* and incubated for 120min at 37ºC. All the tested strains were able to sustain their viability during the complete time-course with no significant reduction or increase. Strain 415 is *Acinetobacter* gensp. 3.; 418 and 1097 are *A. calcoaceticus;* 1095, 1096, 1098, and 1099 are *A. baumannii*. Error bars represent the standard deviation of two independently repeated experiments.

6.3.4 Bile salts tolerance

The presence and absence of recovery in 0.125, 0.25, 0.5, 1, and 2% porcine bile was examined. The specified concentrations had no effect on the viability of the evaluated strains, when tested after 0, 5, 10, 15, 30, 45, 60, 90, and 120 min of inoculation, as positive recovery was observed on the agar plates at these time points. Consequently, the concentration was increased to 4% and the viable counts were accordingly determined at the time points indicated in **Figure 6.6**. The order magnitude of viability remained the same for the complete exposure time. When data for the test strains at the first (T_0) and the last (T_{120}) sampling time were compared, not only there was 0.0% inhibition but also cell counts of 7 of 8 strains increased by 0.4 to 0.8 $\log_{10}$ cfu/ml. However, that of strain 1102 (*A. baumannii* ATCC 19606^T) remained the same.

Figure 6.6 Survival curves of *Acinetobacter* strains in 4% porcine bile solution.



The viable counts of *Acinetobacter* strains in 4% porcine bile were determined at the time points specified in the figure. The order magnitude of viability remained the same for the complete exposure time. Strain 415 is *Acinetobacter* gensp. 3.; 418 and 1097 are *A. calcoaceticus;* 1095, 1096, 1098, 1099, and 1102 are *A. baumannii*. Error bars represent the standard deviation of two independently repeated experiments.

6.4 DISCUSSION

In order for *Acinetobacter* to colonize the gastrointestinal tract and cause infections following ingestion of a contaminated enteral feed, it has to survive the multi-environments existing in this system starting in the stomach. The stomach environment is generally effective in microbial eradication (Wilson *et al.*, 2002). However, a portion of some microorganisms survive. The levels of sensitivity to gastric acidity vary in bacteria as some are more acid tolerant than others and thus infectious at lower doses (Blaser and Newman, 1982; Lin *et al.*, 1996). Low acid levels are believed to be associated with an increase in infection potential (Martinsen *et al.*, 2005). *Acinetobacter* in this study was considered resistant to pHs greater than 2.5, while 30-60 min of acid tolerance was shown at this pH level. Strains were only monitored for 2h at 37^{o}C in accordance with the two hourly feeding intervals set for the nasogastric nutrition **(Chapter 5)**. The exposure to low acidity was not gradual so it is unknown whether higher survival rates specifically at pH 2.5 could have been obtained. However, this is likely as the adaptive stress response is a well-known mechanism enabling microorganisms to withstand extreme acidity (pH < 4.0) through pre-exposure to sublethal pH values (4.5-5.5) (Seputiene *et al.*, 2006). Pathogens such as *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes*, and *E. coli* are examples of bacteria possessing the acid tolerance response (ATR) systems and whose survival at low pHs is promoted by the expression of acid shock proteins (ASPs) which is induced by sublethal levels of acidity (Bang *et al.*, 2000; Davis *et al.*, 1996; Seputiene *et al.*, 2006).

Previous studies demonstrated marked inhibition of bacteria at pHs below 2.0 (Zhu *et al.*, 2006) while those between 4.0 and 7.0 did not have such impact on bacterial survival (Waterman and Small, 1998). Maintaining the pH of the stomach at low levels (< 4.0) is therefore critical in terms of the extent of the bactericidal effect. Exposure to high acidity for 20 to 30 min can achieve this damage (Zhu *et al.*, 2006). Nevertheless, this level of acidity is rarely sustained in the neonate stomach as the pH tends to be above 4.0 for prolonged periods of time (Mitchell *et al.*, 2001). Milk is known for its neutral pH and buffering activity (Washington *et al.*, 1999). In NICUs, infant formula feeding is frequent and usually every 2 to 4 h (Hurrell *et al.*, 2009a; Mitchell *et al.*, 2001; Washington *et al.*, 1999). Mitchell *et al.*, (2001) reported gastric pH greater than 4 for 29 to 212 min after feeds. In another study

(Omari and Davidson, 2003), the pH was lower than 4, in healthy premature neonates who received feeding every 4 h, for an average of 52.7% of the time. Within the first hour of feeding the mean pH was as high as 6.9, 6.0, and 5.7. Those recorded in the next hour dropped to 6.0, 3.7, and 3.5. According to Sondheimer's investigation (1985), the mean gastric pH of healthy premature babies less than 7 days old was greater than 4 for 3h before and following infant formula feeding. Another group of 7- 15 days old neonates who were also premature but healthy had an average fasting stomach pH of 2.9 which rose to 5.2 within 15 min of receiving the feed and remained above 3.0 for the 3h before the next feeding point was due (Sondheimer *et al.*, 1985). This meant longer maintenance of high pH levels with the highest reached at feeding times and this is specifically when the bacteria in the contaminated feed are ingested.

The composition of the milk consumed has also been shown to offer some degree of protection against low pHs (Charteris *et al.*, 1998; Conway *et al.*, 1987; Kos *et al.*, 2000). The gastric transit tolerance of some organisms such as *Bifidobacterium* and *Lactobacillus* sp. was generally improved and with two strains in particular exhibiting 100.0% gastric tolerance. This was when milk proteins (whey protein, and sodium caseinate) were added singly or in combination to simulated gastric juice containing 0.5% sodium chloride and 0.3% pepsin and adjusted to pH 2.0 (Charteris *et al.*, 1998). Kos *et al.* (2000) also described 45.0% transit survival of *Lactobacillus acidophilus* from gastric juice (pH 2.0) similar to that described by Charteris *et al.* (1998) as a result of adding whey proteins concentrate. This was in contrast to the 15.0% in the absence of this type of milk protein. Lactic acid bacteria (*Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Lactobacillus acidophilus*) were also shown to survive better in gastric juice (pH 1.0 – 5.0) in the presence of skimmed milk (Conway *et al.*, 1987).

Even during the pH decrease before the next feed is due, organisms can still survive if they exit the stomach environment due to gastric emptying. This relies on the time taken by the stomach to gradually empty its contents. In other words, fast gastric emptying from the stomach may enable the bacterium to be delivered into the small intestines before the pH decreases and hence enhances the likelihood that a larger number of live cells reach the duodenum. According to (Cavell, 1981, & 1982), the average time for half of the stomach

contents to be emptied after receiving infant formula feeds was 51.9±9.8 min and 78.0±14.0 min in healthy preterm and term neonates respectively. These values may however vary between different infant formula according to their compositions of fat and carbohydrates (Siegel *et al.*, 1985). The short gastric emptying time may accordingly be associated with longer time the pH of the stomach is below 4.0 (Vandenplas *et al.*, 1988).

Although acidity provides an effective antibacterial activity against infections, critically ill neonates can be at risk of acute bleeding resulting from gastric mucosal lesions, which are induced by the low acidity of the stomach (i.e. pH 2.05) (Kuusela, 1998). Histamine 2 receptor blockers such as ranitidine and cimetidine are used in these cases as a prophylactic or a therapeutic option by suppressing the production of acid in order to increase the pH in the stomach (Kuusela, 1998). The main effect of these medications is the increase of the gastric pH which permits the colonization of this area by opportunistic pathogens including *A. baumannii* (Garrouste-Orgeas *et al.*, 1997; Zavros *et al.*, 2002).

Proteolysis has a bactericidal effect that is associated with the pH. A combination of pepsin and acid has proved to be more effective than the pH alone as it may increase the susceptibility of bacterial cells to this enzyme (Zhu *et al.*, 2006). Incubating *E. coli* K12 in solutions containing pepsin (0.5, 1.0, and 2.0mg/ml) and pre-acidified to a range of pHs decreased the viability of the strain by 100.0%, relative to the control (without pepsin), after 100 min at pH 3.5. On the other hand, the incubation in 1.0mg/ml pepsin (pH 2.5, 3.0, and 3.5) reduced the viable counts of *H. pylori* by 50.0% after 20 min (Zhu *et al.*, 2006). In our study, there was no apparent kill observed with *Acinetobacter* strains in the pepsin solution. The pH of this solution was left neutral and as a result, the enzyme which only works at acidic pHs (Wilson, 2005), was deactivated. Therefore, strains are required to be re-tested in the same pepsin solution described but at different acidic pHs before an accurate statement about the resistance of *Acinetobacter* to this enzyme could be made.

The enzymes in the pancreatic juice did not exhibit a real challenge to survival of *Acinetobacter*. The findings showed that *Acinetobacter* strains are resistant to many pancreatic enzymes, including protease, lipase, trypsin, amylase, and ribonuclease which composed the porcine pancreatin and trypsin used in this study. The absence of apparent loss

of viability in the pancreatic juice was not surprising as it is likely to be attributed to the neutral pH of this environment (Naim *et al.*, 2004). Other organisms (e.g. *E. coli* and *Streptococcus thermophilus*) reported in the literature varied in terms of their sensitivity to pancreatic juice (Barmpalia-Davis *et al.*, 2008; Charteris *et al.*, 1998; Marteau *et al.*, 1997; Naim *et al.*, 2004), but cannot to be compared to the findings of the study herein due to experimental differences (compositions, conditions, or dynamics).

Towards the ileum region conditions become less destructive for bacterial existence although the ones that survived would still have to avoid being swept away, oxygen restriction, limited nutrients and resist the antibacterial effect of bile salts (Engleberg *et al.*, 2007). The pH in the ileum of an adult is approximately neutral, while that of the duodenum is between 5.7 and 6.4 and it ranges between 5.9 and 6.8 in the jejunim. Along the different parts of the colon (caecum, ascending, transverse, descending, and sigmoid colon), the mean pH varies from 5.7, 5.6, 5.7, 6.6, and 6.6 respectively (Wilson, 2005). These optimal pHs, compared to that in the stomach, may allow bacterial multiplication (Naim *et al.*, 2004). Bacteria in the small intestines are also provided with a variety of nutrients from the chyme in the lumen. In addition, microbes themselves produce molecules which can be considered as a source of nutrients for other microbes, whether resident or transient to this region, by breaking down nutrients obtained from dietary compositions, or derived from gastrointestinal secretions or from the epithelial cells shed from the intestinal mucosa (Wilson, 2005). Regarding the oxygen availability, the condition in the lumen of the colon is anaerobic. However, the mucosal surface is supplied with high levels of oxygen content by the underlying tissue (Wilson, 2005).

The awareness that a large proportion of sublethally injured cells may have been able to pass through the intestines where they could successfully survive and resume their growth activity suggests that they can potentially cause infections even at low ingestion doses. *E. coli* O157:H7, for example, is well known for its capacity to survive in the gastrointestinal tract, resume its growth after undergoing a lag phase of adaptability and reparation, and then cause subsequent illnesses at 10 to 100 ingested cells/g (Abdul-Raouf *et al.*, 1993; Choi *et al.*, 2000; Lin *et al.*, 1996; Marteau *et al.*, 1997).

It has been argued that survival in the intestinal tract and the development of subsequent infection requires toleration of the effects of the biles secreted (Hofmann, 1994). Bile tolerance is a common feature of Gram-negative intestinal pathogens as for the commensal intestinal microbiota (Pace *et al.*, 1997; Pope *et al.*, 1995; Prouty *et al.*, 2002; Thanassi *et al.*, 1997). Such bacteria produce stress proteins including molecular stress response chaperones (Breton *et al.*, 2002; Flahaut *et al.*, 1996; Leverrier *et al.*, 2003; Sanchez *et al.*, 2005), proteins for oxidative stress relief (Bernstein *et al.*, 1999; Lechner *et al.*, 2002), efflux proteins to transport bile salts out of the cell (Begley *et al.*, 2002; Hung and Mekalanos, 2005; Lin *et al.*, 2005; Prouty *et al.*, 2004), and exporter proteins (Bron *et al.*, 2004).

The reported colonization of the gut by *Acinetobacter* spp. (Das *et al.*, 2011; Parm *et al.*, 2010, & 2011a, & b; Roy *et al.*, 2010; Thom *et al.*, 2010) indicates the ability of the organism to resist the antimicrobial activity of bile salts. This was confirmed by the findings of this study, as all the *Acinetobacter* strains tested were capable of maintaining their viability at various concentrations of porcine bile. The type of bile solution used herein was a porcine bile extract which contained glycine and taurine conjugates of deoxycholic acid and other unspecified bile salts (Sigma-Aldrich, UK). The response could be different towards un-conjugated forms of bile which may also be produced in the intestinal lumen and were not included here (Wilson *et al.*, 2002). According to Begley *et al.* (2006), un-conjugated bile salts have greater antimicrobial activity than the conjugated compounds.

The gastrointestinal survival of the *Acinetobacter* strains examined in this study may not be precisely reflective of the complex and sequential stresses as well as the changing dynamics that exist during the gastrointestinal passage. However, the physiological responses described in this study are the first to be explored in the literature and indicated the capacity of *Acinetobacter* strains to occupy different niches within the gastrointestinal system and successfully respond and adjust to stressful conditions faced during their passage. This is ultimately related to their virulence as opportunistic human pathogens and their potential risk of colonization and the development of a neonatal infection. Elucidation of the mechanisms underlying their survival in such environments is importantly required. Equally important was to follow up this study with an investigation of the potential consequences of this

survival, in the intestinal tract, through the organism's interaction with the epithelial and phagocytic cells, which is represented in the next chapter.

CHAPTER 7. *IN VITRO* STUDIES OF *ACINETOBACTER* INFECTION MODELS

7.1 INTRODUCTION

Mucosal surfaces become colonized with potentially pathogenic Gram-negative bacteria immediately after birth (Parm *et al.*, 2011b). According to Metsvaht *et al.* (2010) and Parm *et al.* (2010), 52–83% of high-risk neonates in NICUs are colonized by Gram-negative microorganisms by the end of the first week of life. By 30 days, this percentage increases to almost 100% (Almuneef *et al.*, 2001; Goldmann *et al.*, 1978; Parm *et al.*, 2010). Disturbance of the barrier integrity may cause the colonization state to turn into a case of an invasive infection (Graham *et al.*, 2007). The documented colonization of the gastrointestinal tract with *Acinetobacter* spp. (Das *et al.*, 2011; Garrouste-Orgeas *et al.*, 1997; Kim *et al.*, 2011; Ma *et al.*, 1999; Parm *et al.*, 2010, & 2011a, & b; Roy *et al.*, 2010; Thom *et al.*, 2010) is indicative of the successful establishment of the organism in this environment. In order to achieve this, the bacterium has to be able to adhere to the epithelial cells as a first step of the interaction with the host cell before subsequent colonization (Beachey *et al.*, 1988). An important function of the epithelial layer coating the surfaces of the gastrointestinal tract is to protect the underlying tissues from inhabitant bacteria on the surfaces (Wilson *et al.*, 2002). Adhesion to this layer can only be the starting point to a pathological process involving the invasion of the superficial layers of the tissue or deeper, in some cases, leading to dissemination of the bacterium throughout the body which can be life threatening (Wilson *et al.*, 2002). An important risk factor for bloodstream infections in neonates is in fact considered to be the colonization of the gastrointestinal tract with opportunistic bacteria (Donskey, 2004; Graham *et al.*, 2007; Westerbeek *et al.*, 2006). It has been previously shown that the gut colonization with imipenem-resistant *A. baumannii* preceded the occurrence of bacteraemia in adult patients (Thom *et al.*, 2010). A genetic relationship was found between the perirectal cultures and blood cultures in 6 of 7 cases with the clinical infection (Thom *et al.*, 2010).

In order to understand the pathogenicity of *Acinetobacter*, various animal models of infection have been studied. These included a rabbit endocarditis model (Rodriguez-Hernandez *et al.*, 2004), a rat soft tissue infection model (Pantopoulou *et al.*, 2007), guinea pig (Bernabeu-Wittel *et al.*, 2005), murine (Knapp *et al.*, 2006; Renckens *et al.*, 2006), and rat pneumonia

models (Jacobs *et al.*, 2010b; Russo *et al.*, 2008). A range of epithelial cell lines have also been used as *in vitro* models to study the adhering capacity of *A. baumannii*. For example, *A. baumannii* 19606^T was able to attach to human alveolar epithelial cells (A549) (Gaddy *et al.*, 2009a), and to human bronchial epithelial cells NCI-H292 (de Breij *et al.*, 2009). *A. baumannii* ATCC 19606^T as well as four clinical *A. baumannii* strains were also positive for the adhesion to and invasion of cervical carcinoma cells (HeLa, CCL-2) (Choi *et al.*, 2008b; Jacobs *et al.*, 2010b; Lee *et al.*, 2001) and human laryngeal epithelial cells (HEp-2, CCL-23) (Choi *et al.*, 2008b; Choi *et al.*, 2005). Adherence of clinical *A. junii*, *A. calcoaceticus*, *Acinetobacter* gensp. 13TU, and *Acinetobacter* gensp. 3 strains to human bronchial epithelial NCI-H292 and BEAS-2B cells was observed by de Breij *et al.* (2010) and Jacobs *et al.* (2010a) respectively. In addition, the same cell line was used to evaluate clinical *A. baumannii* strains in terms of their adhesion capacity (de Breij *et al.*, 2010; Lee *et al.*, 2008a; Lee *et al.*, 2006). Epithelial cells from human buccal mucosa also represented an *in vitro* infection model to assess the interaction of *A. calcoaceticus* RAG-1 (ATCC 31012) (Rosenberg *et al.*, 1981), *A. baumannii* ATCC 14606 and five other *A. baumannii* strains found on intravascular catheter tips (Costa *et al.*, 2006). Small pieces of rat bladder tissue (Sepulveda *et al.*, 1998), and rat tracheal tissue (Ruiz *et al.*, 1998) were employed for the same purpose using clinical *A. baumannii* strains.

Strains examined in the above-mentioned studies all showed the ability to adhere to different types of cell lines. However, these infection models are clinically irrelevant to gastrointestinal illnesses and hence are unsuitable for studying factors contributing to the pathogenicity of *Acinetobacter* in the gastrointestinal tract. Relevant studies were mainly case reports and *in vivo* infection models. Zavros *et al.* (2002) reported gastric mucosal inflammation, gastritis, hypergastrinemia and increases in gastric epithelial cell numbers caused by *A. lowffii* that was similar to that associated with the pathogenic organism *Helicobacter pylori* in infected mice. The latter is well-known as the causative agent of gastric adenocarcinoma, peptic ulceration, and chronic atrophic gastritis (Marshall and Warren, 1984; Nomura *et al.*, 1991; Parsonnet *et al.*, 1991). The inflammation described above represented a case of invasion with *Acinetobacter*. In such scenarios, epithelial cells respond by secreting cytokines involved in the activation of the immune system. However, sustained release or overproduction of these cytokines involved in the activation of the immune system may result in mucosal

inflammatory response. It is this response that may lead to modifications and damage to the mucosa through the cascade of events triggered (Forman, 1996; Rathinavelu *et al.*, 2003). Kim *et al.* (2011a) reported a case of a 66 year old male who presented with abdominal pain, hematemesis, nausea, and vomiting, and who was diagnosed with acute phlegmonous gastritis. The gastric wall showed severe inflammation of the mucosa with purulent exudate covering necrotic areas (intramural abscess). This condition is often fatal and associated with high mortality rate (Kim *et al.*, 2011). Both *A. lwoffii* and *Klebsiella oxytoca* were detected in the associated gastric tissue culture. According to Ofori-Darko *et al.* (2000), intubation of *Acinetobacter* spp. into the mouse stomach stimulates the secretion of the hormone gastrin, which is a growth factor for the oxyntic mucosa and regulates acid secretion (Dockray, 1999). The result is a subsequent proliferation of the epithelial cells and predisposed gastritis. *Acinetobacter* spp. has been isolated, amongst other Gram-positive and negative bacteria, from mice stomachs diagnosed with chronic gastritis (Stockbruegger, 1985; Stockbruegger *et al.*, 1984). Grotiuz *et al.* (2006) reported the presence of *A. haemolyticus* as the cause of bloody diarrhoea in a 3 month old infant.

With regard to *In vitro* studies involving the human colonic adenocarcinoma cell line (Caco-2), Sechi *et al.* (2004) showed positive cell adhesion of 9 of 20 clinical isolates of *A. baumannii*. However, further virulence aspects such as the subsequent invasion were not examined in this study. Pavlov *et al.* (2004), on the other hand, assessed the adherence and invasive potential of various bacteria, including *Acinetobacter*, cultured from treated and untreated drinking water supplies, using the Caco-2 and HEp-2 cells. Amongst other isolates, *Acinetobacter* spp. was one of the most adherent Gram-negative bacteria on both cell lines. Of the 79 invasive isolates, 4.4% *Acinetobacter* spp. were able to invade HEp-2 cells, while 2.8% invaded Caco-2 cells (Pavlov *et al.*, 2004).

Bacterial dissemination throughout the body following invasion of deep tissues can generally be achieved via the lymphatic system, blood stream, or survival within phagocytes (macrophages and neutrophils) (Wilson *et al.*, 2002). Tuberculosis, meningitis, and typhoid diseases, for example, are all characterized by the dissemination of their causative pathogens from the original site of entry to other tissues (Wilson *et al.*, 2002). Monocytes differentiated into macrophages play a key role in recognizing and killing invading bacteria by

oxidative/non-oxidative damage and phagocytosis as a part of the host innate immunity (Madigan *et al.*, 2012). Microorganisms have developed mechanisms which enable them to evade this antibacterial defense. This depends principally on the inhibition of phagocytosis. Another strategy adopted by bacteria to evade the phagocytic destruction is to survive within phagocytes. *Salmonella* Typhimurium, and *Yersinia* spp. are examples of pathogens possessing this capacity (Hueck, 1998).

The cytokine production by macrophages in response to infections with *Acinetobacter* has already been described in the literature (de Breij *et al.*, 2010). Binding of *A. baumannii* ATCC 19606^T to the surface of monocyte-derived macrophages (U937) and subsequent apoptosis has been identified by Choi *et al.* (2008b). However, a larger number of *Acinetobacter* strains and further studies regarding the interaction with macrophages and the consequences of this encounter on the bacterium and the phagocytic cell would be useful in developing a wider picture and in-depth understanding of the potential virulence of the organism.

Clinical evidence of the damage caused to the epithelial tissues of different regions of the gastrointestinal tract as a result of the invasion process of *Acinetobacter* are already documented as described earlier. What is required though is to support this evidence with extensive research to characterize the pathogenesis of *Acinetobacter* spp. and its attributes. This has not been sufficiently explored in the literature. Implications of such studies in the context of consuming a contaminated feed would potentially signify the risk of exposure to this opportunistic pathogen which principally formed the aim of this present study. To accomplish this goal, the research objectives were defined as a set of criteria. These were adhesion, invasion, persistence within epithelial cells, cytotoxicity, macrophages uptake and subsequent internal survival.

7.2 MATERIALS & METHODS

7.2.1 Bacterial strains and growth conditions

In the preliminary experiments, 29 *Acinetobacter* strains were assessed. This number was then reduced to 9 strains for the remaining parts of the studies. **Table 1** in **Chapter 1** shows the *Acinetobacter* strains under evaluation, their sources and culture collection numbers. Invasive *Salmonella* Enteritidis (strain 358) was used in this study as a positive control whereas *Escherichia coli* K12 (strain 1230) was the negative control due to it inability to invade epithelial cells. *Acinetobacter* cultures were grown in TSB and incubated for 18h at 37^{o}C.

7.2.2 Caco-2 cells and storage conditions

The human colorectal epithelial cells, Caco-2 (ATCC; USA), were used to study bacterial attachment and invasion. Stock cultures of Caco-2 in the freeze mix fetal bovine serum: dimethyl sulfoxide (FBS: DMSO- 95:5; v/v) were kept in cryotubes in a liquid nitrogen store for long term preservation.

7.2.3 Caco-2 growth and infection media

The minimum essential medium (MEM) (Sigma, M4655) was used as the growth medium and contained 2mM L-glutamine, 1.5g/l sodium bicarbonate, 1.0mM sodium pyruvate. The MEM was also mixed with 1% non-essential amino acids solution (M7145, Sigma), 10% fetal bovin serum (FBS) (Sigma, F7524), and 1% penicillin streptomycin solution (P4333). The infection medium consisted of MEM, 10% FBS, and 1% non-essential amino acids solution.

7.2.4 Caco-2 preparation and maintenance process

When needed, a stock vial of Caco-2 cells was thawed rapidly (to prevent the toxicity, to cells, of the dimethyl sulfoxide (DMSO) in the freezing medium) by holding in the hands. The vial was wiped with 70% ethanol and dispensed into 25 cm^2 flask (Sigma, CLS3056) containing 5ml of pre-warmed growth MEM. Fifty percent of the medium was replaced every two days until the cells were activated and growing. Cells were then transferred to 75 cm^2 flasks (Sigma, CLS430641) containing 20ml growth MEM. The medium was replaced and excess cells were removed twice a week to maintain the exponential growth of cells. The cell confluence was checked, on a daily basis, using an inverted microscope.

7.2.5 Sub-culturing of mammalian cell line Caco-2

When 70-80% confluence was reached, the cells were split into new flasks. To harvest the cells, the old medium was discarded using a sterile pipette, and the flask was washed twice with 2ml sterile warm Dulbecco's phosphate buffered saline (PBS) (Sigma, D8662). To detach cells from the flask, 5ml trypsin was added and the flask was placed in a 37°C incubator for 5-8 min in a 5% CO_2 atmosphere. Detachment was observed under the inverted microscope to ensure dispersion of the cell layer. The flask was gently rocked to aid detachment. Detached cells were transferred into a sterile universal tube for centrifugation at 1200rpm for 5 min to remove the trypsin. The supernatant was discarded and the pellet was re-suspended in 5ml fresh growth MEM in order to perform a 1:5 split. The pellet was broken up and the cells were mixed thoroughly but gently by pipetting up and down. One millilitre of the suspension was transferred into 19 ml growth MEM.

7.2.6 Caco-2 cell count

The number of cells obtained per 1ml of medium was determined as follows using a haemocytometer. A cover slip was positioned onto a haemocytometer, and then ~20 µl of the cell suspension was loaded into both chambers in the haemocytometer. The number of cells in 10 medium squares of the grid, and in each chamber was counted and averaged. This

average was then multiplied by $2.5x10^5$ to calculate the total number of cells per ml. $2x10^5$/ml was the required cell concentration per well.

In order to obtain the required concentration of cells, the following formula was applied:

$C_1 V_1 = C_2 V_2$

Where C_1 is the multiplication of $2.5x10^5$ by the average numbers of cells in both chambers in the haemocytometer, giving the actual concentration of the Caco-2 cells.

C_2 is the required cell concentration per ml, which was $4x10^4$ cells providing the doubling time for Caco-2 cells is ~20h and the final concentration needed for the assay after 48h incubation is $2x10^5$ cells per well.

V_2 is the final volume of the cell suspension in the growth MEM to be dispensed into the 24-well plates. Usually extra 2ml of the medium were added to allow for loss. For example, if 48ml were required for 2 control strains and 6 test strains (triplicate wells/strain, and 0.5ml/well, as well as 1 plate for the association assay and 1 for the invasion assay). Then, in this case V_2 would be 50ml.

Thus: V_1 (the volume of cell suspension to be added to the growth MEM) =

$$\frac{4x10^4 \text{ cells}}{\text{cells/ml}} \text{ X } 50\text{ml}$$

7.2.7 Seeding 24-well plates

Collagen-coated 24-well plates were seeded with 0.5ml of the cell suspensions adjusted to the desired concentration per well. The plates were incubated at 37°C under in 5% CO_2 for 2-3 days until 80% confluence was achieved.

7.2.8 Preparation of inocula

On the day of the tissue culture assays, the optical density of the overnight (18h) bacterial cultures was measured using the spectrophotometer at 620nm wavelength. The overnight cultures were then spun down for 1 min at 14,000 rpm. The TSB medium was discarded and the pellet was re-suspended in 1ml pre-warmed MEM (growth medium). The bacterial suspensions were diluted to OD 0.5, 0.05, or 0.005 according to the desired concentration. These optical densities equated to ~10^8, 10^7, and 10^6 cfu/ml respectively, which were determined experimentally (viable plate count) for all the test strains. The size of the inocula used to infect Caco-2 cells was also confirmed retrospectively by performing the Miles and Misra viable count method on the day of the experiment.

7.2.9 The association assay

When Caco-2 cells were 80% confluent, the medium in the wells was replaced with 0.5ml of the desired bacterial concentrations. These concentrations were 10^6, 10^7, 10^8 cfu/ml to achieve a multiplicity of infection (MOI) of 10, 100, and 1000 respectively. The test and control strains were all assigned 3 wells each. The infected cells were then incubated for 3h at 37°C in a 5% CO_2 atmosphere. After which, these cells were washed three times with 0.5ml pre-warmed PBS which was aspirated. Next, Caco-2 cells were lysed using 0.1ml of 0.5% Triton X-100 solution. Lysis of the cells was assisted by repeatedly pipetting up and down. The lysis procedure took approximately 10 min during which, according to the preliminary experiments, there was no reduction in the viability of bacteria. Viable counts of the associated cells (attached and internalised) were performed by serially diluting the lysate (1:10) in 96-well plates. Dilutions were then plated onto TSA plates applying the Miles and Misra method. Data are presented as the average log values of the experiments repeated at least 3 independent times.

7.2.10 The gentamicin protection assay

The infection procedure for the invasion assay used the same method as that of the association assay but with added steps. Cells were infected with the bacteria followed by a 3h incubation period and then 3 washes with 0.5ml PBS. However, instead of adding the Triton X-100 next, the 24-well plates assigned for the invasion assay required the addition of 250μg/ml gentamicin. Plates were then further incubated for 2h at 37°C in 5% CO_2. Aspirates were plated onto TSA and incubated for 24h at 37°C to check for complete bacterial inhibition by gentamicin. Caco-2 cells were washed 3 times with 0.5ml PBS. To lyse cells, 0.1ml Triton X-100 was added into each well and repeatedly pipetted. Finally, having added gentamicin to kill the external bacteria, only the internalised bacterial cells were counted by the Miles and Misra method. All the invasion assays in this study were conducted independently at least two to three times. In each experiment, wells were infected in triplicate with individual strains.

7.2.11 Preliminary experiments

7.2.11.1 Determination of the bactericidal concentration of gentamicin

Before tissue culture assays could be carried out, preliminary experiments were needed in order to determine the susceptibility of test strains to gentamicin as this is used to discriminate between attached and internalized bacterial cells. Strains were tested at the gentamicin concentrations of 50 and 100μg/ml. Due to the resistance of some strains, this concentration was increased to 250μg/ml and strains were re-tested. The antibiotic sensitivity testing was conducted in accordance with the British Society for Antimicrobial Chemotherapy guidelines (BSAC, 2001). The inhibitory effects of the gentamicin were determined by the micro-dilution method (BSAC, 2001). Bacterial cultures were grown in TSB for 18-20h at 37°C. Their turbidity was adjusted to McFarland Standard 0.5 (~ $1.5x10^8$ cfu/ml) before being diluted to 1:10 and 1:100. Equal volumes of these dilutions and the gentamicin solution were added at a final concentration of 50, 100, and 250μg/ml to 96-well plates. Blank bacterial suspensions (without gentamicin) were also included as controls for

each strain. Plates were incubated for 20h at 37°C. Aliquots (~10µl) of these overnight suspensions were inoculated into TSA plates. The presence or absence of growth recovery was checked 24h later. Duplicate results were recorded for each strain.

7.2.11.2 Determination of bacterial viability in 0.5% Triton X-100

The effect of 0.5% Triton X-100 solution (T9284) on *Acinetobacter* strains was examined. 0.5% Triton X-100 was prepared in distilled water. The solution was then filter sterilised using 0.2 µm pore diameter cellulose filter. Viable counts were performed directly after the addition of the Triton-X solution at T_0. Aliquots (15µl) of *Acinetobacter* 18h cultures were added to 135µl of the Triton-X solution and incubated at 37°C. At each time point (0, 15 and 30 min), 20µl of the ten-fold serial dilutions were inoculated, in triplicate, onto TSA plates using the Miles and Misra method. Reduction in the viable count (cfu/ml) was calculated in comparison to T_0 for each test strain. The experiment was performed once.

7.2.12 Effect of infection time course on the attachment and invasion levels of *Acinetobacter*

Cells were infected with 18h *Acinetobacter* cultures at 10^7cfu/ml and incubated for different durations (0, 90, 120, 150, 180 min) at 37°C in the presence of 5% CO_2. The same association-invasion assay procedure, described above, was followed. Cells were washed 3 times with PBS and lysed with Triton X-100, and finally the Miles and Misra plate count method was performed. For the gentamicin protection assay, 250µg/ml gentamicin was added and then plates were incubated for 2h at 37°C in 5% CO_2. The Miles and Misra technique was also carried out on the invaded cells which were lysed with Triton X-100. The numbers of the associated bacterial cells were subtracted from the internalised ones in order to calculate the amount of colony forming units attached to Caco-2 cells. These were expressed as the percentage of the initial bacterial cell number, added to the Caco-2 cells at the start of the experiment, which was able to attach to the epithelial cells (attachment efficiency). The efficiency of invasion was defined as the percentage of the inoculum that was found to invade

Caco-2 cell monolayer. Both the attachment and invasion assays were carried out at least two to three times.

7.2.13 Effect of different MOIs on the attachment and invasion levels of *Acinetobacter*

The association and invasion levels of *Acinetobacter* were examined at different infectious doses. The ratios of the infected Caco-2 cells to the bacterial cells were 1:10, 1:100, and 1:1000. This was achieved by infecting 10^5 Caco-2 cells with 10^6, 10^7, 10^8 cfu of each strain per ml. The association and gentamicin assays were conducted as detailed above.

7.2.14 Intracellular survival

The intracellular survival of *Acinetobacter* strains was also studied. Confluent Caco-2 cells cultivated in 24-well plates were infected in the order of 10^6 cfu/ml for 3h at 37°C in 5% CO_2. The gentamicin protection assay was carried out as described earlier **(Section 7.2.10)**. Briefly, bacterial suspensions were aspirated and Caco-2 cells were washed with PBS. Infection MEM containing 250µg/ml gentamicin was added and then incubated for 2h at 37°C. Afterwards, a second washing step was carried out three times. Then, 10µg/ml gentamicin was added and 24-well plates were incubated at 37°C in 5% CO_2 for the duration desired. Twenty four hours later, the persistent bacteria were counted using the Miles and Misra technique on TSA plates. To determine the original number of internalised bacteria (T_0), the lysates, which were treated with Triton-X 100 following the addition of 250µg/ml gentamicin and 2h incubation, were serially diluted (tenfold) and plated on TSA applying the Miles and Misra method. Viable counts were calculated for triplicate wells per strain. The assay was separately repeated twice. The values of the persistent bacteria were expressed as the percentage of the inoculum that survived for 24h inside the epithelial Caco-2 cells.

7.2.15 Cytotoxicity studies

The aim for this part of the tissue culture studies was to assess the cytotoxic impact of an *Acinetobacter* infection on the colonic epithelial cells (Caco-2). The correlation between mitochondrial activity and the viability of eukaryotic cells was applied to measure this cytotoxicity. For this purpose, methylthiazolyldiphenyl-tetrazolium bromide (MTT) was used as a means of quantitatively assessing the cytotoxicity levels in these eukaryotic cells, whereby only metabolically active cells can reduce the yellow MTT to formazan crystals (purple). Hence, the darker the blue the more viable cells that are present which can be measured spectrophotometrically.

7.2.15.1 Preparation of MTT

MTT solution (Sigma-Aldrich, M2128) was prepared by dissolving 5mg in 1ml distilled water. The solution was then sterilised by filtration using a 0.2 µm pore diameter cellulose filter. Aliquots of the MTT stock solution were finally stored at -20°C.

7.2.15.2 MTT viability assay

Eighteen hour cultures of *Acinetobacter* in TSB were diluted to the equivalent of OD_{620nm} 0.005 (~10^6cfu/ml) in MEM. Duplicate wells containing 80% confluent Caco-2 cells were inoculated with 0.5ml of each bacterial suspension prepared in the infection MEM. Plates were incubated for 3h at 37°C in the presence of 5% CO_2. Cells were then washed with PBS (three times), and 250µg/ml gentamicin was added. One hour later, the washing step was repeated and a fresh medium containing 10µg/ml gentamicin was added. Plates were incubated at 37°C for 24h before the spent medium was aspirated and cells were washed twice with 1ml PBS. For each tested strain, 50µl of the 5mg/ml MTT solution was dispensed into duplicate wells containing 450µl infection MEM for a final concentration 0.5mg/ml MTT. Plates were then incubated for 1h at 37°C. The medium was aspirated and 200µl DMSO was added to each well. Plates were then placed on a shaker for 5 min at 200rpm. 150µl of the solubilised solution was transferred to 96-well plates and the absorbance was

read at 570nm using a spectrophotometer. Control wells contained uninfected Caco-2 (zero killed cells). The readings obtained were compared to that of the control and the percentages of MTT reduction (viability) of the infected Caco-2 cells were calculated. This was separately repeated twice and the average was taken.

7.2.16 Macrophage (U937) uptake and survival studies

7.2.16.1 U937 cells and storage conditions

The human monocyte cells U937 were used to examine the survival of *Acinetobacter* inside macrophages. These macrophages resulted from the maturation and differentiation of the monocytes U937 induced by soluble stimuli such as phorbol 12-myristate 13-acetate (PMA) used also in this study. Stock cultures of U937 cells in the freeze mix fetal bovine serum : dimethyl sulfoxide (FBS : DMSO- 95:5; v/v) were kept in cryotubes in a liquid nitrogen store for long term preservation.

7.2.16.2 U937 growth and infection media

The U937 growth medium consisted of RPMI-1640 (Sigma-Aldrich, R8758), 1% non-essential amino acid solution, 1% penicillin streptomycin solution, and 10% fetal bovine serum. The infection medium is composed of RPMI-1640, 1% non-essential amino acid solution, and 10% FBS.

7.2.16.3 U937 subculturing

The cell density was maintained at ~10^5 cell/ml. The process of preparing, counting and maintaining U937 cells was the same as that of Caco-2 cell line. Cells were replenished twice a week with a fresh medium replacing 50% of the spent medium. The cells were harvested by centrifugation at 1200 rpm for 5 min. Re-suspension of the pellet was in RPMI-1640 growth medium containing 0.1µg/ml PMA. The density of the U937 cells was counted using the haemocytometer as described in **Section 7.2.6**. To seed the 24 well-plates, 0.5ml of the cell

suspension (~10^5 cell/ml) were dispensed in each well and incubated for 48h at 37°C in the presence of 5% CO_2. This was to allow the fixed monocytes to mature and grow.

7.2.16.4 Preparation of PMA

The stock solution of PMA (Sigma-Aldrich, P1585) was prepared at the concentration of 0.1mg/ml by dissolving in DMSO. It was then divided into 200µl aliquots before stored at -20°C.

7.2.16.5 Bacterial strains and growth conditions

The same strains involved in the previous tissue culture studies were also included in the macrophage survival assays. *Citrobacter koseri* (48) and *Escherichia coli* K12 (1230) represented the positive and negative control respectively. Cultures of the test organism were grown in TSB and incubated for 18h at 37°C.

7.2.16.6 Preparation of inocula

The optical density of the overnight (18-20h) cultures in TSB was measured using the spectrophotometer at a wavelength of 620 nm. The overnight cultures were then centrifuged for 1 min at 14,000 rpm. The TSB medium was discarded and the pellet was re-suspended in 1ml pre-warmed RPMI-1640 infection medium. The bacterial suspensions were diluted to the equivalent of the desired OD 0.005 (~10^6 cfu/ml). The size of the inocula used to infect U937 was also confirmed retrospectively by performing the Miles and Misra viable count method on the day of the experiments.

7.2.16.7 Macrophage uptake and persistence assay

On the day of the experiment, the spent medium containing PMA was removed by aspiration and then the cells were washed with 0.5ml PBS to eliminate any residual PMA. The bacterial suspensions in RPMI-1640 were then added at an OD 0.005 (~10^6 cfu/ml). Each strain, including the controls, was used to infect 2 wells each. The infected cells were incubated at

37°C in 5% CO_2 for 3 hours. After which these cells were washed three times with 0.5ml pre-warmed PBS which was then removed. Next, 250µg/ml gentamicin was added and plates were incubated for 2h at 37°C in a 5% CO_2 atmosphere. Wells were washed three times with 500µl pre-warmed PBS. To lyse the macrophages, 0.1ml of the 0.5% triton-X solution was used. Lysis of the cells was assisted by repeated pipetting. Cell lysates were serially diluted 1:10 in 96-well plates. Dilutions were then plated onto TSA applying the Miles and Misra method to determine the number of internalised bacteria during the 3h infection time course (T_0).

With respect to macrophage persistence, bacterial cells detected after 24h of exposure to macrophages were counted using the same procedure described above. The gentamicin, however, was added twice, firstly at 250µg/ml for 2h as previously mentioned, and secondly at 10µg/ml following three washes with 0.5ml PBS. Next, plates were incubated at 37°C under the standard 5% CO_2 conditions for 24h. Cells lysed with 0.5% Triton-X were serially diluted and plated on TSA for 24h at 37°C. Assays in this study were conducted individually twice and the results obtained were averaged and expressed as the percentage of the inoculum persistent within macrophages.

7.3 RESULTS

7.3.1 Determination of the bactericidal concentration of gentamicin

The invasion assay required an optimisation of the gentamicin concentration. This was to ensure a complete kill of the external bacteria and hence eliminate false or inaccurate invasion levels. Bacterial suspensions were first prepared in 50 and 100μg/ml gentamicin and were incubated for 18h at 37°C. Aliquots (~10μl) of these overnight suspensions were plated out. The presence or absence of growth recovery was recorded 24h later. The bacterial load exposed to gentamicin was at 10^6 and 10^7 cfu/ml. These concentrations of *Acinetobacter* matched the sizes of inocula intended for the association and invasion protection assays. At 10^6 cfu/ml, 11 out of 29 strains were found to be inhibited in the presence of 50 and 100μg/ml gentamicin **(Table 7.1)**. One of these inhibited strains (1102) was able to grow at the same gentamicin concentrations when the bacterial count was 10^7 cfu/ml. Resistant strains, at either of the specified concentrations, were excluded from further studies and the 10 remaining sensitive strains (at 10^7 cfu/ml) were then used for a real time gentamicin sensitivity testing. Five of the included strains were *A. baumannii* (1112, 1099, 1102, 1111, 1183, 1440). Two strains were *A. calcoaceticus* (418, 1097), and the others were *Acinetobacter* gensp. 3 (415, 1182).

Table 7.1 Sensitivity of *Acinetobacter* strains to gentamicin.

Species	Strain number	Gentamicin concentration			
		50μg/ml		100μg/ml	
A. baumannii	1095	++ *	++ **	++ *	++ **
A. baumannii	1096	++	++	++	++
A. baumannii	1098	++	++	++	++
A. baumannii	1099	--	--	--	--
A. baumannii	1102	--	--	--	--
A. baumannii	1109	++	++	++	++
A. baumannii	1111	--	--	--	--
A. baumannii	1112	--	--	--	--
A. baumannii	1113	++	++	++	++
A. baumannii	1114	++	++	++	++
A. baumannii	1115	++	++	++	++
A. baumannii	1116	++	++	++	++
A. baumannii	1117	++	++	++	++
A. baumannii	1118	++	++	++	++
A. baumannii	1119	++	++	++	++
A. baumannii	1120	++	++	++	++
A. baumannii	1121	++	++	++	++
A. baumannii	1122	++	++	++	++
A. baumannii	1123	++	++	++	++
A. baumannii	1124	++	++	++	++
A. baumannii	1125	--	++	--	++
A. baumannii	1126	++	++	++	++
A. baumannii	1127	++	++	++	++
A. baumannii	1183	--	--	--	--
A. baumannii	1440	--	--	--	--
A. calcoaceticus	418	--	--	--	--
A. calcoaceticus	1097	--	--	--	--
Acinetobacter gensp. 3	415	--	--	--	--
Acinetobacter gensp. 3	1182	--	--	--	--

For the optimisation of the gentamicin concentration, the positive and negative recovery bacterial cells in 50 and 100μg/ml gentamicin was examined. Concentrations of bacterial cells exposed to gentamicin were 10^6 and 10^7 cfu/ml corresponding to those intended for the association and invasion protection assays. At 10^6 cfu/ml, 11 out of 29 strains were found to be inhibited in the presence of 50 and 100μg/ml gentamicin. One of these inhibited strains (1102) was able to grow at the same gentamicin concentrations when the bacterial count was 10^7 cfu/ml. * Bacterial concentration (10^6 cfu/ml), ** Bacterial concentration (10^7 cfu/ml), ++ duplicate positive recovery, -- duplicate negative recovery.

To confirm the above-mentioned results, *in-situ* testing was carried out. The gentamicin protection assay was performed and supernatants (0.5ml) were plated out at the end of the 5h incubation of the infected epithelial cells at 37°C. Complete inhibition of the external bacteria present in the supernatant was checked. Six of the 10 test strains were found to be gentamicin resistant to 100µg/ml at both bacterial concentrations **(Table 7.2)**. Therefore, this concentration was increased to 250µg/ml and strain susceptibility was validated again using the same real-time method. This time the infection doses were $10^7$and 10^8 cfu/ml to ensure there was no resistance associated with the increase in bacterial cells at MOI 100 and MOI 1000 respectively. Strain 1112 (*A. baumannii*) sustained its resistance while the other nine strains were completely inhibited at all inocula sizes. The type *A. baumannii* strain NCTC 12156^T, however, was no longer sensitive to gentamicin (250µg/ml) when the bacterial suspension contained 10^8 cfu/well (MOI 1000).

Table 7.2 *In-situ* gentamicin sensitivity testing of *Acinetobacter* strains.

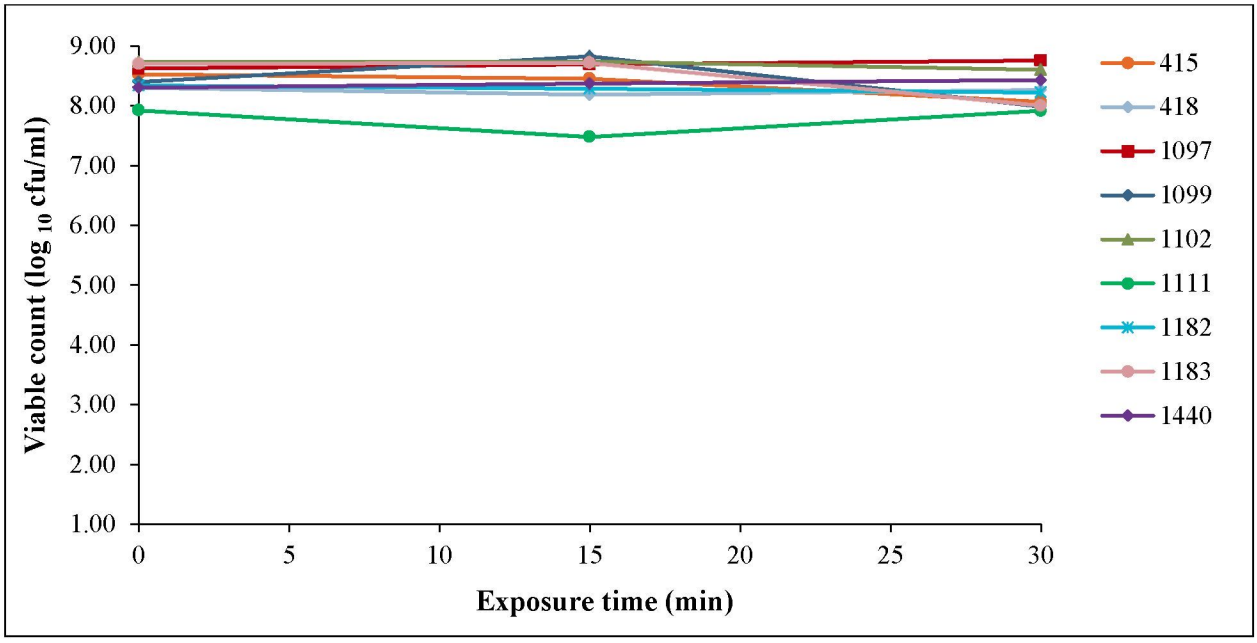
Species	Strain number	Gentamicin concentration			
		100µg/ml		250µg/ml	
A. baumannii	1099	+++ *	+++ **	--- *	--- **
A. baumannii	1102	+++	+++	---	+++
A. baumannii	1111	+++	+++	---	---
A. baumannii	1112	+++	+++	+++	+++
A. baumannii	1183	---	---	---	---
A. baumannii	1440	---	---	---	---
A. calcoaceticus	418	---	---	---	---
A. calcoaceticus	1097	+++	+++	---	---
Acinetobacter gensp. 3	415	---	---	---	---
Acinetobacter gensp. 3	1182	+++	+++	---	---

Acinetobacter strains that were sensitive to 100µg/l gentamicin (as shown by the micro-dilution method) were used for a real-time susceptibility testing. Six of the 10 test strains were found to be gentamicin resistant to 100µg/ml at MOI 100 and MOI 1000. Therefore, this concentration was increased to 250µg/ml and strain susceptibility was validated again using the same real-time method. Strain 1112 (*A. baumannii*) sustained its resistance while the other nine strains were completely inhibited at all inocula sizes. The type *A. baumannii* strain NCTC 12156^T, however, was no longer sensitive to gentamicin (250µg/ml) when the bacterial suspension contained 10^8 cfu/well. * Bacterial concentration (10^7 cfu/ ml), ** Bacterial concentration (10^8 cfu/ ml), +++ triplicate positive recovery, --- triplicate negative recovery.

7.3.2 Determination of bacterial viability in 0.5% Triton X-100

The quantitation of the internalised acinetobacters was achieved by using Triton X-100 0.5% (v/v). This detergent was employed in the gentamicin protection assays to lyse the eukaryotic cells and consequently release the internal bacteria. The sensitivity of *Acinetobacter* strains to Triton X-100 was tested. Bacterial suspensions were prepared in Triton X-100 solution and incubated for 30 min at 21°C (room temperature). Aliquots (15µl) of *Acinetobacter* were plated out at 0, 15, and 30 min. The number of viable cells was calculated and survival rates in relation to T_0 were established for each test strain. No apparent reduction in viability was observed for 7 of the 9 strains over 30 min of exposure **(Figure 7.1)**.

Figure 7.1 Effect of 0.5% Triton X-100 on the viability of *Acinetobacter* strains.



The number of viable cells in 0.5% Triton X-100 was calculated using the Miles and Misra method at the time points indicated in the figure. The experiment was performed once, and the results showed no apparent reduction in the viability of 7 of the 9 strains over 30 min of exposure. Strains 1099, 1102, 1111, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3.

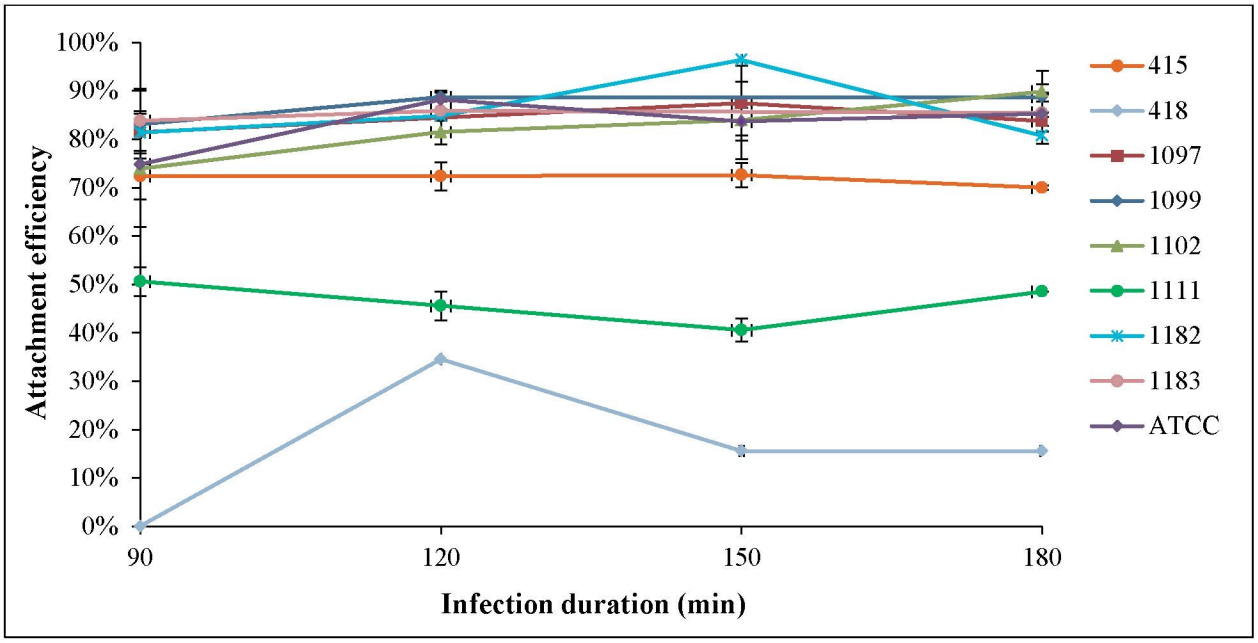
7.3.3 Effect of the infection time course on the attachment and invasion levels

The effect of infection duration on the levels of cells attached and internalised was examined. Caco-2 cells were infected with *Acinetobacter* cultures at MOI 100, and then were incubated for different durations (90,120, 150,180 min) at 37°C. Six of the nine test strains displayed an increase in their attachment efficiency 120 min post infection **(Figure 7.2)**. This increase, however, was only significant ($P < 0.05$) with *A. baumannii* ATCC 17978 (1440) and *A. calcoaceticus* (418). The latter was particularly interesting in that associated cells went from undetected, after 90 min, to 35% attachment efficiency 120 min later. This was followed by a notable yet non-significant ($P > 0.05$) decrease of 19% (T_{150}) before it plateaued by the end of the experiment. Cell association of strain 1099, and 1183 (both *A. baumannii*) levelled after 120 min of infection, whereas that of strain 1440 decreased marginally. Adhered cell numbers of strain 1097 (*A. calcoaceticus* NCTC 7844) and 1182 (*Acinetobacter* gensp. 3) both declined to different extents after 150 min, but this was not considered statistically significant ($P > 0.05$). A steady increase in the attachment levels was exhibited by the type strain *A. baumanii* NCTC 12156^T (1102) all the way through the assay from 74% up to 90% ($P > 0.05$). On the other hand, strain 415 (*Acinetobacter* gensp. 3) decreased from 72% to 70% as the infection period increased.The highest value for *A. baumannii* 1111 was recorded at 90 min ($P > 0.05$). In general, the attachment of strains 415, 418, and 1111 to the Caco-2 cells appeared to be lower than that of the remaining strains.

The impact of the infection time-course on the levels of invasiveness was also investigated as a means of optimising the gentamicin protection assay. Eight out of the nine test strains were able to invade the colonic epithelial cells **(Figure 7.3)**. In contrast, no invasion was detected with *A. calcoaceticus* (418) despite its ability to adhere. The increase in the invasion efficiency was observed in 7 strains 120 min post-infection. It ranged between 4% and 26%, and was particularly significant ($P < 0.05$) with strain 1182. The internalised cell counts of the invasive strains 1097, 1099, 1102, and 1182 continued to increase for up to 180 min. The invasion efficiency of strain 1183, however, plateaued after 150 min while that of strain 415 non-significantly decreased by 1%. The invasion rates for strain 1111 increased to 35% (120 min) and then steadily dropped to 27% ($P > 0.05$) by the end of the assay. Although strains

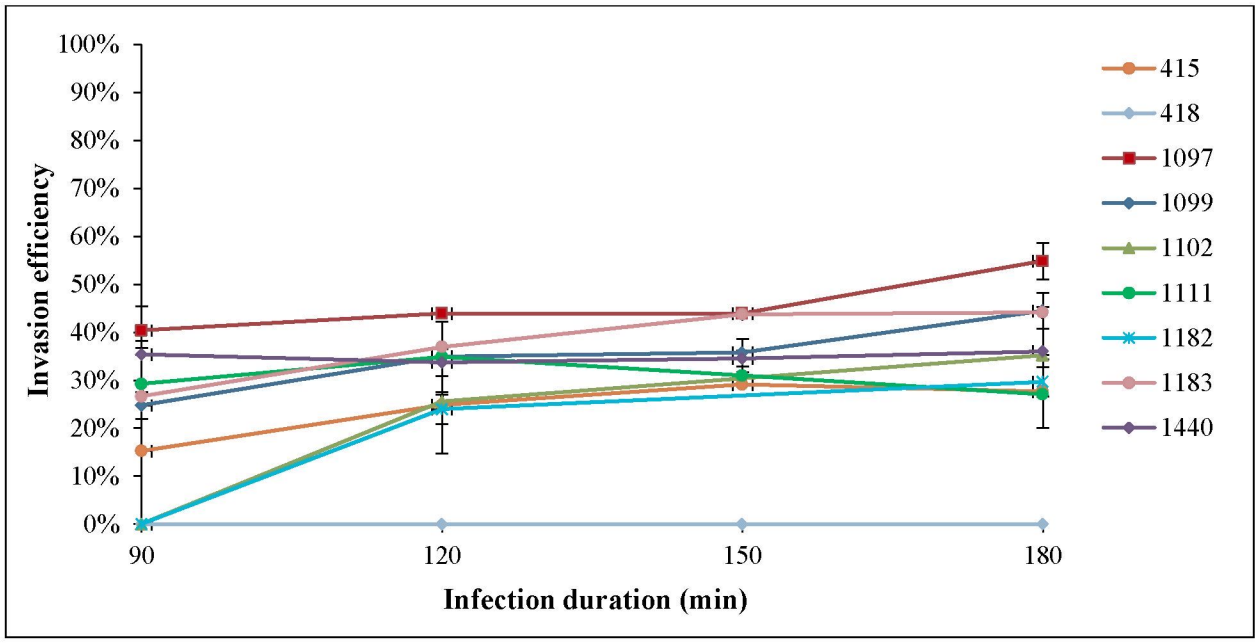
415 and 1111 had low levels of attachment as indicated in **Figure 7.2**, their invasiveness was not distinctively different from those with higher attachment values. In comparison with the majority of *Acinetobacter* test strains, the length of infection time-course did not seem to have a notable impact on the invasion levels of strain 1440. Its internalised counts remained stable for the first 150 min of incubation. It then rose by 1% the next 30 min. In general, the highest increases in invasion efficiencies of the majority of strains (5 out of 8) were recorded at 180 min. Three of which (1099, 1102, and 1182) were statistically significant ($P < 0.05$). Therefore, the infection time-course was designed to last for 180 min as a means of maximizing the invasion levels.

Figure 7.2 Effect of infection time course on the attachment levels of *Acinetobacter* strains.



The figure shows the percentages of the inocula that attached to Caco-2 cells over 180 min of infection. The highest increases in attachment efficiencies of the majority of strains (4 out of 9) were recorded at 120 min. Three of which were statistically significant. Strains 1099, 1102, 1111, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3. Inoculum size was 10^7cfu/ml (MOI 100). Error bars represent the standard deviation of two independently repeated experiments.

Figure 7.3 Effect of infection time course on the invasion levels of *Acinetobacter* strains.



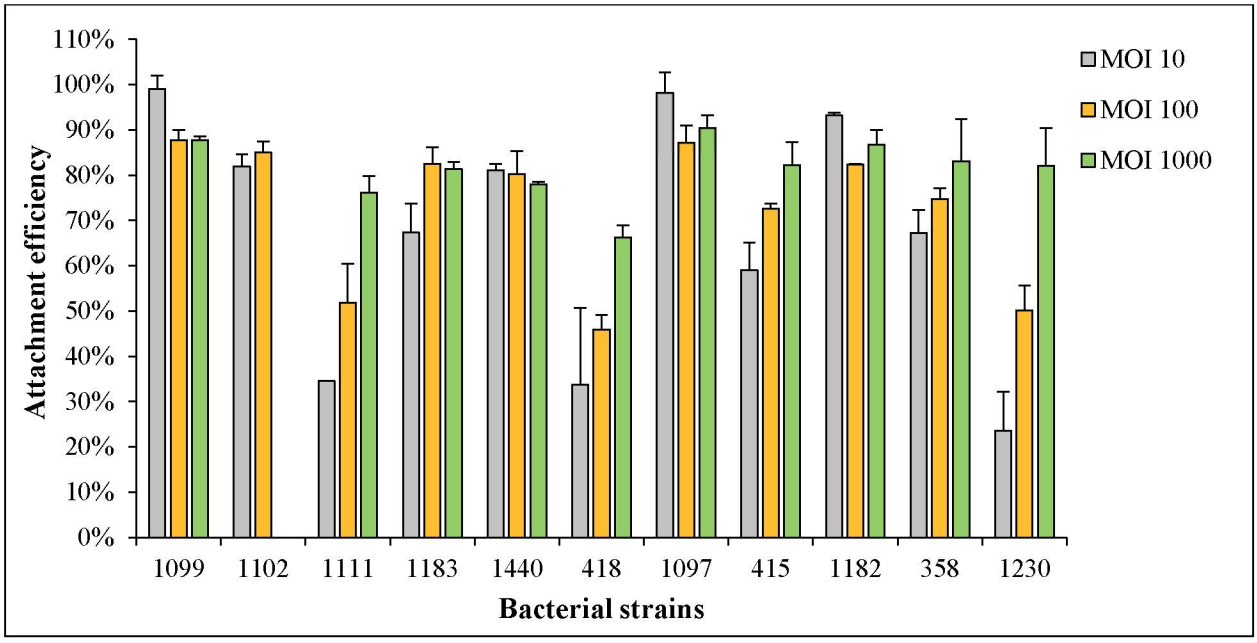
The figure shows the percentages of the inocula that invaded the Caco-2 cells over 180 min of infection. Eight of the nine strains proved to be invasive. The increase in the invasion efficiency was observed in seven strains 120 min post-infection. In general, the highest increases in invasion efficiencies of the majority of strains (5 out of 8) were recorded at 180 min. Strains 1099, 1102, 1111, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3. Inoculum size was 10^7 cfu/ml (MOI 100). Error bars represent the standard deviation of two independently repeated experiments.

7.3.4 Effect of different MOIs on the attachment levels of *Acinetobacter*

Following the optimisation of the gentamicin concentration and the infection duration, the attachment trends of *Acinetobacter* were examined at different infection doses. The MOIs employed were 10, 100, and 1000. The lowest attachment efficiency was exhibited by strain 418 at all 3 MOIs **(Figure 7.4)**. Strain 1099, on the other hand, showed the highest attachment levels at MOI 10 (99%). At MOI 100, the maximum percentage (90%) of adhered cells was obtained by strain 1097. Four out of the nine *Acinetobacter* strains; *Acinetobacter* gensp. 3 (415), *A. calcoaceticus* (418), *A. baumanii* NCTC 12156^T (1102), and *A. baumannii* (1111); increased in adherence efficiency as the inoculum size increased. This also applied to both *E. coli* K12 1230 and *Salmonella* Enteriditis 358. These increases in numbers from MOI 10 to 1000 were statistically significant ($P < 0.05$) with strain 415, 418, 1111 and 1230 but

not with strain 1102 and 358. In contrast to these progressive increases shown by the above-mentioned strains, strain *A. calcoaceticus* NCTC 7844 (1097), *A. baumannii* (1099), *Acinetobacter* gensp. 3 (1182), and *A. baumannii* ATCC 17978 (1440) were at their highest attachment values at the MOI of 10. These were significantly ($P < 0.05$) higher than the other MOIs with strain 1099, and 1182 (greater than MOI 100 only) but not with strains 1097 and 1440 ($P > 0.05$). Strain 1183 (*A. baumannii*), on the other hand, showed the maximum attachment levels at MOI 100. However, it was not significantly different to that at MOIs 10 and 1000.

Figure 7.4 Effect of MOIs on the attachment levels of *Acinetobacter* strains.



The figure shows that the highest attachment levels were observed with 4, 2, and 3 of the *Acinetobacter* strains at MOI 10, 100, and 1000 respectively. These were calculated as the percentages of the inocula that were able to adhere to Caco-2 cells. Strain 1102 was not tested at MOI 1000 due to its gentamicin resistance at this level, as shown by the preliminary experiments. Strains 1099, 1102, 1111, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3. *Salmonella* Enteritidis (358) and *Escherichia coli* K12 (1230) were used as positive controls for attachment. Error bars represent the standard deviation of at least two independent experiments.

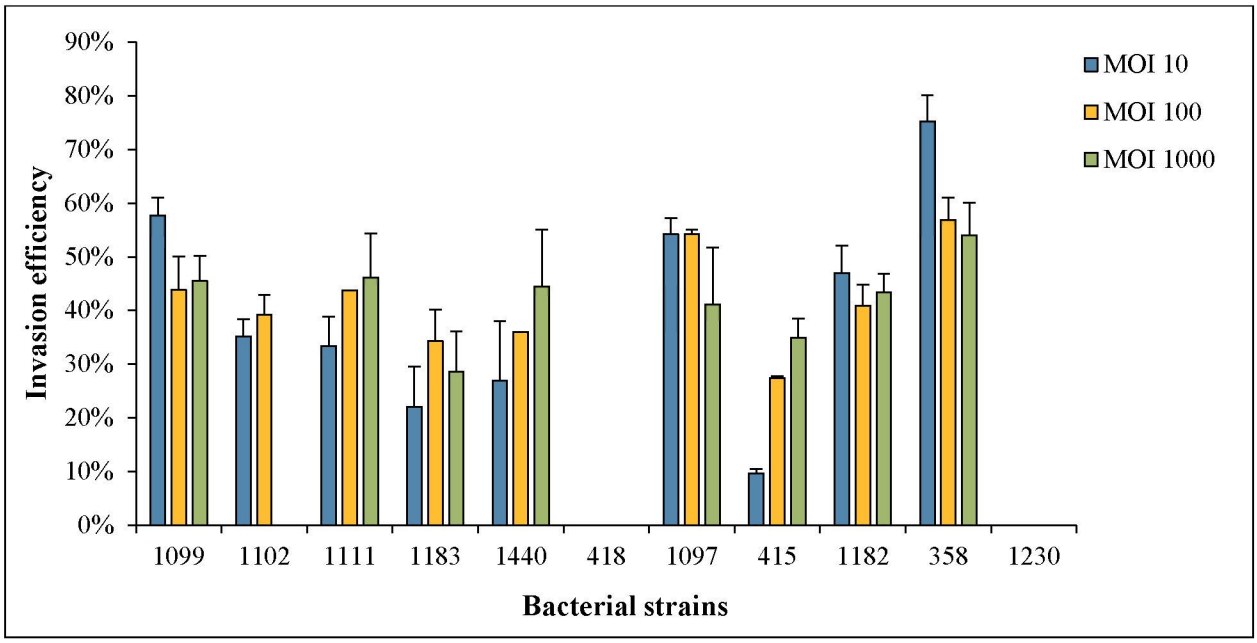
7.3.5 Effect of different MOIs on the invasion levels of *Acinetobacter*

With regard to the levels of invasion when applying different bacterial concentrations, there was no general observation that applied to all strains or strains from certain species owing to the strain-specific variations **(Figure 7.5)**. Five strains (415, 1102, 1111, 1183 apart from at MOI 1000, and 1440) were seen to increase as the MOI increased. The rate of increase from an MOI of 10 (18%) to MOI 1000 (25%) was most significant with strain 415 ($P < 0.05$). Strains 1099 and 1182 exhibited the highest numbers of internalized cells (58, and 47% respectively) at an MOI of 10 and then stabilized at higher MOIs. *A. calcoaceticus* NCTC 7844 (1097) exhibited higher numbers of internalized cells ($P > 0.05$) at an MOI of 10 and 100 (54% each) compared to MOI 1000.

The highest invasion efficiency (58%) was achieved by strain 1099 at MOI 10 and was 18% lower than that of the positive control (358). Conversely, strain 415 was the lowest in the invasion numbers (10%) at the same MOI. Overall, all invasive *Acinetobacter* strains (8/8) were above the invasion efficiency 30% at MOI 1000. At MOI 100, all strains excluding (415) were also above the level of 30% at MOI 100. Two (415, 1183) out of the 8 strains were below the above mentioned percentage at MOI 10. The positive control (358) remained above 50% regardless of the MOI, while strain 415 was the only strain whose invasion was below 20%.

When the efficiency of the attachment and invasion were compared **(Figures 7.4 & 7.5)**, internalised *Acinetobacter* cells appeared to be on average 44, 43, and 44% less than the adhered cells for 7 out of the invasive test strains at MOI 10, 100, and 1000 respectively. In comparison to all the invasive *Acinetobacter* strains tested, strain 1111 showed distinctively the smallest difference between the percentages of the internalized cells and those of cell-adhered. The attachment efficiency for this strain was 1, 8, and 30% higher than the invasion efficiency at MOI 10, 100, and 1000 respectively.

Figure 7.5 Effect of MOIs on the invasion levels of *Acinetobacter* strains.

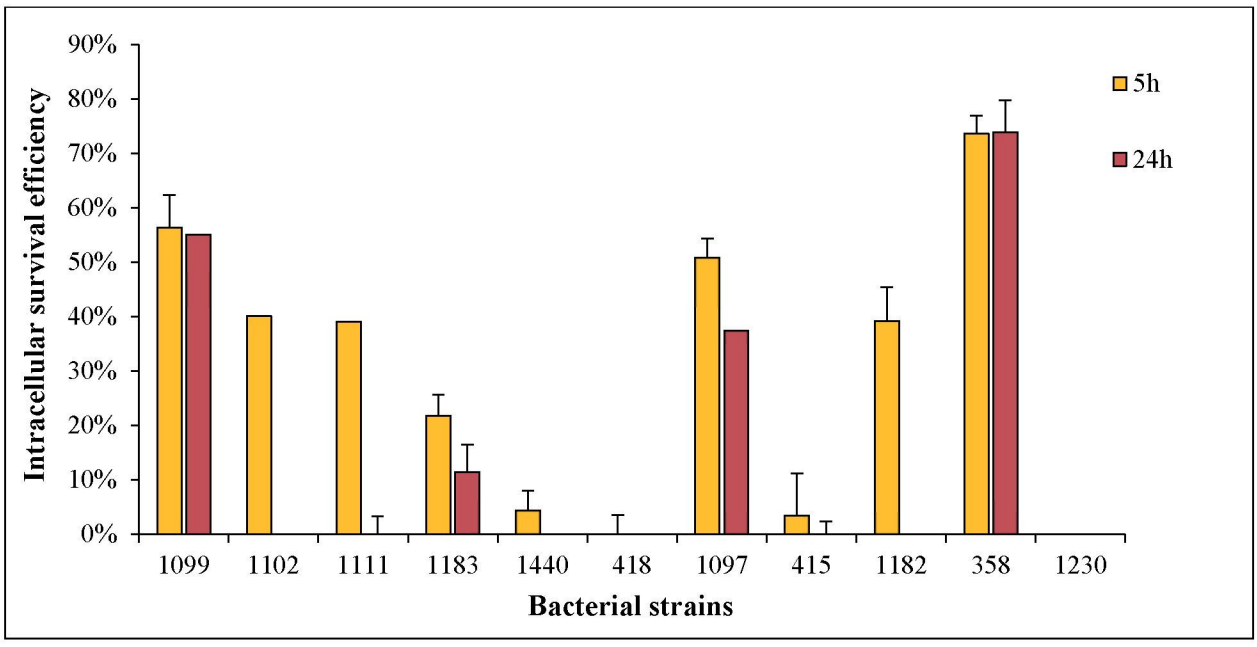


The percentages illustrated indicate the efficiencies of the inocula in invading the Caco-2 cells. *Acinetobacter* 418 was the only strain unable to invade the epithelial cells. Overall, the invasion levels of five of the nine *Acinetobacter* strains increased with the increase in the bacterial dose (except at MOI1000 with strain 1183). Strains 1099, 1102, 1111, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3. The invasive *Salmonella* Enteritidis (358) was used as a positive control while *Escherichia coli* K12 (strain 1230) was the negative control. Error bars represent the standard deviation of at least two independent experiments.

7.3.6 Intracellular survival

To study the intracellular survival of *Acinetobacter* strains, the gentamicin protection assay was used and the bacterial counts were determined 5h (T_0) and 24h post-infection. Of the 8 invasive strains used in this study, strain 1097 (*A. calcoaceticus* NCTC 7844), 1099 and 1183 (both *A. baumannii*) were able to persist inside the epithelial cells (Caco-2) for 24h **(Figure 7.6)**. The highest survival was shown by strain 1099 (55%), which was 19% lower than the positive control (358), followed by strain 1097 (37%), and 1183 at 11%. When compared to the values at T_0, it was found that the survival efficiency of the positive control (358) was 100.4%. In other words, all the internalised cells managed to maintain their viability for up to 24h of infection duration. This was not observed with the *Acinetobacter* strains. Strain 1097 dropped significantly by 13% ($P < 0.05$), whereas the viable counts of strain 1099 and 1183 decreased by 1% and 10% respectively ($P > 0.05$).

Figure 7.6 Levels of intracellular survival of *Acinetobacter* strains inside Caco-2 epithelial cells.

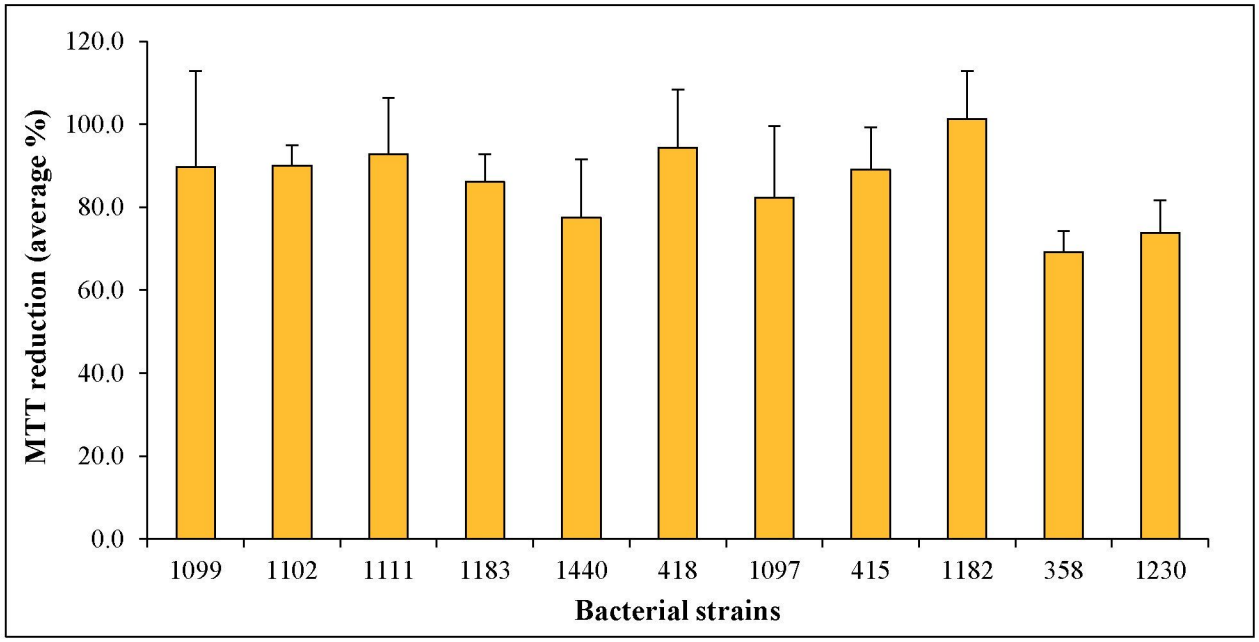


The results show the ability of three of the invasive strains to survive for up to 24h of infection duration in Caco-2 cells. Internalised bacteria were determined after 5h of inoculation, while the values of the persistent bacteria were taken after 24h and were expressed as the percentages of the inocula that survived for 24h inside the epithelial cells. Strains 1099, 1102, 1111, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3. The invasive *Salmonella* Enteritidis (358) was used as a positive control while *Escherichia coli* K12 (1230) was the negative control. Inoculum size was 10^6 cfu/ml. Error bars represent the standard deviation of at least two independent experiments.

7.3.7 Cytotoxicity of *Acinetobacter*

The effect of *Acinetobacter* strains on the viability of the epithelial Caco-2 cells was indicated by the percentage of the MTT reduction. This was shown to be greater than 80% in relation to the uninfected cells **(Figure 7.7)**. Cells infected with *A. baumannii* 1440 (ATCC 17978) showed the lowest reduction percentage (78%) which was 8% higher than that infected with *Salmonella* Enteriditis (358). The highest was calculated at 101% and colonized with *Acinetobacter* gensp. 3 (1182). Of note is the non-significant difference between the invasive 358 and non-invasive 1230 strains. The toxicity of *Escherichia coli* K12 (1230) appears to be attributed to the attachment of this organism to the epithelial cells and not a result of the invasion process.

Figure 7.7 Levels of *Acinetobacter* cytotoxicity.

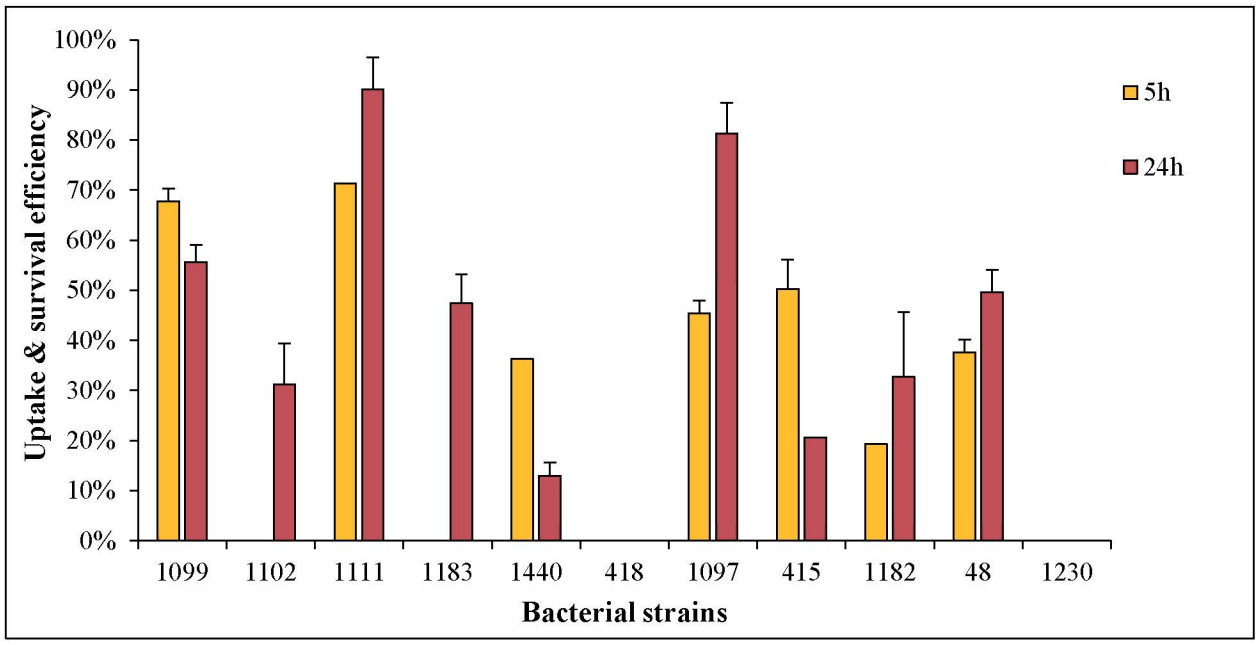


The average MTT reduction percentages were calculated in relation to the uninfected epithelial Caco-2 cells (MTT reduction = 100.0%). MTT was used to measure cytotoxicity levels of *Acinetobacter* where only the viable eukaryotic cells can reduce this substance to purple formazan crystals. Cells infected with strain 1440 showed the lowest reduction percentage (77.5%). The highest was calculated at 101.19% and colonized with strain 1182. Strains 1099, 1102, 1111, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3.; 358 and 1230 are *Escherichia coli* K12 and *Salmonella* Enteritidis respectively. Inoculum size was 10^6 cfu/ml. Error bars represent the standard deviation of two independent experiments.

7.3.8 Persistence within U937 human macrophage cells

Macrophage uptake of *Acinetobacter* and subsequent persistence was observed with 8 of the 9 *Acinetobacter* strains (**Figure 7.8**). Survival of *A. baumannii* (1099 and 1111), and *A. calcoaceticus* NCTC 7844 (1097) was above the 50% exhibited by the positive control (*Citrobacter koseri* 48). The highest percentage was observed with strain 1111 at 90% ($P < 0.05$). This was 41% greater than the positive control. In comparison, strain 1440 (*A. baumannii* ATCC 17978) showed the lowest number of surviving cells (13%). The intracellular counts of *A. calcoaceticus* NCTC 7844 (1097), *A. baumannii* NCTC 12156^T (1102), *Acinetobacter* gensp. 3 (1182), and *A. baumannii* (1183 and 1111) increased within 24h. Compared to those determined at 5h, the numbers of 1097, 1111, and 1182 rose by 36% ($P < 0.05$), 19% ($P = 0.05$), and 13% ($P > 0.05$) respectively. Strain 1102 and 1183, in particular, were not detected after 5h of infection, but their survival at 31% and 47% was evident after 24h respectively. While the five strains mentioned above proliferated within 24h of incubation, strains 1099 (*A. baumannii*), 1440 (*A. baumannii* ATCC 17978), and 415 (*Acinetobacter* gensp. 3) exhibited a decrease in survival by 12% ($P > 0.05$), 23% ($P < 0.05$), and 30% ($P < 0.05$) respectively.

Figure 7.8 Levels of *Acinetobacter* persistence within macrophages.



Levels of uptake of *Acinetobacter* strains by macrophages were calculated at 5h, while that of the persistence was determined at 24h and was expressed as the percentage the inoculum that was able to survive within the macrophage. Eight of the nine *Acinetobacter* strains were detected after 24h of inoculation. Five of the *Acinetobacter* strains survived in higher counts than the positive control. *Citrobacter koseri* 48 was used as the positive control, whereas *Escherichia coli* K12 (1230) was the negative control. Inoculum size was 10^6 cfu/ml. Strains 1099, 1102, 1111, 1183, and 1440 are *A. baumannii*; 418 and 1097 are *A. calcoaceticus;* 415 and 1182 are *Acinetobacter* gensp. 3. Error bars represent the standard deviation of two independent experiments.

7.4 DISCUSSION

The human colonic adenocarcinoma cell-line (Caco-2) used in this study mimics the intestinal crypts, caecum, and the lower small intestinal sites (Lee *et al.*, 1986; Newell and Pearson, 1984). When they are differentiated, Caco-2 cells express intestinal-like microvilli and form monolayers with tight junctions resembling the gut epithelia (Pinto *et al.*, 1983). For this reason, it is considered a suitable *in vitro* model for studying the bacterial effects and interactions with the intestinal epithelial cells (Bolton *et al.*, 2000) while avoiding the extensive use of animal models. For qualitative and quantitative infection studies, the gentamicin protection assay has been commonly applied based on the principle that the negligible amounts of the antibiotic pass into eukaryotic cells and therefore intracellular bacteria are protected. In this study, the detection of viable bacterial cells, despite the 2h exposure to 100µg/ml gentamicin, shows that they were partially protected from the gentamicin effect. This was possibly due to biofilm formation or clumping (Hertle and Schwarz, 2004) which hindered the complete access of the antibiotic to those aggregates and hence achieving complete kill of the bacterial population. Lee *et al.* (2008b) observed clusters of *A. baumannii* cells forming microcolonies at localized areas of the human bronchial cells (NCI-H292) as well as dispersed cells on surface of the monolayer.

The findings of this study demonstrated the ability of all *Acinetobacter* strains to attach to the intestinal epithelial cells. The levels of adherence were, however, strain-specific. The food isolates *Acinetobacter* gensp. 3 (415) and *A. calcoaceticus* (418), as well as *A. baumannii* (1111) isolated from a clinical specimen, were generally the lowest in their adhesion capacity. Following the colonisation of the host cells, the majority (8/9) of *Acinetobacter* strains was able to enter the epithelial cells; including 415 and the isolates form follow-up formulas (1182 and 1183). Strain 418 was the only *Acinetobacter* strain to adhere but not show any invasion capacity. The invasion values varied according to strain; with the highest (*A. baumannii* 1099) being 0.6% of the initial inoculum. This was greater than those recorded with other neonatal invasive pathogens such as *Cronobacter sakazakii* implicated in cases of meningitis, sepsis, bacteraemia, and necrotizing enterocolitis (Townsend *et al.*, 2008). In contrast to the other *Acinetobacter* strains, *A. calcoaceticus* (1097), *A. baumannii* (1099), and

Acinetobacter gensp. 3 (1182) were the most invasive since the smallest MOI (MOI 10) was sufficient for maximum invasion. Overall, the adherence did not correlate to the invasiveness of strains since comparable invasion levels was obtained with both the strongly and the less adherent strains.

Strain specificity was not the sole factor accounting for the differences in the attachment and invasion efficiencies between the *Acinetobacter* strains, as the MOI was also a contributory factor. Increasing the infection dose was found to lead to the detachment of the HEp-2 cell monolayer consequently to the acidification of the medium caused by the metabolism of *Salmonella* Typhimurium (Douce *et al.*, 1991). Hertle and Schwarz (2004) also described that at an MOI greater than 10 for *Serratia marcescens* caused the epithelial RT112 and HEp-2 cells to lyse within 15 min of exposure as a result of hemolysin production. An association between the higher concentration of *Acinetobacter* and possible epithelial cell damage can be represented by the various degrees of reduction in the invasion levels of *A. calcoaceticus* (1097), *A. baumannii* (1099 and 1183), and *Acinetobacter* gensp. 3 (1182) at MOI 100, 1000, or both.

The numbers of bacteria adhering to and invading epithelial cells can also be influenced by the exposure duration (Friis *et al.*, 2005). For example, *A. baumannii* strains infecting NCI-H292 cells required an incubation period ranging from 60 to 90 min to reach their maximum adherence, while at 120 min the decrease in adherence values was evident (Lee *et al.*, 2006). In the present study, the attachment levels of the majority of strains did not significantly change from 120 and 180 min of incubation. The exceptions were *A. calcoaceticus* (418) and *Acinetobacter* gensp. 3 (1182) whose efficiencies to adhere to Caco-2 cells decreased after 120 and 150 min of exposure respectively. With regard to invasion, Choi *et al.* (2008c) described a consistent increase in the internalisation of *A. baumannii* ATCC 19606^T into NCI-H292 cells for up to 7h. In comparison, the maximum invasion obtained here was reached at 180 min for five of the eight invasive *Acinetobacter* strains tested. However, no significant ($p \geq 0.05$) reduction was shown by any of the remaining strains.

The invasion ability of *Acinetobacter* may be indicative of their pathogenic potential to the neonatal host. According to Townsend *et al.* (2008) *Cronobacter sakazakii* strains which caused fatal meningitis and necrotizing enterocolitis in neonates, also showed the highest

percentages of invasion in intestinal cells (Caco-2). However, the levels of invasion are not always an indicator for the virulence capacity. *Campylobacter jejuni* can be used as another example in this respect. Although the organism is the most common cause of bacterial foodborne diarrhoeal disease worldwide, internalization of only 1 to 3 cells per intestinal INT-407 cell has been detected even with the most invasive strains (Biswas *et al.*, 2000; Biswas *et al.*, 2004).

Comparisons with *Acinetobacter* adhesion and invasion data from previous studies cannot always be accurately made. This is not only due to experimental differences, strains, and bacterial inoculation size, but also because of the variability of the presentation and interpretation of the data. Bacterial invasion may be expressed as the number of the internalised bacteria per cell or per well. Others represent their findings as the percentage of the epithelial cells infected. In this study, however, data were shown as percentages of the inocula found intracellularly or attached.

Invasion of intestinal epithelial cells is a crucial characteristic of many pathogens. It allows them to reach a protected environment where they can subsequently proliferate and /or spread from cell to cell and be less subject to therapeutic interventions or the host immune defenses (Pereira *et al.*, 2008). Three of the eight invasive *Acinetobacter* strains were found to survive for 24h inside the Caco-2 cells. The internalised cells of *A. baumannii* (1183) and *A. calcoaceticus* (1097) decreased by 10% and 13% respectively. On the other hand, *A. baumannii* (1099) maintained its persistence for the time-course. Therefore, it seems reasonable to speculate that the intracellular survival of these strains after 24h of infection may provide a reservoir for prolonged colonization of the infected neonate even if an appropriate antibiotic treatment is used. A persistent diarrhoeal disease caused by enteroaggregative *Escherichia coli* (EAEC) has been associated with the ability of this organism to survive for long periods (72h) within the human colorectal epithelial cells (T84), and in some cases the intracellular multiplication (Pereira *et al.*, 2008). *Helicobacter pylori* and uropathogenic *Escherichia coli* (UPEC) are examples of extracellular pathogens which transiently colonise the intracellular environment of mucosal epithelia as a means for prolonged persistence (Mulvey *et al.*, 2001; Necchi *et al.*, 2007).

The absence of internalised cells, observed with some *Acinetobacter* strains at 24h, could be the result of their inability to survive prolonged periods of time inside the epithelial cells due to fusion with endosomes or lysosomes (Kahl *et al.*, 2000). On invasion, bacteria become enclosed within a vacuole in which they remain, or are able to escape and persist in the cytoplasm (e.g. *Listeria monocytogenes* (Tang *et al.*, 1994)) or extracellularly (e.g. *Neisseria meningitidis* (Join-Lambert *et al.*, 2010)). Intracellular *A. baumannii* ATCC 19606^T has been reported to persist in a membrane-bound vacuole compartment for up to 5h (Choi *et al.*, 2008c). The risk of being inside the membrane-bound vacuole it that it may fuse with endosomes which then matures to an acidic lysosome (~ pH 5.0) containing hydrolytic enzymes which can lead to degradation of bacteria (Wilson *et al.*, 2002). However, according to Choi *et al.* (2008c), the majority of pathogenic bacteria are able to evade the fusion with endosomes or lysosomes as a means of survival. Whether this is the case or not with the *Acinetobacter* tested here is unknown. Further elucidation is required in this respect.

The effect of the *Acinetobacter* invasion on the intestinal epithelial cells has been assessed in this study, by measuring the viability of eukaryotic cells through mitochondrial activity, using the MTT assay. The maximum loss of viability of the infected Caco-2 cells was 20% relative to controls. Although the nature of the damage has not been illustrated by imagery, this percentage indicated some degree of cell death. Loss of viability of the epithelial cells may be caused by the consumption of the available nutrients in the medium, the accumulation of bacterial waste products, or the medium becoming acidic (Wilson *et al.*, 2002). Cell death can also occur in a controlled mechanism termed 'programmed cell death' or 'apoptosis', which is distinguishable from necrotic cell death by the characteristic changes of the cell (presence of apoptotic bodies, chromatin fragmentation and condensation, membrane blebbing). Induced apoptosis as a consequence of bacterial invasion including *Acinetobacter* has been previously described. Lee *et al.* (2001) have, for example, shown that HeLa epithelial cells undergo apoptosis 8h following infection with *A. baumannii*. Cell death was also observed, by Choi *et al.* (2005), after 12h as the result of *A. baumannii* ATCC 19606^T targeting the mitochondria of the human laryngeal epithelial cells (HEp-2). In addition, the interaction of *A. baumannii* ATCC 19606^T with human alveolar epithelial cells (A549) has been shown to cause the apoptotic death of these cells (Gaddy *et al.*, 2009a). The death of intestinal epithelial cells may have important implications for the development of infections. The

release of the bacteria following the disruption of the mucosal lining may allow them to enter the deep tissues (Choi *et al.*, 2005). Therefore, it can be expected that the potential pathology of *Acinetobacter* infections might be extended in this respect.

The mechanism responsible for the adherence and the interaction of *Acinetobacter* spp. with the intestinal epithelial cells facilitating the entry to the host cell was beyond the scope of this study. However, Lee *et al.* (2006) has demonstrated that *A. baumannii* bind to the smooth areas of the epithelial NCI-H292 cell surface. The bacterial surface displayed a thin fimbrial-like structure which extended and firmly anchored to the surface of the epithelial cells. Gohl *et al.* (2006) has also reported the involvement of thin pili in the adherence of *Acinetobacter* strain BD413 to the biotic surface of erythrocytes. De Breij *et al.* (2010), on the other hand, found that the pilus-like structure and other long extensions, which were irregularly distributed over the cell surface of *A. baumannii* strains and occasionally connected the cells, did not always associate with the adherence capacity.

Choi *et al.* (2008c) identified the zipper-like mechanism by which *A. baumannii* invade the epithelial NCI-H292 cells. Once loosely attached, the bacterium was wrapped with the extended membrane of the epithelial cells and then internalised. The cell membrane was finally closed at the invasion site. Based on these cellular changes, the invasion mechanism was characterised as a zipper-like process rather than a trigger mechanism. The former requires the direct contact between the bacterial ligands and the host cell receptors to cause a local cytoskeletal rearrangement instead of injecting bacterial effector proteins which trigger dramatic rearrangements, such as membrane ruffles, of the cytoskeleton (Choi *et al.*, 2008c). Further research work needs to be undertaken in order to confirm Choi *et al.*'s (2008) observation using the strains and intestinal cell line used here.

Survival of the bacteria when facing host defenses within the circulation is a prerequisite for diseases such meningitis or septicemia. One important defense of the innate immune system is microbial uptake by macrophages, which exhibit antibacterial strategies such as nutrient deprivation, acidic compartmentalization, and oxidative burst (Townsend *et al.*, 2007). The ability of pathogens to evade phagocytic killing either by the inhibition of the uptake or survival within the macrophage represents a substantial virulence mechanism against one of

the major innate immune defenses (Wilson *et al.*, 2002). The interaction of *Acinetobacter* with the innate immunity, represented by phagocytosis as a classical model (Greenberg, 1999), was studied. The human macrophage U936 was employed for this purpose. Despite strain to strain variations, the detection of internalised acinetobacters inside the macrophages, at T_0 (5h), using the gentamicin protection assay shows that *Acinetobacter* did not block the phagocytosis process and that the organism was actually phagocytosed.

A. calcoaceticus 418 (food isolate) was the only strain unable to escape the phagocytic killing as no viable cells were detected at any sampling point. The same strain also did not have the capacity to invade the epithelial Caco-2 cells. *A. baumannii* NCTC 12156^T (1102) and 1183 were not detected at the first time point (T_0) as opposed to after 24h possibly because the internalised cells were below the detection limit. *A. calcoaceticus* NCTC 7844, *Acinetobacter* gensp. 3 (1182), and *A. baumannii* (1102, 1111, 1183) did not only persist within macrophages but also replicated indicating their potential to cause systematic infections in neonates (Fields *et al.*, 1986; Ohl and Miller, 2001). Maintaining the organism's viability inside the macrophage renders it safe from the activity of humoral components such as antibiotic peptides, antibodies, and complement. It also protects the bacterium from being washed away, and saves it the need to adhere to host cells (Wilson *et al.*, 2002). However, the intracellular bacterium would still be susceptible to macrophage activation (Wilson *et al.*, 2002). The prolonged persistence of *Acinetobacter* within macrophages suggests that the organism has developed a mechanism to avoid being killed when living intracellularly.

Overall, intra-macrophage survival data showed wide differences between strains of the same species. This may be reflective of the levels of pathogenicity in neonates. According to Schwan *et al.* (2000), *Salmonella* Enteritidis and *S.* Typhimurium, which generally tend to cause localized infections in humans, such as mild gastroenteritis, do not survive well in human macrophages when tested *in vitro*. The multiplication in the number of internalized cells within the macrophage also suggests that the strains did not have a cytotoxic effect on the phagocytic cells. In contrast, the reduction observed with *A. baumannii* ATCC 17978 (1440) and 1099 could be attributed to either an activation of phagocytic killing or a programmed death (Wilson *et al.*, 2002). Explaining this and understanding the mechanism underlying the persistence within macrophages are areas for future research.

Establishing an infection in the small intestinal tract requires invasive and immune evasion capabilities. The different mechanisms potentially contributing to intestinal infections in neonates was investigated in this study and provided a novel insight into the pathogenicity of *Acinetobacter*. A human colonic adenocarcinoma cell line (Caco-2) was used to elucidate the interaction of *Acinetobacter* spp. with the complex epithelia similar to that of the gut (Bolton *et al.*, 2000). The *in vitro* infection assays were optimized and the findings obtained provide clear evidence that *Acinetobacter* not only have the ability to adhere to and invade the intestinal epithelial cells but also demonstrate that these capacities are widespread across the *Acinetobacter* genus and not restricted to strains from clinical sources. Furthermore, the pathogenic advantage some strains have over others was shown by their persistence inside the epithelial cells. Human macrophages U937 were also used to determine the interaction with phagocytic cells. The results represent an original contribution to our knowledge about the fate of the *Acinetobacter* as a consequence of encountering a major player in the innate immune defense system. To our knowledge, this study is also the first in the field to characterize the pathogenic potential of strains isolated from desiccated milk formulas (1182 and 1183). The implications of this research support and deepen our concern regarding the risk of the neonatal exposure to *Acinetobacter* through the ingestion of contaminated feed.

CHAPTER 8. DETECTION OF PHOSPHOLIPASE GENES: PRELIMINARY GENETIC ANALYSIS

8.1 INTRODUCTION

In the previous Chapter, *Acinetobacter* exhibited a number of virulence traits including adherence to the intestinal (colonic) epithelial cells, invasion, and associated cytotoxicity. The latter can be defined as the damage inflicted on the host cells during the infection process. Such an effect may be direct by the impairment of the functionality of the cell or by causing it to die (Wilson *et al.*, 2002). On the other hand, indirect damage can be caused by the over-induced or prolonged host responses such as the overexpression of pro-inflammatory cytokines in response to, for example, survival and growth of the bacterium (Wilson *et al.*, 2002). The first category is represented by exotoxins which are exported by some disease causing organisms or released on cell lysis (Wilson *et al.*, 2002). The exposure to these toxins produces an array of pathogenic effects that is responsible for the symptoms of the diseases (Hacker and Heesemann, 2002) which vary in severity depending on the activity of the toxin involved. Membrane disturbing toxins for instance may enzymically cause membrane damage either by acting as pore-forming toxins or hydrolases (Wilson *et al.*, 2002). The second category includes enzymes, such as lecithinases and phospholipases (A, C, and D), which degrade the phospholipids of the cell membrane and thus destroys its integrity (Hacker and Heesemann, 2002).

Bacterial phospholipases comprise a range of proteins classified based on the site of action on the phospholipid molecule (Wilton and Waite, 2002). The phospholipases A (PLAs) hydrolyses the acyl group and are categorised according to the position of the acyl ester group hydrolysed (l or 2-acyl ester; PLA1 and PLA2). Phospholipase B hydrolyses both acyl groups. Phospholipase C (PLC), on the other hand, cleaves the glycerophosphate bond of the phospholipids while the phospholipase D (PLD) catalyzes the hydrolysis of the phosphatidylcholine to phosphatidic acid and choline (Exton, 1997; Jones *et al.*, 1999; Waite, 1999; Wilton and Waite, 2002).

The activities of the phospholipases indicate their importance as virulence factors of many bacterial pathogens (Titball, 1998). Phospholipase A2 has been implicated in the inflammation and mucosal damage associated with peptic ulcer formation caused by *Helicobacter pylori* (Langton and Cesareo, 1992). The colonisation of the gastric tissues of

mice infected with this organism has also been associated with the presence of the PLA gene (Dorrell *et al.*, 1999). Phospholipase C protein has also been involved in the virulence of many pathogenic bacteria most notably *Clostridium perfringens* (α toxin), *Listeria monocytogenes*, and *Pseudomonas aeruginosa* (Songer, 1997; Titball, 1998, & 1993). The necrotic and cytolytic activity of the enzyme (α toxin) produced by *C. perfringens* is a characteristic trait of this pathogen (Boquet *et al.*, 1998). The expression of the acidic phospholipase C type H has also been linked to the strong hemolytic activity produced by *P. aeruginosa* (Vasil *et al.*, 2009). Furthermore, PLC mediates the escape of *L. monocytogenes* from the phagosomes in the macrophages (Camilli *et al.*, 1993; Poussin *et al.*, 2009) and in the epithelial cells (Grundling *et al.*, 2003; Marquis *et al.*, 1995) hence contributing to the organism's intracellular existence. Another phospholipase protein with a major role in the pathogenesis of bacteria is the phospholipase D. It contributes to the hematogenous dissemination of *Corynebacterium pseudotuberculosis* within infected animals (Batey, 1986) as well as the macrophage death by this pathogen (McKean *et al.*, 2007). The involvement of this exotoxin in the invasion of epithelial cells has also been observed with *Neisseria gonorrhoeae* during the infection of cervical epithelial cells where it was required for the adherence and potentiation of bacterial internalisation by the host cell via the membrane ruffling mechanism (Edwards *et al.*, 2003).

Although genes encoding for phospholipases A, C, and D have been found in sequenced genomes of *A. baumannii* (AB0057, AB900, AB307-0294, AYE, ACICU, SDF, and ATCC 17978) (Adams *et al.*, 2008; Antunes *et al.*, 2011c; Camarena *et al.*, 2010; Jacobs *et al.*, 2010a; Vallenet *et al.*, 2008), the role of phospholipases in the virulence of *Acinetobacter* has received little attention. One of the earliest studies concerning these enzymes in acinetobacters was conducted by Lehmann (1973) who described the ability of phospholipase C to release 69% of the red cell membrane-bound acid soluble phosphorus by hydrolysing the sphingomyelin, phosphatidylethanolamine and lecithin of the cell membrane (Lehmann, 1973). The enzyme also contributes to the cytotoxicity of *A. baumannii*. A deletion mutant for this protein (PLC1) has been shown to decrease the cytotoxic effect on the FaDu epithelial cells (Camarena *et al.*, 2010). The PLD protein has also been recognised as a virulence factor of the wild type *A. baumannii* as it is believed to be involved in both epithelial cell invasion and human serum proliferation (Jacobs *et al.*, 2010a).

Given the role of bacterial phospholipases in the pathogenicity of *A. baumannii* and other pathogens in terms of facilitating their invasion, persistence, and collectively circumventing the host defenses, it was of interest to explore the presence of phospholipase genes in *Acinetobacter* strains, using a PCR detection analysis. This examination could potentially be correlated to the invasion ability exhibited previously by those strains which may serve as a prospect for future in-depth virulence studies.

8.2 MATERIALS & METHODS

8.2.1 Bacterial strains and culture preparation

A. baumannii (1099, NCTC 19606^T/1102, 1111, 1183, 1440/ATCC 17978), *A. calcoaceticus* (418, and 1097), and *Acinetobacter* gensp. 3 (415, and 1182) that were involved in the virulence *in vitro* studies **(Chapter 7)** were also included for the genetic screening. *A. baumannii* strain ATCC 17978/ NTU1440 (accession numbers GenBank: CP000152 and Refseq: NC_009085) was used as a positive control for the validity of the screening procedure. This strain was isolated from a fatal case of meningitis in a 4 month old infant (Smith *et al.*, 2007). Bacterial cultures were grown in TSB at 37°C for 18h before the DNA extraction.

8.2.2 Isolation of genomic DNA

Pure DNA extraction was achieved using the GenElute^{TM} Bacterial Genomic DNA Kit (Sigma-Aldrich, NA2120). The manufacturer's protocol was followed. Overnight *Acinetobacter* cultures (10^8-10^9 cfu/ml) were centrifuged for 2 min at 12000g. The supernatant was discarded and the pellet was resuspended in 180µl Lysis Solution T (Sigma-Aldrich, B6678). For RNA-free genomic DNA, 20µl RNase Solution (Sigma-Aldrich, R6148) was added, mixed, and then incubated at room temperature for 2 min. Twenty microliters of 20mg/ml Proteinase K Solution (Sigma-Aldrich, P2308) was added and mixed with the sample before 30min incubation at 55°C. To lyse bacterial cells, the added Lysis Solution C (Sigma-Aldrich, B8803) was mixed thoroughly for 15 sec using vortex mixing. The mixture was then incubated at 55°C for 10 min. The following step required adding 500µl of the Column Preparation Solution (Sigma-Aldrich, C2112) to a pre-assembled GenElute Miniprep Binding Column (Sigma-Aldrich, C9471), seated in a 2ml Collection Tube (Sigma-Aldrich, T7813), followed by 1 min centrifugation at 12000g. The eluate was discarded and 200µl of 100% ethanol was added to the lysate which was then mixed thoroughly by vortexing for 10 sec. The contents of the tube were transferred into the Binding Column, using a wide bore pipette tip to reduce shearing the DNA, and then centrifuged at

6500g for 1 min. The collection tube containing the eluate was discarded and the column was placed in a new 2ml Collection Tube. For washing, 500µl Wash Solution 1 (Sigma-Aldrich, W0263) was added to the column and centrifuged at 6500g for 1 min. The collection tube containing the eluate was again discarded and the column was placed in a new 2ml Collection Tube. 500µl Wash Solution 1 was added to the column, for the second washing, and centrifuged for 3 min at 12000g to dry the column. If residual ethanol was seen, then the column would be centrifuged for an additional 1 min at 12000g. The Collection Tube containing the eluate was discarded and the column was placed in a new 2ml Collection Tube. Next, to elute the DNA, 200µl of the Elution Solution (Sigma-Aldrich, B6803) was pipetted onto the centre of the column. This was then incubated at room temperature for 5 min before centrifuged for 1 min at 6500g. The same elution step was repeated for a second time in the same Collection Tube in order to improve the yield. For short-time storage, the eluate containing the genomic DNA was stored at 4^{o}C.

8.2.3 Targeted genes and primer design

A set of 3 phospholipase genes was selected based on their presence in the sequenced *A. baumannii* strain ATCC 17978. The nucleotide sequences of the desired genes were obtained from the National Center for Biotechnology Information using the Refseq accession number (NC_009085) and the name of the gene. The primers were then designed and provided by Sigma-Aldrich. The details for the selected genes are presented in **Table 8.1**.

Table 8.1 Details of the phospholipase genes selected for PCR analysis in *Acinetobacter*.

Gene	Accession Number	Gene ID	Primer	Sequence (5' - 3')	Annealing Temp (^{0}C)
Phospholipase A1 precursor *	A1S_1919	4920063	pla-F	GCCGACACCTTAGCCCCTGT	66.4
			pla-R	TCCGCGCAGCTTGCCACTAA	
Phospholipase C *	A1S_2055	6234072	plc-F	AACCCAGCAACGGCAGACCG	68.0
			plc-R	GACCCAACCGCCACGACTCC	
Putative phospholipase D *	A1S_2989	4920117	pld-F	GGTTGCGCTTATTGGTGGGCG	59.3
			pld-R	ACGCCGAACGTGGGTCAAAGT	

* (Iacono *et al.*, 2008; Smith *et al.*, 2007).

8.2.4 Polymerase chain reaction (PCR) procedure

The PCR mix consisted of: 5µl of 5× GoTaq Green Flexi buffer (Promega, M891A), 5µl of 25mM $MgCl_2$ (Promega, M351B), 1µl of deoxynucleoside triphosphate (dNTP) mix (10mM each) (Promega, U1330), 2.5µl of each primer, 0.25µl of 1x GoTaq DNA polymerase (Promega, M830B), 1µl DNA samples, and 8.75µl of nuclease free water. To generate the amplicons of interest, the following settings for the polymerase chain reactions (PCR) were applied: Initial denaturation 95°C for 5 min followed by 95°C for 1 min, annealing ranged (according to the probes) from 59.3 to 68.0°C for 1 min, and extension at 72°C for 1 min, final extension at 72°C for 5 min. The numbers of cycles of these PCRs were 35. To separate the DNA fragments into different band sizes, agarose gel electrophoresis was performed. One percent of agarose (Sigma-Aldrich, A9539) was prepared in 1X TAE buffer from a 50X ready-made TAE buffer (Fisher Scientific, FERB49), and then heated up until completely dissolved. 10% SYBR safe stain (Sigma-Aldrich, S9430) was added to the agarose and mixed. The agarose gel was poured and left to set before loading 4µl of the DNA samples as well as the 1kb ladder (Promega, M7541). The gel was allowed to run at 100V for 40 min. Finally, the amplified products were visualised under the UV light and photo images were captured.

8.3 RESULTS

The results of the PCR detection of the phospholipase genes in the 9 *Acinetobacter* strains are summarised in **Table 8.2**. **Figure 8.1** showed that the gene encoding for the PLA protein (A1S_1919) was observed in 6 of the *Acinetobacter* strains. Exceptions were *Acinetobacter* gensp. 3 (415), *A. calcoaceticus* (418), and 1182 (*Acinetobacter* gensp. 3). The *plc* gene was detected in all the strains apart from 415 and 418 **(Figure 8.2)**. Strains 415, 418, and 1182 also appeared to be negative for the *pld* gene (A1S_2989) **(Figure 8.3)**.

Table 8.2 Detection of phospholipase genes in *Acinetobacter* strains.

Gene	*Acinetobacter* strains								
	1099	1102	1111	1183	1440	415	1182	418	1097
Phospholipase A1 precursor	+	+	+	+	+	-	-	-	+
Phospholipase C	+	+	+	+	+	-	+	-	+
Putative phospholipase D	+	+	+	+	+	-	-	-	+

Strains 1099, 1102, 1111, 1183, and 1440 are *A. baumannii*; 415 and 1182 are *Acinetobacter* gensp. 3; 418 and 1097 are *A. calcoaceticus*.

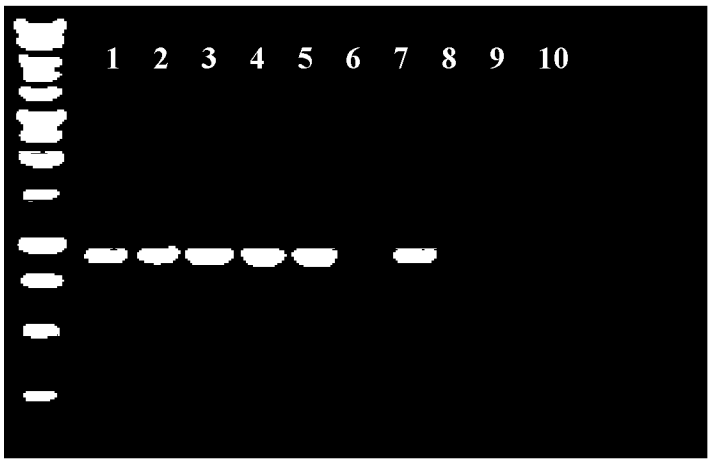
Figure 8.1 Detection of phospholipase A gene.

Lane 1 in the agarose gel represents the positive control 1440 (*A. baumannii* ATCC 17978). Lane 2: strain 1099. Lane 3: strain 1102. Lane 4: strain 1111. Lane 5: strain 1182. Lane 6: strain 1183. Lane 7: strain 1097. Lane 8: strain 415. Lane 9: strain 418. Lane 10: the negative control (with no bacterial DNA). Strains 1099, 1102, 1111, 1183, and 1440 are *A. baumannii*; 415 and 1182 are *Acinetobacter* gensp. 3; 418 and 1097 are *A. calcoaceticus*.

Figure 8.2 Detection of phospholipase C gene.

Lane 1 in the agarose gel represents the positive control 1440 (*A. baumannii* ATCC 17978). Lane 2: strain 1099. Lane 3: strain 1102. Lane 4: strain 1111. Lane 5: strain 1182. Lane 6: strain 1183. Lane 7: strain 1097. Lane 8: strain 415. Lane 9: strain 418. Lane 10: the negative control (with no bacterial DNA). Strains 1099, 1102, 1111, 1183, and 1440 are *A. baumannii*; 415 and 1182 are *Acinetobacter* gensp. 3; 418 and 1097 are *A. calcoaceticus*.

Figure 8.3 Detection of phospholipase D gene.



Lane 1 in the agarose gel represents the positive control 1440 (*A. baumannii* ATCC 17978). Lane 2: strain 1099. Lane 3: strain 1102. Lane 4: strain 1111. Lane 5: strain 1183. Lane 6: strain 1182. Lane 7: strain 1097. Lane 8: strain 415. Lane 9: strain 418. Lane 10: the negative control (with no bacterial DNA). Strains 1099, 1102, 1111, 1183, and 1440 are *A. baumannii*; 415 and 1182 are *Acinetobacter* gensp. 3; 418 and 1097 are *A. calcoaceticus*.

8.4 DISCUSSION

The PCR method was used in this study to screen for three phospholipase genes in three different species of *Acinetobacter* that were isolated from clinical and non-clinical sources (*A. baumannii*, *A. calcoaceticus*, and *Acinetobacter* gensp. 3). Those obtained from food samples; *Acinetobacter* gensp. 3 (415), and *A. calcoaceticus* (418); did not appear to have the *plc* gene. Strain 418, in particular, was also unable to invade the epithelial Caco-2 cells as described in the previous chapter (**7**). Camarena *et al.* (2010) has shown that a phospholipase C (*plc*; A1S_0043) mutant decreased the cytotoxic effect of *A. baumannii* ATCC17978 on FaDu epithelial cells. The wild type, on the other hand, caused the host cell monolayer to become permeable before undergoing extensive detachment and eventually death after 18h of infection. This is consistent with previous reports describing several physiological and morphological changes, such as cell shrinkage, monolayer detachment, and loss of epithelial cell viability due to colonization by *A. baumannii* (Choi *et al.*, 2005; Lee *et al.*, 2001). However, according to results of the MTT assay in **Chapter 7**, the viability loss of Caco-2 cells infected with both the strains positive and negative for this gene was similar. Vallenet *et al.* (2008) stated that the hemolytic activity of the *plc* genes in *A. baumannii* could not be conclusively confirmed. This is since, on one hand, the sequences of two 50% identical copies of *plc* genes found in the genome of *A. baumannii* AYE and SDF are best matched with PLC-N encoded by the *plcN* gene which does not have any hemolytic activity as opposed to its counterpart PLC-H encoded by the *plcS* gene which is strongly hemolytic (Titball, 1993). These proteins, on the other hand, have a pI lower than seven which is a PLC-H feature. Adams *et al.* (2008) also identified two PLC encoding genes (A1S_0043 and A1S_2055) that shared 72% similarity (Camarena *et al.*, 2010) and were present in all the clinical *A. baumannii* strains (AB0057, AB900, AB307-0294, AYE, ACICU, and ATCC 17978) but absent from the non-pathogenic soil bacterium *A. baylyi* ADP1 (Adams *et al.*, 2008). These two genes have 75% homology with those from *Burkholderia pseudomallei* (Rossier and Cianciotto, 2005). One of the two *plc* genes (*plc*-2) carried by *Burkholderia pseudomallei* contributes significantly to the cytotoxicity towards HeLa cells (cell membrane damage) compared to the minor activity of *plc*-1 (Korbsrisate *et al.*, 2007).

Examination of the *Acinetobacter* strains did not indicate the presence of the genes encoding for the PLA (A1S_1919) and PLD proteins (A1S_2989) in strains 415, 418, and 1182. Jacobs *et al.* (2010a) revealed that the A1S_2989 gene contributes to the ability of *A. baumannii* to colonise the pericardial membrane of infected mice, and to invade the human bronchial epithelial BEAS-2B cells, as well as to proliferate in human serum during infection. Strain 415 and 1182 herein did not appear to have the gene but had an apparent invasion capacity **(Chapter 7)**. One to several copies of *pla* and *pld* have been found in the genomes of *A. baumannii* AYE, SDF, ADP1 (Vallenet *et al.*, 2008) and ATCC 17978 (Jacobs *et al.*, 2010a). In the *A. baumannii* AYE genome, one copy was speculated to be necessary for utilizing phosphorous sources while the other three, although possibly active, are likely to be employed only when under certain environmental conditions such as the infection process (Vallenet *et al.*, 2008). The disruption of one *pld* gene (A1S_2989) resulted in the inability to invade (Jacobs *et al.*, 2010a). These multiple copies could presumably be a way of avoiding the host's immune response, which accordingly gives more significance to the activity of this gene during invasion.

In summary, this preliminary study exploring a sample of phospholipases genes showed that all the clinical isolates of *A. baumannii* (1099, 1102, 1111, and 1440) and the follow on formula isolate (1183) harbored the target genes. These were also found in the clinical isolate of *A. calcoaceticus* (1097), but on the other hand, may be lacking from strain 418 obtained from food. Similarly, all the phospholipase genes were not detected in *Acinetobacter* gensp. 3 (415), which is also from a food origin, while the *plc* was present in strain 1182 (follow on formula). Confirmation of the genes' absence may be required. It is, therefore, of interest to investigate this further at the molecular level, and search for trends potentially separating the clinical and non-clinical isolates of various *Acinetobacter* species using larger numbers of strains and various virulence determinants.

CHAPTER 9. CONCLUSION

The nutrient compositions of infant formulas aim to provide the necessary nutrient requirements of growing infants. The Codex Alimentarius Commission, established by the WHO and FAO developed a global safety, quality, and compositional standard for infant formula. However, despite these 'good manufacturing practices' regulations, bacterial contamination of powdered infant formulas remains an issue since sterilization cannot be achieved during production. Together the early stages of the development of the immune system and the intestinal flora as well as the external attenuating factors such as administration of antibiotics, mentioned above, all present neonates in ICUs as a high-risk group among hospitalized patients, due to their increased susceptibility to infections. An important way of preventing exogenous infections is to prevent their exposure to pathogens. In order to control this risk, an analysis of whether the pathogen would be able to cause a potential infection through a specific route of exposure is deemed necessary (FAO-WHO, 2006).

Although the ingestion route has not been epidemiologically linked to acquiring *Acinetobacter* infections, clinical features of gastrointestinal and bloodstream *Acinetobacter* infections has been reported in the literature (Grotiuz *et al.*, 2006; Mader *et al.*, 2010; Mishra *et al.*, 1998; Thom *et al.*, 2010). Some of these reports were unable to identify the contamination source despite comprehensive clinical and environmental surveillance (Mader *et al.*, 2010). Therefore, it was sensible not to exclude the ingestion factor although it was unclear what the feeding methods was and whether these were indeed investigated. This might not always be possible if the potentially contaminated batch is no longer available for analysis. The possibility of neonatal infections resulting from consuming enteral feeds contaminated with *Acinetobacter* have not been explored before and is believed to be worthy of consideration because of the particular vulnerability of the neonates in ICUs. The data presented in this research project began to fill in this knowledge gap and give a new insight into the organism's activities that can potentially result in recognisable pathology locally in the gastrointestinal environment and systematically. Moreover, it provides scientific research evidence that complement the existent reports describing the clinical manifestations of the relevant *Acinetobacter* infections. This research work also highlights the wider picture of the risk imposed on the neonate right from the persistence of *Acinetobacter* in desiccated infant formula, the consumption of the contaminated feed, and the multiplication of the bacterial

cells inside the enteral feeding tubes, followed by gastrointestinal survival and finally consequences of the interaction with the epithelial cells and the response to an immune mechanism. This understanding provides a critical platform for future work where the molecular and genetic mechanisms underlying different aspects of interaction between the bacterium and the host during the gastrointestinal infection process can be elucidated.

The knowledge of risk factors presented by this work scientifically justifies and supports the inclusion of *Acinetobacter* in the list of pathogens posing a risk to neonates (Food and Agriculture Organization-World Health Organization FAO-WHO, 2006). It also stresses the importance of following strict hygienic practices by health-care personnel when preparing, handling, and storing of the powdered infant formula feeds as a means of controlling potential *Acinetobacter* infections in NICUs. Although the intrinsic susceptibility of the neonates may not be controlled, what can be done to prevent potential *Acinetobacter* infections is to reduce the risks of exposure to this organism. For safe consumption of powdered infant formula, the guidelines issued by the Codex Alimentarius Commission (2008) state the use of sterilized equipment for the preparation of the powdered infant formula feed and that that feed should be rehydrated at 70°C or above and only be prepared once needed. Feeds should never be stored in the fridge for longer than 24 hours must also be thrown away if not consumed within two hours. To prevent heavy colonization and subsequent invasive diseases in high-risk infants, avoiding the long placement of enteral feeding tubes is also recommended. However, since it can be costly to replace these tubes very frequently, the risk control target has to be the quality of the feeds given enterally.

In summary, the contributions of this research work to the existing knowledge of *Acinetobacter* are represented by the following:

1. Developed, and evaluated the DDA medium and other *Acinetobacter* selective-differential media.
2. First to study the persistence of clinical and food isolates of *Acinetobacter* strains in desiccated infant formula and to demonstrate their prolonged survival over 2 years period.
3. Determined the ability of the organism to form biofilms on nasogastric feeding tubes as a risk factor for repeated and increased contamination.

4. First to demonstrate the *Acinetobacter* survival in simulated gastrointestinal conditions that can influence the probability of infection.
5. First to examine the adherance of *Acinetobacter*, to human colonic epithelial cells, and the consequent invasion using optimised assays.
6. Established the effect of the infection duration and MOI on the attachment and invasion levels of *Acinetobacter*.
7. Showed the moderate levels of cytotoxicity of invasive *Acinetobacter*.
8. First to prove the capacity of some *Acinetobacter* strains to survive inside the colonic epithelial cells as a consequence of the invasion process.
9. First to demonstrate the persistence and replication within macrophages as a means of evading the adaptive immune response by the host.
10. Detected the presence of genes encoding phospholipases in all the clinical isolates tested.

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