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MODELING DIARRHEAGENIC *E. coli* INFECTIONS AND CO-INFECTIONS: SPECIFIC ROLES OF DIET AND PATHOGEN

by

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS OF DOCTOR OF PHILOSOPHY (PHD), IN MICROBIOLOGY

in the

DEPARTMENT OF MICROBIOLOGY

SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES

at the

UNIVERSITY OF VENDA

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MARCH 2020

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TABLE OF CONTENTS

Page

Declaration viii

Dedication ix

Acknowledgements x

Abstract xii

List of Abbreviations xv

List of Figures xix

List of Tables xxi

Chapter 1: General Introduction 1

1.1. Introduction 1

1.2. Study Rationale 3

1.3. Research questions 5

1.3. Objectives of The Study 5

1.3.1. Primary Objective 5

1.3.2. Secondary Objectives 5

1.5. References 6

Chapter 2: Literature Review 15

2.1. *Escherichia coli* 15

2.2. Enteropathogenic *E. coli* 16

2.2.1. EPEC Pathogenesis Mechanism 16

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2.2.2. *In vitro* Models of EPEC....................19

2.2.3. *In Vivo* Models of EPEC....................19

2.3. Enterotoxigenic *E. coli* 22

2.3.1. ETEC Pathogenesis Mechanism 23

2.3.2. *In Vitro* Models of ETEC 25

2.3.3. *In Vivo* Models of ETEC 25

2.4. Enteroaggregative *E. coli* 28

2.4.1. EAEC Pathogenesis Mechanism 28

2.4.2. .*In Vitro* EAEC Studies....................29

2.4.3. *In Vivo* EAEC Studies....................30

2.5. Occurrence of Co-infections 31

2.5.1. Models of Co-infections 32

2.5.1.1. Diarrheagenic *E. coli* co-infection models....................33

2.6. Inflammatory Responses 34

2.6.1. Faecal Biomarkers of Inflammation 36

2.6.1.1. Lipocalin-2 (LCN-2) 36

2.6.1.2. Myeloperoxidase (MPO) 37

2.6.2. Acute-Phase Systemic Response 38

2.6.2.1. Serum Amyloid A 38

2.6.2.2. C-Reactive Protein 39

2.7. Metabolomics 40

2.8. Summary of Literature Review 41

2.9. References 44

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Chapter 3. Objective 1: To Develop an Infectious EPEC Murine Model Using C57BL/6 Mice Treated with Antibiotic Cocktail 75

3.1. Abstract 75

3.2. Introduction 76

3.32. Materials and Methods 77

3.3.1. Animal Husbandry 77

3.3.2. Rodent Diet and Antibiotics 78

3.3.3. Preparation of EPEC For Mouse Infection 78

3.3.4. Stool Shedding and Tissue Burden 80

3.3.5. EPEC Adherence on the Intestine 80

3.3.6. Intestinal Inflammatory Response 81

3.3.7. Systemic Inflammation 81

3.3.8. ^{1}H NMR Spectroscopy Based Metabolic Profiling 82

3.3.9. Statistical Analysis 83

3.4. Results 83

3.4.1. EPEC Infection Leads to Weight Loss and Diarrhoea 83

3.4.2. Shedding and Tissue Burden in Weaned Mice Infected with EPEC 85

3.4.3. Infection Leads to Disruption of Epithelial Function and Acute Systemic Response 88

3.4.4. EPEC Derived Metabolic Perturbations 93

3.5. Discussion 96

3.6. References 102

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Chapter 4. Objective 2: Determine the Interaction Between EPEC AND ETEC Co-infections 112

4.1. Abstract 112

4.2. Introduction 113

4.3. Materials and Methods 114

4.3.1.*In Vitro* Analysis 114

4.3.1.1. HCT-8 Cells growth 114

4.3.1.2. Preparation of Bacterial Strains and Infection on HCT-8 Cells..........................115

4.3.1.3. Measurement of IL-8 levels..116

4.3.1.4. DNA extraction and Real-Time PCR 116

4.3.1.5. RNA extraction and Real-Time PCR 116

4.3.2. *In Vivo* Analysis 118

4.3.2.1. Animal Husbandry 118

4.3.2.2.Rodent Diet and Antibiotics 118

4.3.2.3. Preparation of Bacterial Strains and Mice Infection 118

4.3.2.4. Stool Shedding and Tissue Burden 119

4.3.2.5. Intestinal Inflammatory Responses 119

4.3.2.6. Systemic Inflammation 120

4.3.2.7. RNA Extraction and Real-Time PCR................. 120

4.3.3. Statistical Analysis 120

4.4. Results 121

4.4.1. EPEC and ETEC *In Vitro* Analysis 121

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4.4.1.1. Adherence and Inflammatory responses in HCT-8 cells co-infected with P/T strains 121

4.4.1.2. Expression of EPEC and ETEC virulence genes in HCT-8 cells....................122

4.4.2. EPEC and ETEC *In Vivo* Analysis 124

4.4.2.1. Clinical Outcomes of C57BL/6 mice Infected with P/T Co-infections 124

4.4.2.2. Stool Shedding and Tissue Burden of Mice Infected with P/T Co-infections 126

4.4.2.3. Inflammatory responses in mice co-infected with EPEC and ETEC strains....................128

4.4.2.4. Expression of EPEC and ETEC virulence genes *In Vivo* 130

4.5. Discussion 134

4.6. References 139

Chapter 5. Objective 3: Determine the Interaction Between EAEC and EPEC Co-infections 148

5.1. Abstract 148

5.2. Introduction 149

5.3. Materials and Methods 150

5.3.1. *In Vitro* Analysis 150

5.3.1.1. HCT-8 Cells Growth 150

5.3.1.2.Preparation of Bacterial Strains and Infection on HCT-8 cells 150

5.3.1.3. Measurement of IL-8 levels... 151

5.3.1.4. HeLa Cells Growth 151

5.3.1.5. Preparation of Bacterial Strains and Infection on HeLa Cells 152

5.3.1.6. DNA Extraction and Real-Time PCR 153

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5.3.1.7. RNA Extraction and rea;-time PCR............................ 153

5.3.1.8. Adherence Assay Using Immunofluorescence Microscopy 153

5.3.2. *In Vivo* Analysis 154

5.3.2.1. Animal husbandry...154

5.3.2.2. Rodent Diet and Antibiotics 154

5.3.2.3. Preparation of Bacterial Strains and Mice Infection 154

5.3.2.4. Stool Shedding 155

5.3.2.5. Intestinal Inflammatory Response 155

5.3.3. Statistical Analysis 155

5.4. Results 156

5.4.1. EAEC and EPEC *In Vitro* Analysis 156

5.4.1.1. Adherence and Inflammatory Responses in HCT-8 cells Infected with A/P Co-infections 156

5.4.1.2. Adherence Traits of A/P Co-infections on HeLa Cells 157

5.4.1.3. Expression of EPEC Virulence Genes on HCT-8 Cells 160

5.4.2. EAEC and EPEC Co-infections *In Vivo* Analysis...161

5.4.2.1.Clinical Outcomes of Mice Infected with A/P Co-infections 161

5.4.2.2. Stool Shedding of A/P co-infected mice 163

5.4.2.3. Biomarkers of Inflammatory Responses in Mice Infected with A/P Co-infections 164

5.5. Discussion 166

5.6. References 170

Chapter 6. Summative Comments and Recommendations 179

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6.1. Summative Comments 179

6.2. Limitations 182

6.3. Recommendations and Future Studies 182

6.4. References 183

Appendix...186

Appendix A: Supplementary Data for Chapter 3...185

Appendix B: Lists of Abstracts and Conferences Attended...........................188

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DECLARATION

I SOLANKA ELLEN LEDWABA (Student Number: 11640355), declare that the thesis hereby submitted to the University of Venda for the degree PhD (Microbiology) has not previously in its entirety or in part been submitted to any university for a degree. I am the sole author of the abstract, introduction, literature review and summative comment sections.

Signed ______________ , on the 3rd day of March 2020

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DEDICATION

I dedicate this work to my loving family

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ACKNOWLEDGEMENTS

First of all, I would like to thank God my saviour for being there for me and helping me survive the hard times that I have encountered throughout my life until this moment.

I would like to express my sincere gratitude to my promoter Prof Pogieter for being my mentor throughout my postgraduate studies. Thank you for your guidance, advice, support and allowing me to carry-out my research. Thank you for always believing in me and grooming me as a young upcoming researcher.

I also would like to express sincere gratitude to Prof Traore-Hoffman for being my co-promoter and offering me words of encouragement and always having faith in me.

I express my profound gratitude to Dr Guerrant for allowing to be my co-promoter and taking me under his wing during my stay in USA. Thank you for believing in me and allowing me to carry-out my co-infections study using the animal models in your lab. Your words of encouragement and support throughout my research project and assistance with the manuscript preparations have greatly shaped my work. Thank you for going an extra mile for me to obtain funds to finish my project. It was a great pleasure working under you.

I would like to express my sincere gratitude to Dr Nataro for also allowing me to work in his lab and carryout my co-infection projects. Thank you for your advice throughout the project and contributing greatly to my manuscript. I am truly thankful for your support and for offering funds to extend my stay in USA in order to achieve my goals. It was an honour working under you and believing in a young researcher like me.

I am grateful to Dr AE Santiago for her supervision of my daily work in the cell culture. Thank you for your assistance in designing the experiments.

I am also grateful to Mr DT Bolick for being my mentor throughout the animal studies and assisting in manuscript preparations. Thank you for your patience and taking time to teach me animal work, offering technical support and interpretation of results. I also thank you

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for assisting with my experiments especially those that required working late nights and during the weekends. You have always been there for me in my academic and non-academic life. Your advice, support and training have really shaped my work and my path as a researcher.

It was a great honour to be the recipient of the Global infectious disease research training (GIDRT) program. This fellowship program gave me the opportunity to travel to USA and work under Dr Guerrant and Dr Nataro at University of Virginia. The program has provided extensive training on cell cultures and animal models and this has enabled me to carry out my co-infections project. Thank you to all the committee members that were part of the GIDRT program, their continuous advice has had a great impact in my study.

Thank you to the National Research foundation (NRF) scholarship for offering me funding to carry-out my research

A lot of people have taken their time to help with my experiments. I would like to thank everyone from Nataro's lab and Guerrant's lab for welcoming me and for offering their assistance when I needed it. Thank you for making me part of your family and always inviting me to spend time with you after work. Thanks to the following: Dr Jorge Giron for offering advice on the cell culture work and with microscopy. Rachel Smith and Abby Mickey for assisting with interpretation of results and for being great friends. Mike Yan for providing training on HeLa cells. Abraham for helping with immunofluorescence microscopy. Pedro Medeiros Deiziane Costa for helping with the animal work, manuscript preparations and offering support. Dr G Kolling your advice and help in the lab is greatly appreciated.

All the members of the Department of Microbiology, thanks for your technical support.

Lastly, I want to thank my parents (Piet and Lydia Ledwaba) and my siblings (Patricia, Faith, Martina and Juwaki), my nephew Thabiso, my spouse (Mpumelelo) and my son (Kevin) for always offering their unconditional love and words of encouragement. Thanks for always supporting me and not giving up on me. I will forever be thankful.

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ABSTRACT

Diarrhoea is still a major problem worldwide. Enteric pathogens such as Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC) and Enterotoxigenic *E. coli* (ETEC) have been reported to cause diarrhoea in children under the age of 5 years. The incidences of these pathogens are due to factors such as poor water quality, sanitation and hygiene practices. Infections with these pathogens result in diarrhoea and have been reported to result in severe disease outcomes more especially in children under 2 years of age.

EPEC infections have been well studied using *in vitro* analyses, with studies highlighting the adherence traits, proteins and virulence genes involved in pathogenesis and inflammatory responses. EPEC is characterized by localized adherence with microcolony formation at the site of infection. *In vivo* studies have reported on human EPEC infection. However, the current animal models have not been able to replicate clinical outcomes (such as diarrhoea and weigh loss) of EPEC infection similar to humans. Therefore, there is still a need for a suitable small animal model that mimic clinical outcomes of human EPEC infections *in vivo*.

Children living in poor environmental conditions are more susceptible to diarrhoeal pathogens. Furthermore, the incidences of children being exposed to co-infections (more than one pathogen at the same time) is relatively high. The EAEC/EPEC (A/P) and EPEC/ETEC (P/T) co-infections have been increasingly detected in children with and without diarrhoea. It has been suggested that patients infected with these co-infections might result in severe disease outcome than those infected with single pathogens. Pathogens are constantly evolving and the microbe-microbe interaction in the host can result in these pathogens competing for the same niche and thus result in increased virulence. Interaction of co-infections can lead to increased inflammatory responses thus affecting the infected host.

The first objective of this study was to develop an EPEC murine model using weaned C57BL/6 mice that have been pretreated with antibiotic cocktail. Mice were orally infected

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with wild-type (WT) typical EPEC, *bfp*- and *escN* mutant strains. The WT had transient weight loss and wet stools with mucous; and the *bfp*- infected mice also had transient weight loss and bloody stool appearance. Increase in inflammatory biomarkers MPO, LCN-2, CRP, IL-6 and SAA were observed in the WT and *bfp*- infected mice. The mice infected with *escN* mutant did not exhibit any weight changes and the stools were similar to the uninfected mice. Furthermore, no inflammatory biomarkers were observed in mice infected with the *escN* mutant. Metabolic perturbations were observed in WT EPEC infected mice at day 3 post infection with the TCA cycle metabolites (reduced succinate, citrate, fumarate, cis-aconitate) being excreted at lower quantities indicating that the energy production in the infected mice was greatly affected.

The second objective of this study was to determine the interaction between the P/T co-infections using *in vitro* and *in vivo* analyses. *In vitro*, human colorectal tumour 8 (HCT-8) cells were infected with single strains of ETEC, EPEC and both the pathogens and incubated for 3 hours. After infection the cells were analysed for bacterial adherence using real-time PCR. The single strains adhered at the same rate similar to the P/T co-infected cells. IL-8, as a marker of inflammatory response, was measured using ELISA. The results indicated that the P/T co-infected cells had a significant increase in IL-8 response higher than the single infections. The P/T co-infections were further analysed *in vivo* using the EPEC murine model developed in this study. Interestingly, mice infected with P/T co-infections developed severe diarrhoea accompanied with significant increased weight loss and some mice died during the 3-day infection period. The inflammatory responses MPO, LCN-2 and SAA were higher in the co-infected mice indicating a synergistic effect. The *bfp* and *eltA* virulence genes were significantly increased in the P/T co-infections.

The third objective of this study was to determine the interaction between A/P co-infections using *in vitro* and *in vivo* analyses. HeLa cells and HCT-8 cells were infected with EAEC, EPEC and both the pathogens at the same time in order to determine adherence and inflammatory responses. EAEC adherence was higher than EPEC and A/P co-infections adherence. A/P co-infections did not have increased IL-8 response in HCT-8 cells when compared to EAEC alone. The virulence genes involved in EPEC

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adherence and Type 3 Secretion System (*bfp*, *eae*, *tir*, *ler*, *per*, *espB* and *espA*) were significantly reduced in A/P co-infected cells. An interesting adherence trait was observed between the A/P co-infections in HeLA cells, EAEC was found to adhere around EPEC altering the localized adherence pattern. The A/P co-infections were further analysed using the EPEC murine model developed in this study. The A/P infected mice had diminished weight changes and EAEC shedding was enhanced when EPEC was present. Faecal inflammatory biomarkers MPO and LCN-2 in A/P infected mice did not have any additive effect.

The findings of this study contributed significantly to the knowledge of human EPEC infection in weaned C57BL/6 mice, highlighting clinical outcomes, inflammatory responses and metabolic perturbations. Furthermore, this study also highlighted the interaction of P/T and A/P co-infections using *in vitro* and *in vivo* analyses in order to determine the disease severity and outcomes. It was observed in this study that co-infections can result in either synergistic or antagonistic effects. Further studies are therefore, required in order to understand the underlying mechanisms that are involved during co-infections and this can further assist in the development of therapeutic interventions.

Key words: C57BL/6 mice, co-infection, Enteroaggregative *E. coli*, Enteropathogenic *E. coli*, Enterotoxigenic *E. coli*, murine model, diarrhoea, enteropathy, inflammation, metabolic perturbation

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LIST OF ABBREVIATIONS

A/E	Attaching and Effacing lesions
A/P	EAEC and EPEC
A-SAA	Acute-phase serum amyloid A
AAALAC	Assessment and Accreditation of Laboratory Animal Care, International
AA	Aggregative Adherence
AAF	Aggregative adherence fimbria
AggR	Aggregative regulator
ATP	Adenosine triphosphate
Bfp	Bundle forming pili
Caco-2	Colon carcinoma-2
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
Cfa	Colonization factor antigen
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
CRP	C-reactive protein
DA	Diffuse adherence
DAPI	4',6-diamidino-2-phenylindole
DEC	Diarrheagenic *E. coli*
DMEM	Dulbecco’s modified Eagle’s medium

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DNA	Deoxyribonucleic acid
eae	intimin
Esp	EPEC secreted proteins
Esc	EPEC secreted components
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EAST-1	EAEC heat-stable enterotoxin-1
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ELISA	Enzyme-linked immunosorbent assay
F	Forward
FBS	foetal bovine serum
GAA	Guanidinoacetic acid
HCT-8	Human colorectal tumour 8
H&E	Haematoxylin and eosin
Hep-2	Human Epithelial type 2
Ig	Immunoglobulin
IL	Interleukin
LCN-2	Lipocalin-2
LEE	Locus of enterocyte effacement
Ler	LEE-encoded regulator

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LA	Localized Adherence
LAL	Localized-like adherence
LT	Heat-liable enterotoxin
MOI	Multiplicity of infection
MPO	Myeloperoxidase
NFκB	Nuclear factor kappa-light-chain of activated B cells
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal projection to latent structures discrimination analysis
PCA	Principal component analysis
P/T	EPEC and ETEC
Per	Plasmid-encoded regulator
Pi	Post infection
PO	Propylene oxide
qPCR	Quantitative Polymerase Chain Reaction
R	reverse
RNA	Ribonucleic acid
RPMI	Rose Park Memorial Institute
SAA	Serum Amyloid A
SEM	Standard error of the mean
ST	Heat-stable enterotoxin
T2SS	Type 2 secretion system
T3SS	Type 3 section system
TCA cycle	Tricarboxylic acid cycle

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TEM	Transmission Electron Microscope
Tir	Translocated intimin receptor
TJ	Tight junction
T_m	Mixing time
TNF-α	Tumour necrosis factor alpha
USA	United States of America
WT	Wild type

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LIST OF FIGURES

Page

Figure 2.1: Typical EPEC showing localized adherence on HeLa cells following 3-hour infection viewed using Zeiss microscope 17

Figure 2.2: EPEC 4-stage pathogenesis model 18

Figure 2.3: ETEC pathogenesis model...23

Figure 2.4: EAEC aggregative adherence (AA) showing stacked-brick-like pattern on HeLa cells following 3-hour infection viewed using Zeiss microscope...29

Figure 3.1: Schematic presentation of the animal experimental procedure with the age of mice, and the infection cycle...79

Figure 3.2: Change in weight and clinical outcomes of EPEC infection in C57BL/6 mice............85

Figure 3.3: Stool shedding in EPEC infected mice...86

Figure 3.4: Tissue burden in EPEC infected mice...87

Figure 3.5: Adherence on intestinal microvilli..88

Figure 3.6: Biomarkers of intestinal epithelial damage in stools and cecum contents of EPEC infected mice..90

Figure 3.7: Acute phase inflammatory responses...92

Figure 3.8: Metabolomic analysis of urinary specimens collected from age-matched, control and EPEC infected mice..95

Figure 3.9: Proposed model of EPEC infection in weaned C57BL/6 mice................................100

Figure 4.1: Schematic presentation of the experimental procedure of EPEC and ETEC infections in C57BL/6 mice..119

Figure 4.2: Adherence and inflammatory cytokines in P/T infected HCT-8 cells.........................122

Figure 4.3: Relative expression of EPEC virulence genes in HCT-8 cells infected with P/T co-infections...123

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Figure 4.4: Relative expression of ETEC virulence genes in HCT-8 cells infected with P/T co-infections....124

Figure 4.5: Change in weight and survival outcomes of C57BL/6 mice infected with P/T co-infections....126

Figure 4.6: Stool shedding of mice infected with P/T co-infections....127

Figure 4.7: Tissue burden of P/T co-infections....128

Figure 4.8: MPO and LCN-2 measured in stool specimen collected at day 2 pi....129

Figure 4.9: Acute phase inflammatory response of P/T co-infections....130

Figure 4.10: Relative expression of EPEC virulence genes in the ileum....131

Figure 4.11: Relative expression of ETEC virulence genes in the ileal tissue....132

Figure 4.12: Relative expression of EPEC virulence genes measured in the colonic tissue....133

Figure 4.13: Relative expression of ETEC virulence genes measured in the colonic tissue....134

Figure 5.1: Schematic experimental presentation of EPEC, EAEC and A/P co-infections on HCT-8 and HeLa cells....151

Figure 5.2: Schematic experimental design of EPEC, EAEC and A/P co-infections in C57BL/6 mice....155

Figure 5.3: Adherence assay and inflammatory responses in HCT-8 cells infected with A/P co-infections....157

Figure 5.4: Adherence assay in HeLa cells infected with A/P co-infections....158

Figure 5.5: Immunofluorescence microscopy on HeLa cells....159

Figure 5.6: EPEC virulence gene expression on HCT-8 cells infected with A/P co-infections 161

Figure 5.7: Change in weight of C57BL/6 mice infected with A/P co-infections....162

Figure 5.8: Stool shedding of mice infected with A/P co-infections....163

Figure 5.9: Inflammatory biomarkers MPO and LCN-2 in mice infected with A/P co-infections 165

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LIST OF TABLES

Page

Table 4.1: Primer sequence used to detect EPEC and ETEC..116

Table 4.2: Primers used for EPEC virulence gene expression....................................117

Table 4.3: Primers used for ETEC virulence gene expression..118

Table 5.1: Primer sequence used to detect EPEC and EAEC...153

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CHAPTER 1

GENERAL INTRODUCTION

1.1. INTRODUCTION

Gastroenteritis in children is still a major problem globally (Liu et al, 2015) and is estimated to cause 1.3 million deaths in developing countries (Troeger et al, 2017). The Global Burden Disease Study have reported that diarrhoea is the eighth leading cause of deaths among adults, and the fifth leading cause of death among children less than 5 years of age (Troeger et al, 2018). In low-income settings, the incidences of childhood diarrhoea are higher due to factors such as poor water quality, sanitation and hygiene aspects (Brown et al, 2013; Jensen et al, 2017; Cumming et al, 2019). In addition, many of these children have recurrent diarrhoea due to their less developed immune system (Jensen et al, 2017; Fang et al, 2018), and this contributes to the infection-malnutrition cycle (Guerrant et al, 2013; Walson and Berkley, 2018). This cycle in children results in long term effects such as stunted growth, cognitive impairment and metabolic syndromes (Guerrant et al, 2013; Nataro and Guerrant, 2017).

Diarrhoea is caused by enteric pathogens that invade the intestines contributing to several disease outcomes (Navaneethan and Giannella, 2008). There are several known bacterial, parasitic and viral enteric pathogens that cause diarrhoeal infections in children worldwide (Farrar et al, 2013). In developing countries, bacterial pathogens have been reported to mostly infect young children (Kotloff et al, 2017; Platts-Mills et al, 2018). Of these bacterial pathogens, belong to the family of *Escherichia coli (E. coli)*. *E. coli* are harmless commensal strains that colonize the human intestines from birth. However, there are certain *E. coli* strains that have the ability to produce virulence genes resulting

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in intestinal and extra-intestinal infections (Kaper et al, 2004). The Diarrheagenic *E. coli* (DEC) pathotypes are primarily transmitted via faecal-oral route (Farrar et al, 2013) and have been reported to cause diarrhoea accompanied with several clinical outcomes (Kotloff et al, 2017; Platts-Mills et al, 2018; Ledwaba et al, 2018). Infections caused by Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), and Enteroaggregative *E. coli* (EAEC), have been reported to cause moderate to severe diarrhoea in children residing in developing countries (Platts-Mills et al, 2015; Kotloff et al, 2017).

Enteric pathogens are evolving, and for decades studies have reported on the occurrence of dual pathogen infections in both humans and animals (Adhikari et al, 1985; Mathewson et al, 1987; Wada et al, 1996; Albert et al, 1999; Ortega et al, 2017; Dutta et al, 2018; Ledwaba et al, 2018; Mathew et al, 2019). In epidemiological studies, co-infections with more than one pathogen in infected patients have been reported for many years (Adhikari et al, 1985; Vilchez et al, 2009; Bonkoungou et al, 2012; Shrivastava et al, 2017; Dutta et al, 2018; Ledwaba et al, 2018; Mathew et al, 2019). The presence of different pathogens within a host affects the optimal level of the pathogen's virulence (López-Villavicencio et al, 2011). In the infected host, co-infecting pathogens often compete with each other for nutrients resulting in increased virulence. The metabolic waste products of these pathogens are secreted directly into the host, and this alters the metabolic state of the host resulting in increased immune response (Olive and Sassetti, 2016). Epidemiological studies have therefore suggested that patients infected with co-infections are more likely to develop more severe diarrhoeal disease outcomes due to the interaction of more than one pathogen (Vichez et al, 2009; Pereira et al, 2010; Ledwaba et al, 2018; Mathew et al, 2019).

The diagnosis of DEC pathotypes in clinical studies is through biochemical, O:H serotyping and molecular methods (Croxen et al, 2013). In order to better understand the mechanism of the pathogens and interaction with the host, *in vitro* and *in vivo* analysis are considered good models. These models offer great background due to the closed controlled environment. *In vitro* models (using tissue cultures) have been used extensively to study the adherence patterns, virulence traits, the secretion systems and

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immune responses produced during EAEC, EPEC and ETEC infections (Crane et al, 2006; Morin et al, 2010; Liévin-Le Moal et al, 2013; Law et al, 2013). These pathogens have also been studied *in vivo* to further understand the pathogenesis mechanisms and disease outcomes during infection using different animal models (Morin et al, 2010; Bolick et al, 2013; Law et al, 2013; Bolick et al, 2018). Bolick and colleagues recently developed ETEC murine model, showing the importance of LT enterotoxin and infection resulted in clinical outcomes of diarrhoea and weight loss with increased inflammatory responses (Bolick et al, 2018). These models help in understanding human diseases and further assist in development of vaccines (Croxen et al, 2013, Law et al, 2013).

1.2. STUDY RATIONALE

Diarrhoeal diseases are a major problem globally. The incidence of diarrhoeal pathogens is high in low income settings due to factors such as poor water quality, sanitation and hygiene aspects (Brown et al, 2013). Young children are often exposed to various pathogens due to their less developed immune system (Jensen et al, 2017; Fang et al, 2018). Exposure to diarrhoeal pathogens often leads to increased medical costs of infected patients. Diarrhoea is caused by pathogens that enter the intestine leading to disruption of intestinal barrier function, adherence or invasion of microbes into the intestinal lumen, increased inflammatory responses, and damage to the villous resulting in malabsorption of nutrients (Guerrant et al, 2016). This viscous cycle of disrupted gut function is referred to as environmental enteropathy in asymptomatic patients with malnutrition (Guerrant et al, 2016).

DEC pathotypes have been reported to cause moderate to severe diarrhoea in low income settings (Kotloff et al, 2017; Platts-Mills et al, 2018). This study focused on ETEC, EPEC and EAEC pathogens respectively. Studies have shown that children infected with these pathogens result in series of clinical outcomes such as diarrhoea, vomiting and abdominal cramps (Vilchez et al, 2009; Dutta et al, 2018; Ledwaba et al, 2018; Platts-

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Mills et al, 2018). EPEC infections cause infantile diarrhoea and have been associated with deaths in children under the age of 1 year (Kotloff et al, 2017). *In vitro* studies showing the pathogenesis mechanism of EPEC have been reported extensively (Nataro et al, 1985; Nataro et al, 1987; Knutton et al, 1989; Law et al, 2013, Garcia et al, 2016, Sanchez-Villamil et al, 2016; Morampudi et al, 2017). These studies have reported on the distinct adherence traits (Nataro et al, 1985; Nataro et al, 1987; Garcia et al, 2016; Vieira et al, 2019), importance of the Type 3 secretion system (T3SS) and the proteins involved (Cleary et al, 2004), pedestal formation (Knutton et al, 1989), tight junction disruption (Morampudi et al, 2017) and inflammatory responses (Sanchez-Villamil et al, 2016) produced during EPEC infection. *In vivo* studies have also been reported using small animal models (such as piglets, mice and rabbits) and humans in order to understand the pathogenesis mechanism of EPEC (Law et al, 2013). However, these animal models have not been able to fully replicate the clinical aspects as a result of EPEC infections as observed in humans (such as diarrhoea and weight loss). Therefore, this study was aimed to develop an EPEC murine model showing the clinical outcomes and increased inflammatory responses as a result of EPEC infection.

Co-infections commonly occur naturally in the host (Read and Taylor, 2001). Interaction of multiple pathogens within a host is different from single infections. During co-infections, interaction can result in pathogens competing with each other for the same niche resulting in increased virulence, or the pathogens utilizing the same nutrients resulting in a beneficial relationship (López-Villavicencio et al, 2011). Co-infections between bacteria, parasites and viruses have been reported (Wada et al, 1996; Madhi et al, 2000; Manko et al, 2017; Ortega et al, 2017; Reynolds et al, 2017; Dutta et al, 2018; Ledwaba et al, 2018; Lima et al, 2018; Mathew et al, 2019). In clinical studies, DEC pathotypes (such as EAEC, EPEC and ETEC) have been increasingly reported in to occur as co-infections in children (Adhikari et al, 1985; Itoh et al, 1997; Yatsuyanagi et al, 2002; Vilchez et al, 2009; Iijima et al, 2017; Andersson et al, 2018; Dutta et al, 2018; Broujerdi et al, 2018; Ledwaba et al, 2018; Lima et al, 2018). These epidemiological studies have also suggested that patients infected with more than one pathogen at the same time might result in more severe complicated disease outcome. There are studies that have been done to

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understand the pathogenesis mechanisms of co-infections using *in vitro* and *in vivo* models (Crane et al, 2006; Galván-Moroyoqui et al, 2008; Bartelt et al, 2017; Manko et al, 2017; Reynolds et al, 2017). However, very few studies have been done using *in vitro* and *in vivo* analyses in order to understand the interaction of DEC pathotypes co-infections (Wada et al, 1996; Crane et al, 2006; Toledo et al, 2011). This study also examined the interaction of EAEC/EPEC (A/P) and EPEC/ETEC (P/T) co-infections using *in vitro* and *in vivo* analyses, in order to determine whether the interaction will result in a more serious clinical outcome as suggested by clinical studies.

1.3. RESEARCH QUESTIONS

- What is the disease outcome during EPEC infection in C57BL/6 mice pretreated with antibiotics?
- Does this new EPEC infection model result in increased inflammatory responses?
- Do co-infections *in vitro* and *in vivo* result in increased virulence?
- Do co-infections result in severe disease outcome?

1.4. OBJECTIVES OF THE STUDY

1.4.1. PRIMARY OBJECTIVE

- To establish a model of diarrheagenic *E. coli* infections (EPEC model) and co-infections (EAEC/EPEC and EPEC/ETEC) using *in vitro* and *in vivo* analyses

1.4.2. SECONDARY OBJECTIVES

- To develop an EPEC infection murine model

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- To determine the interaction between EPEC and ETEC co-infections using *in vitro* and *in vivo* analyses
- To determine the interaction between EAEC and EPEC co-infections using *in vitro* and *in vivo* analyses

This study used *in vitro* and *in vivo* analysis. In chapter 3, an infectious EPEC murine model was developed using C57BL/6 mice pretreated with antibiotic cocktail. Weaned mice were orally challenged with EPEC in order to determine clinical outcomes, inflammatory responses and metabolic changes. This study also highlighted the importance of EPEC virulence genes involved in adherence and the T3SS. In Chapter 4, interaction of P/T co-infections a were determined using *in vitro* and *in vivo* analyses. *In vitro*, HCT-8 cells were used to determine adherence, inflammatory responses and expression of virulence genes during these co-infections. In Chapter 5, interaction of A/P co-infections were also determined using *in vitro* and *in vivo* analyses. *In vitro*, HCT-8 cells were also used to determine adherence, inflammatory responses and virulence gene expression. Immunofluorescence microscopy was used to study the adherence traits between A/P co-infections using HeLa cells. The EPEC model developed in Chapter 3 was further used to study the interaction between A/P and P/T co-infections *in vivo*. Clinical outcomes, and inflammatory responses were determined during these interactions. This is followed by summative comments of the study objectives.

1.5. REFERENCES

Adhikari, M., Coovadia, Y. and Hewitt, J., 1985. Enteropathogenic *Escherichia coli* (EPEC) and enterotoxigenic (ETEC) related diarrhoeal disease in a neonatal unit. *Annals of Tropical Paediatrics*, **5**(1), 19-22.

© University of Venda

Albert, M.J., Faruque, A.S.G., Faruque, S.M., Sack, R.B. and Mahalanabis, D., 1999. Case-control study of enteropathogens associated with childhood diarrhea in Dhaka, Bangladesh. *Journal of Clinical Microbiology*, **37**(11), 3458-3464.

Andersson, M., Kabayiza, J.C., Elfving, K., Nilsson, S., Msellem, M.I., Mårtensson, A., Björkman, A., Bergström, T. and Lindh, M., 2018. Coinfection with enteric athogens in east african children with acute gastroenteritis—associations and interpretations. *American Journal of Tropical Medicine and Hygiene*, **98**(6), 1566-1570.

Bartelt, L.A., Bolick, D.T., Mayneris-Perxachs, J., Kolling, G.L., Medlock, G.L., Zaenker, E.I., Donowitz, J., Thomas-Beckett, R.V., Rogala, A., Carroll, I.M. and Singer, S.M., 2017. Cross-modulation of pathogen-specific pathways enhances malnutrition during enteric co-infection with *Giardia lamblia* and enteroaggregative *Escherichia coli*. *PLoS Pathogens*, **13**(7).

Bolick, D.T., Medeiros, P.H.Q.S., Ledwaba, S.E., Lima, A.A., Nataro, J.P., Barry, E.M. and Guerrant, R.L., 2018. Critical role of zinc in a new murine model of enterotoxigenic *Escherichia coli* diarrhea. *Infection and Immunity*, **86**(7), e00183-18.

Bolick, D.T., Roche, J.K., Hontecillas, R., Bassaganya-Riera, J., Nataro, J.P. and Guerrant, R.L., 2013. Enteroaggregative *Escherichia coli* strain in a novel weaned mouse model: exacerbation by malnutrition, biofilm as a virulence factor and treatment by nitazoxanide. *Journal of Medical Microbiology*, **62**(Pt 6), 896.

Bonkoungou, I.J.O., Lienemann, T., Martikainen, O., Dembelé, R., Sanou, I., Traoré, A.S., Siitonen, A., Barro, N. and Haukka, K., 2012. Diarrhoeagenic *Escherichia coli* detected by 16-plex PCR in children with and without diarrhoea in Burkina Faso. *Clinical Microbiology and Infection*, **18**(9), 901-906.

Broujerdi, S.M., Ardakani, M.R. and Rezatofighi, S.E., 2018. Characterization of diarrheagenic *Escherichia coli* strains associated with diarrhea in children, Khouzestan, Iran. *The Journal of Infection in Developing Countries*, **12**(08), 649-656.

© University of Venda

Brown, J., Cairncross, S. and Ensink, J.H., 2013. Water, sanitation, hygiene and enteric infections in children. *Archives of Disease in Childhood*, **98**(8), 629-634.

Cleary, J., Lai, L.C., Shaw, R.K., Straatman-Iwanowska, A., Donnenberg, M.S., Frankel, G. and Knutton, S., 2004. Enteropathogenic *Escherichia coli* (EPEC) adhesion to intestinal epithelial cells: role of bundle-forming pili (BFP), EspA filaments and intimin. *Microbiology*, **150**(3), 527-538.

Crane, J.K., Choudhari, S.S., Naeher, T.M. and Duffey, M.E., 2006. Mutual enhancement of virulence by enterotoxigenic and enteropathogenic *Escherichia coli*. *Infection and Immunity*, **74**(3), 1505-1515.

Croxen, M.A., Law, R.J., Scholz, R., Keeney, K.M., Wlodarska, M. and Finlay, B.B., 2013. Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clinical Microbiology Reviews*, **26**(4), 822-880.

Cumming, O., Arnold, B.F., Ban, R., Clasen, T., Mills, J.E., Freeman, M.C., Gordon, B., Guiteras, R., Howard, G., Hunter, P.R. and Johnston, R.B., 2019. The implications of three major new trials for the effect of water, sanitation and hygiene on childhood diarrhea and stunting: a consensus statement. *BMC Medicine*, **17**(1), 1-9.

Dutta, K., Pal, B., Mandal, R., Mullick, S. and Mandal, P., 2018. Circulating rotavirus types and drug-resistant diarrheagenic *Escherichia coli* causing enteric infection in under-five children in rural west Bengal, India. *Journal of Bioengineering and Biomedical Sciences*, **8**(255), 2.

Fang, Y.H., Luo, Y.Y., Yu, J.D., Lou, J.G. and Chen, J., 2018. Phenotypic and genotypic characterization of inflammatory bowel disease in children under six years of age in China. *World Journal of Gastroenterology*, **24**(9), 1035.

Farrar, J., Hotez, P.J., Junghanss, T., Kang, G., Lalloo, D. and White, N.J., 2013. *Manson's Tropical Diseases E-Book*. Elsevier Health Sciences.

© University of Venda

Galván-Moroyoqui, J.M., Del Carmen Domínguez-Robles, M., Franco, E. and Meza, I., 2008. The interplay between Entamoeba and enteropathogenic bacteria modulates epithelial cell damage. *PLoS Neglected Tropical Diseases*, **2**(7).

Garcia, B.G., Tadasuke Ooka, Yasuhiro Gotoh, Mônica AM Vieira, Denise Yamamoto, Yoshitoshi Ogura, Dennys M. Girão et al., 2016. Genetic relatedness and virulence properties of enteropathogenic *Escherichia coli* strains of serotype O119: H6 expressing localized adherence or localized and aggregative adherence-like patterns on HeLA cells. *International Journal of Medical Microbiology,* **306**(3), 152-164.

Guerrant, R.L., DeBoer, M.D., Moore, S.R., Scharf, R.J. and Lima, A.A., 2013. The impoverished gut—a triple burden of diarrhoea, stunting and chronic disease. *Nature Reviews Gastroenterology and Hepatology*, **10**(4), 220.

Guerrant, R.L., Leite, A.M., Pinkerton, R., Medeiros, P.H., Cavalcante, P.A., DeBoer, M., Kosek, M., Duggan, C., Gewirtz, A., Kagan, J.C. and Gauthier, A.E., 2016. Biomarkers of environmental enteropathy, inflammation, stunting, and impaired growth in children in northeast Brazil. *PLoS One*, **11**(9), e0158772.

Iijima, Y., Oundo, J.O., Hibino, T., Saidi, S.M., Hinenoya, A., Osawa, K., Shirakawa, T., Osawa, R. and Yamasaki, S., 2017. High prevalence of diarrheagenic *Escherichia coli* among children with diarrhea in Kenya. *Japan Journal of Infectious Diseases*, **70**, 80-83.

Itoh, Y., Nagano, I., Kunishima, M. and Ezaki, T., 1997. Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable: H10 associated with a massive outbreak of gastrointestinal illness. *Journal of Clinical Microbiology*, **35**(10), 2546-2550.

Jensen, S.K., Berens, A.E. and Nelson 3rd, C.A., 2017. Effects of poverty on interacting biological systems underlying child development. *The Lancet Child and Adolescent Health*, **1**(3), 225-239.

© University of Venda

Kaper, J.B., Nataro, J.P. and Mobley, H.L., 2004. Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*, **2**(2), 123.

Knutton, S., Baldwin, T., Williams, P.H. and McNeish, A.S., 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infection and Immunity*, **57**(4), 1290-1298.

Kotloff, K.L., Platts-Mills, J.A., Nasrin, D., Roose, A., Blackwelder, W.C. and Levine, M.M., 2017. Global burden of diarrheal diseases among children in developing countries: Incidence, etiology, and insights from new molecular diagnostic techniques. *Vaccine*, **35**(49), 6783-6789.

Law, R.J., Gur-Arie, L., Rosenshine, I. and Finlay, B.B., 2013. *In vitro* and *in vivo* model systems for studying enteropathogenic *Escherichia coli* infections. *Cold Spring Harbor Perspectives in Medicine*, **3**(3), a009977.

Ledwaba, S.E., Kabue, J.P., Barnard, T.G., Traore, A.N. and Potgieter, N., 2018. Enteric pathogen co-infections in the paediatric population from rural communities in the Vhembe District, South Africa. *South African Journal of Child Health*, **12**(4), 170-174.

Liévin-Le Moal, V. and Servin, A.L., 2013. Pathogenesis of human enterovirulent bacteria: lessons from cultured, fully differentiated human colon cancer cell lines. *Microbiology and Molecular Biology Reviews,* **77**(3), 380-439.

Lima, A.A., Soares, A.M., José Filho, Q.S., Havt, A., Lima, I.F., Lima, N.L., Abreu, C.B., Junior, F.S., Mota, R., Pan, W.K.Y. and Troeger, C., 2018. Enteroaggregative *Escherichia coli* subclinical infection and coinfections and impaired child growth in the MAL-ED Cohort Study. *Journal of Pediatric Gastroenterology and Nutrition*, **66**(2), 325-333.

Liu, L., Oza, S., Hogan, D., Perin, J., Rudan, I., Lawn, J.E., Cousens, S., Mathers, C. and Black, R.E., 2015. Global, regional, and national causes of child mortality in 2000–13,

© University of Venda

with projections to inform post-2015 priorities: an updated systematic analysis. *The Lancet*, **385**(9966), 430-440.

López-Villavicencio, M., Courjol, F., Gibson, A.K., Hood, M.E., Jonot, O., Shykoff, J.A. and Giraud, T., 2011. Competition, cooperation among kin, and virulence in multiple infections. *Evolution: International Journal of Organic Evolution*, **65**(5), 1357-1366.

Madhi, S.A., Huebner, R.E., Doedens, L., Aduc, T., Wesley, D. and Cooper, P.A., 2000. HIV-1 co-infection in children hospitalised with tuberculosis in South Africa. *International Journal of Tuberculosis and Lung Disease*, **4**(5), 448-454.

Manko, A., Motta, J.P., Cotton, J.A., Feener, T., Oyeyemi, A., Vallance, B.A., Wallace, J.L. and Buret, A.G., 2017. Giardia co-infection promotes the secretion of antimicrobial peptides beta-defensin 2 and trefoil factor 3 and attenuates attaching and effacing bacteria-induced intestinal disease. *PLoS One*, **12**(6).

Mathew, S., Smatti, M.K., Al Ansari, K., Nasrallah, G.K., Al Thani, A.A. and Yassine, H.M., 2019. Mixed viral-bacterial infections and their effects on gut microbiota and clinical Illnesses in children. *Scientific Reports*, **9**(1), 1-12.

Mathewson, J.J., Oberhelman, R.A., Dupont, H.L., de la Cabada, F.J. and Garibay, E.V., 1987. Enteroadherent *Escherichia coli* as a cause of diarrhea among children in Mexico. *Journal of Clinical Microbiology*, **25**(10), 1917-1919.

Morampudi, V., Graef, F.A., Stahl, M., Dalwadi, U., Conlin, V.S., Huang, T., Vallance, B.A., Hong, B.Y. and Jacobson, K., 2017. Tricellular tight junction protein tricellulin is targeted by the enteropathogenic *Escherichia coli* effector EspG1, leading to epithelial barrier disruption. *Infection and Immunity*, **85**(1), e00700-16.

Morin, N., Tirling, C., Ivison, S.M., Kaur, A.P., Nataro, J.P. and Steiner, T.S., 2010. Autoactivation of the AggR regulator of enteroaggregative *Escherichia coli in vitro* and *in vivo*. *FEMS Immunology and Medical Microbiology*, **58**(3), 344-355.

© University of Venda

Nataro, J.P. and Guerrant, R.L., 2017. Chronic consequences on human health induced by microbial pathogens: growth faltering among children in developing countries. *Vaccine*, **35**(49), 6807-6812.

Nataro, J.P., Kaper, J.B., Robins-Browne, R.O.Y., Prado, V., Vial, P. and Levine, M.M., 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. *The Pediatric Infectious Disease Journal*, **6**(9), 829-831.

Nataro, J.P., Scaletsky, I.C.A., Kaper, J.B., Levine, M.M. and Trabulsi, L.R., 1985. Plasmid-mediated factors conferring diffuse and localized adherence of enteropathogenic *Escherichia coli*. *Infection and Immunity*, **48**(2), 378-383.

Navaneethan, U. and Giannella, R.A., 2008. Mechanisms of infectious diarrhea. *Nature clinical practice Gastroenterology and Hepatology*, **5**(11), 637-647.

Olive, A.J. and Sassetti, C.M., 2016. Metabolic crosstalk between host and pathogen: sensing, adapting and competing. *Nature Reviews Microbiology*, **14**(4), 221.

Ortega, A.F., Martínez-Castañeda, J.S., Bautista-Gómez, L.G., Muñoz, R.F. and Hernández, I.Q., 2017. Identification of co-infection by rotavirus and parvovirus in dogs with gastroenteritis in Mexico. *Brazilian Journal of Microbiology*, **48**(4), 769-773.

Pereira, A.L., Silva, T.N., Gomes, A.C., Araújo, A.C. and Giugliano, L.G., 2010. Diarrhea-associated biofilm formed by enteroaggregative *Escherichia coli* and aggregative *Citrobacter freundii*: a consortium mediated by putative F pili. *BMC Microbiology*, **10**(1), 57.

Platts-Mills, J.A., Babji, S., Bodhidatta, L., Gratz, J., Haque, R., Havt, A., McCormick, B.J., McGrath, M., Olortegui, M.P., Samie, A. and Shakoor, S., 2015. Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). *The Lancet Global health*, **3**(9), e564-e575.

© University of Venda

Platts-Mills, J.A., Liu, J., Rogawski, E.T., Kabir, F., Lertsethtakarn, P., Siguas, M., Khan, S.S., Praharaj, I., Murei, A., Nshama, R. and Mujaga, B., 2018. Use of quantitative molecular diagnostic methods to assess the aetiology, burden, and clinical characteristics of diarrhoea in children in low-resource settings: a reanalysis of the MAL-ED cohort study. *The Lancet Global Health*, **6**(12), e1309-e1318.

Read, A. F., and R. H. Taylor. 2001. The ecology of genetically diverse infections. *Science* **292**, 1099-1102.

Reynolds, L.A., Redpath, S.A., Yurist-Doutsch, S., Gill, N., Brown, E.M., van der Heijden, J., Brosschot, T.P., Han, J., Marshall, N.C., Woodward, S.E. and Valdez, Y., 2017. Enteric helminths promote Salmonella coinfection by altering the intestinal metabolome. *Journal of Infectious Diseases,* **215**(8), 1245-1254.

Sanchez-Villamil, J., Tapia-Pastrana, G. and Navarro-Garcia, F., 2016. Pathogenic lifestyles of *E. coli* pathotypes in a standardized epithelial cell model influence inflammatory signaling pathways and cytokines secretion. *Frontiers in Cellular and Infection Microbiology*, **6**, 120.

Shrivastava, A.K., Kumar, S., Mohakud, N.K., Suar, M. and Sahu, P.S., 2017. Multiple etiologies of infectious diarrhea and concurrent infections in a pediatric outpatient-based screening study in Odisha, India. *Gut Pathogens*, **9**(1), 16.

Toledo, C.C., Arvidsson, I. and Karpman, D., 2011. Cross-reactive protection against enterohemorrhagic *Escherichia coli* infection by enteropathogenic *E. coli* in a mouse model. *Infection and Immunity*, **79**(6), 2224-2233.

Troeger, C., Blacker, B.F., Khalil, I.A., Rao, P.C., Cao, S., Zimsen, S.R., Albertson, S.B., Stanaway, J.D., Deshpande, A., Abebe, Z. and Alvis-Guzman, N., 2018. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of diarrhoea in 195 countries: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet Infectious Diseases*, **18**(11), 1211-1228.

© University of Venda

Troeger, C., Forouzanfar, M., Rao, P.C., Khalil, I., Brown, A., Reiner Jr, R.C., Fullman, N., Thompson, R.L., Abajobir, A., Ahmed, M. and Alemayohu, M.A., 2017. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015 *The Lancet Infectious Diseases*, **17**(9), 909-948.

Vieira, M.A., Dias, R.C., dos Santos, L.F., Rall, V.L., Gomes, T.A. and Hernandes, R.T., 2019. Diversity of strategies used by atypical enteropathogenic *Escherichia coli* to induce attaching and effacing lesion in epithelial cells. *Journal of Medical Microbiology*, **68**(6), 940-951.

Vilchez, S., Reyes, D., Paniagua, M., Bucardo, F., Möllby, R. and Weintraub, A., 2009. Prevalence of diarrhoeagenic *Escherichia coli* in children from Leon, Nicaragua. *Journal of Medical Microbiology*, **58**(5), 630-637.

Wada, Y., Nakaoka, Y., Kondo, H., Nakazawa, M. and Kubo, M., 1996. Dual infection with attaching and effacing *Escherichia coli* and enterotoxigenic *Escherichia coli* in post-weaning pigs. *Journal of Comparative Pathology*, **114**(1), 93-99.

Walson, J.L. and Berkley, J.A., 2018. The impact of malnutrition on childhood infections. *Current Opinion in Infectious Diseases*, **31**(3), 231.

Yatsuyanagi, J., Saito, S., Sato, H., Miyajima, Y., Amano, K.I. and Enomoto, K., 2002. Characterization of enteropathogenic and enteroaggregative *Escherichia coli* isolated from diarrheal outbreaks. *Journal of Clinical Microbiology*, **40**(1), 294-297.

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CHAPTER 2

LITERATURE REVIEW

2.1. *Escherichia coli*

E. coli are gram-negative, non-spore forming bacilli that belong to Enterobacteriaceae family (Kaper et al, 2004). *E. coli* are part of the normal flora of the gastrointestinal lining of warm-blooded animals and humans (Kaper et al, 2004). *E. coli* colonizes the mucosal layer of the large intestine within the first few hours following birth and co-exists with humans for a lifetime with mutual benefit. On the other hand, there are certain *E. coli* strains that were reported to be harmful to both humans and animals (such as pigs, calves and sheep) (Hammerum and Heuer, 2009; Dubreuil et al, 2016; Gomes et al, 2016; Sithara et al, 2017). These microbes are able to produce virulence factors resulting in neonatal meningitis, bacteraemia, intestinal and urinary tract infections (Kaper et al, 2004, Sannes et al, 2004; Croxen et al, 2013). These pathogenic strains are based on the virulence properties, serotypes of distinct somatic “O”, Flagella “H”, and Capsule “K” antigens with different mechanisms of pathogenicity and clinical symptoms (Croxen et al, 2013). The intestinal pathogens are referred to as DEC with six distinct pathotypes namely; ETEC, EPEC, Enteroinvasive *E. coli* (EIEC), EAEC, Enterohemorrhagic *E. coli* (EHEC) and Diffusely Adherent *E. coli* (DAEC) (Kaper et al, 2004; Croxen et al, 2013; Gomes et al, 2016). The focus of the study was based on EPEC, ETEC and EAEC pathotypes respectively.

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2.2. ENTEROPATHOGENIC *E. coli*

EPEC infection has been associated with infantile diarrhoea in low income countries (Kotloff et al, 2013; Platts-Mills et al, 2015). EPEC causes acute diarrhoea in children under 2 years of age and especially those of less than 6 months of age (Kotloff et al, 2013). Infection usually occurs due to ingestion of contaminated food and water. It has been reported that the infective dose is 10^8 CFU (Nisa et al, 2013). Clinically, EPEC produces severe and prolonged watery diarrhoea with mucus accompanied by vomiting and fever (Farrar et al, 2013).

2.2.1. EPEC PATHOGENESIS MECHANISM

EPEC expresses a multitude of genes that assist in its pathogenesis mechanism (Guerrant et al, 2011). EPEC strains are closely related to EHEC and *Citrobacter rodentium* (mouse pathogen) and are all characterized by the attaching and effacing (A/E) lesions and actin accumulation at infection site (Mundy et al, 2005; Mundy et al, 2006; Law et al, 2013). The A/E lesions are induced by the large chromosomal pathogenicity island, Locus of enterocyte effacement (LEE) (Frankel et al, 1998). LEE consists of 5 open reading frames (LEE1 to LEE5) (Dean et al, 2005). LEE1 to LEE3 encodes the Type 3 secretion system (T3SS) and the LEE-encoded regulator (*Ler*) gene, which acts as the major transcriptional regulator of EPEC (Barba et al, 2005; Scaletsky, 2019). The EPEC secreted proteins (Esp), EspA, EspB, EspD are encoded by LEE4 through the T3SS (Clarke et al, 2003; Scaletsky, 2019). Lastly, LEE 5 encodes intimin (eae) and translocated intimin receptor (tir). Other secreted effector proteins include Mitochondrial associated protein, EspF, EspG, EspH and EspZ. These proteins are translocated into the host epithelial cells using the T3SS (Clarke et al, 2003; Dean and Kenny, 2009). The non-LEE encoded effector genes are found outside the LEE region and assist in increased EPEC virulence (Gärtner and Schmidt, 2004). LEE expression is regulated by plasmid-encoded regulator (per) located on the EPEC Adherence Factor plasmid (Scaletsky, 2019). This plasmid occurs exclusively only in typical EPEC strains, while the atypical EPEC strains lack the plasmid (Trabulsi et al, 2002).

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EPEC adherence to epithelial cell occurs via the bundle forming pili (bfp) and is encoded by the *bfpA* gene (Giron et al, 1991; Guerrant et al, 2011). In typical EPEC strains, bfp adherence results in microcolony formation with Localized Adherence (LA) pattern (Figure 2.1); and in atypical EPEC strains adherence results in aggregative adherence (AA), LA-like (LAL) and diffuse adherence (DA) patterns (Pelayo et al, 1999; Trabulsi et al, 2002; Mora et al, 2009).

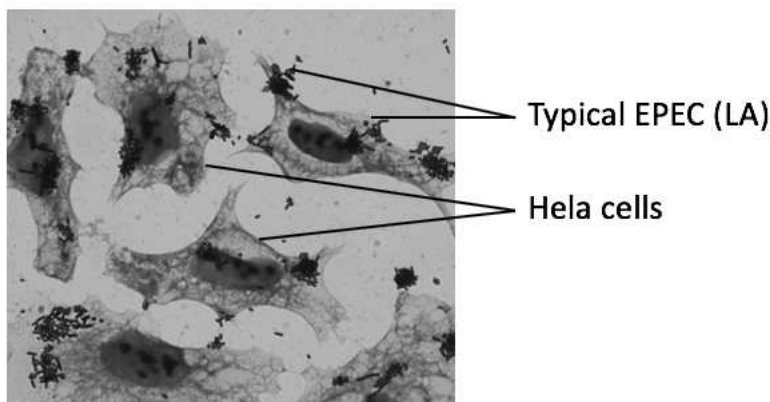

Figure 2.1: Typical EPEC showing localized adherence on HeLA cells following 3-hour infection viewed using Zeiss microscope. Magnification: X100 (Image by Ledwaba SE)

EPEC pathogenesis occurs in 4 stages (Figure 2.2). Following adherence via the bfp, EPEC expresses the T3SS that injects into the host cell via the aid of the filament forming EspA. The tir and the effector proteins (EspB, EspD, EspF, EspG) are then translocated via the T3SS injectosome into the host cell (Clarke et al, 2003; Scaletsky, 2019). These proteins activate the cell-signalling pathways such as tyrosine phosphorylation (which is critical for actin formation), Nuclear factor kappa-light-chain of activated B cells (NFκB) [associated with increased interleukin-8 (IL-8) production], inositol phosphate fluxes (increased calcium from intracellular stores) and increased protein kinase C as a result of intimate adherence (Vallance and Finlay, 2000). Mitochondrial associated protein that targets and disrupts the mitochondrial function, resulting in alteration of the cytoskeletal structure of the host cell and leading to accumulation of actin and loss of microvilli. The translocated EspB and EspD proteins form pores on the surface of the cells. Tir is then

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inserted into the host membrane through the pores, eae then attaches on the surfaces resulting in intimate attachment. The Tir-eae complex assists in pedestal formation. These pedestals assist the bacteria to remain extracellular allowing it to not be recognized by the immune system (Clarke et al, 2003, Bhunia, 2018). Finally, the translocated molecules disrupt the cell functions, this results in loss of tight-junction integrity via EspF (McNamara et al, 2001) and mitochondrial function causing loss of electrolytes and eventually cell death (Clarke et al, 2003; Bhunia, 2018; Scaletsky, 2019).

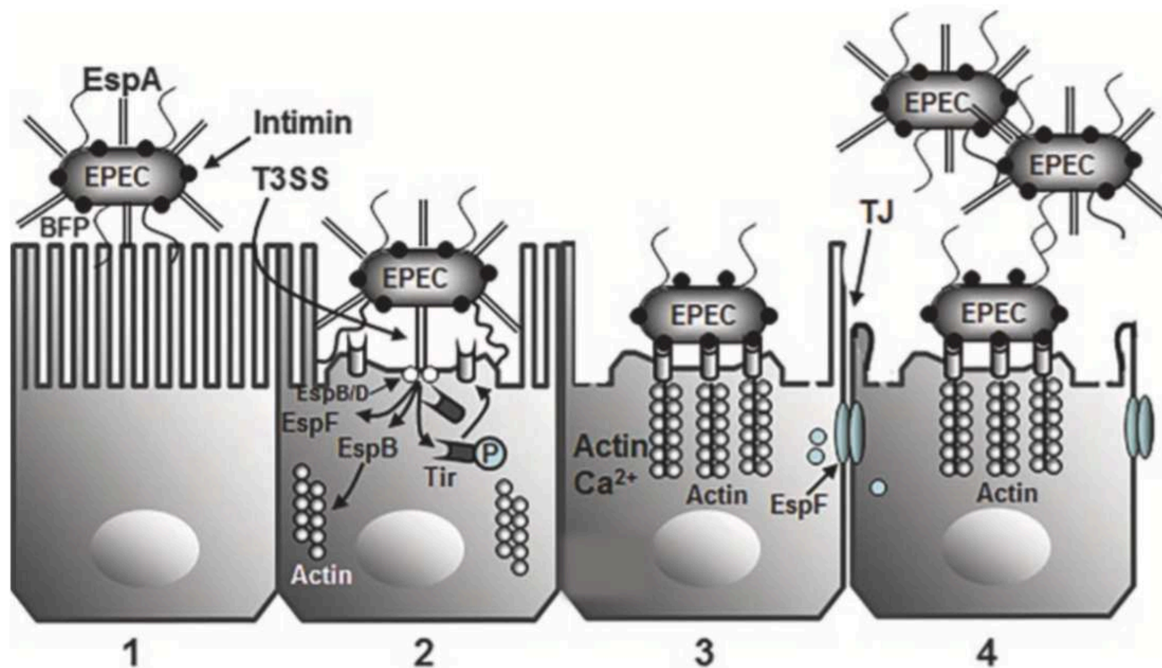

Figure 2.2: EPEC 4-stage pathogenesis model. (Bhunia, 2018). 1. EPEC initial adheres to cells using bundle forming pili (bfp). 2. Effector proteins EspB, D, F and translocated-intimin receptor (tir) are translocated into the host cell via the Type 3 secretion system (T3SS) injectosome. 3. Tir acts as a receptor for intimin leading to actin accumulation at the site of infection and disruption of tight junction (TJ) integrity occurs via EspF. 4. Disruption of the cell-signalling pathways leads to loss of mitochondrial function resulting in electrolyte loss and cell death. Key words: Esp= EPEC secreted protein.

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2.2.2. *IN VITRO* MODELS OF EPEC

EPEC infections *in vitro* have been well studied, exhibiting pathogenic characteristics (Law et al, 2013). In tissue cultures, it is clear to determine the adherence patterns of EPEC infection (Scaletsky et al, 1984; Cleary et al, 2004; Garcia et al, 2016; Vieira et al, 2019). Knutton and colleagues (1989) have provided a foundation in studying actin and pedestal formation during EPEC infection on Human epithelial type 2 (HEp-2), colon carcinoma (Caco-2) and Human Erythroleukemia cell lines (Knutton et al, 1989). Non-polarized cells have also been used to study the T3SS, protein translocation into host cells and functional analyses of injected effectors (Cleary et al, 2004; Leverton and Kaper, 2005; Martinez-Argudo et al, 2007; Nieto-Pelegrin and Martinez-Quiles, 2009; Law et al, 2013; Tapia et al, 2017; Baumann et al, 2018). At the site of infection, EPEC leads to disruption of the brush border (Croxen et al, 2013; Bhunia, 2018). Polarized T84, Caco-2, HT29, Human colorectal tumour-8 (HCT-8) and Madin-Darby Canine Kidney cell lines mimic the intestinal cells by forming the brush border and tight junctions. These polarized cells have been used to assess the physiological properties of EPEC infection with disruption of barrier function on the intestinal brush border (Philpott et al, 1996; McNamara et al. 2001; Dean and Kenny, 2004; Puthenedam et al, 2007; Morampudi et al, 2017). These cells provide good model for determining disruption of tight junctions during infection and determining effectors that are involved (McNamara et al. 2001; Law et al, 2013). EPEC infections have been accompanied by increased inflammatory responses (Ruchaud-Sparagano et al, 2007, Sanchez-Villamil et al, 2016; Baumann et al, 2018).

2.2.3. *IN VIVO* MODELS OF EPEC

The *in vivo* studies assessing the role of genes involved in A/E lesion formation and virulence processes during EPEC infection have been reported (Law et al, 2013). EPEC is an intestinal pathogen, therefore, the *in vivo* system is required in order to determine the pathogenesis mechanism in a living host. Development of an EPEC infection model *in vivo* assists in understanding the pathogen-host interaction, colonization patterns,

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production of virulence genes, inflammatory responses and A/E lesion formations (Law et al, 2013).

Animal species such as *Caenorhabditis elegans* (Anyanful et al, 2005; Mellies et al, 2006), mice (Savkovic et al, 2005; Royan et al, 2010; Manthey et al, 2014; Dupont et al, 2016), rabbits (Milon et al, 1999; Mohamed et al, 2019), pigs (An et al, 2000; Fröhlicher et al, 2008; Malik et al, 2017), sheep (Fröhlicher et al, 2008), chicks and calves (Meyer et al, 1992) have been used to study EPEC infections. These models have provided an understanding in potential hosts that can be used to study EPEC infection. *C. elagans* models have been used to study microbial virulence strategies and factors that are involved in innate immunity (Anyanful et al, 2005; Mellies et al, 2006). EPEC infection in *C. elegans* has been reported to infect and kill using "slow killing "mechanism that leads to accumulation of EPEC in the intestine of *C. elegans* and formation of micro-colonies (Mellies et al, 2006). EPEC has also been characterized by "fast killing" in *C. elegans*, due to production of exotoxins during infection (Anyanful et al, 2005). However, the *C. elegans* model does not have the ability to survive the optimal temperature needed for virulence gene expression of EPEC, and inoculation is often difficult (Mylonakis and Aballay, 2005; Law et al, 2013). In addition, innate immunity of *C. elegans* is different from mammalian vertebrates, therefore cannot be considered a good model for EPEC infection (Law et al, 2013).

Animal models such as rabbits and mice are reported to be good representatives of inflammation as a result of EPEC infection (Law et al, 2013). Mice are considered as good models for studying EPEC infection because they are inexpensive, and the immune system is similar to humans (Law et al, 2013). There are studies that have been done in mice in order to determine EPEC infection (Savkovic et al, 2005; Shifflett et al, 2005; Mundy et al, 2006; Royan et al, 2010; Zhang et al, 2010; Rhee et al, 2011; Dupont et al, 2016). Savkovic and colleagues (2005) developed an EPEC E2348/69 infection model using 6 to 8-week-old C57BL/6 mice. During the 10-day infection period, wild type (WT) EPEC adhered to the cecum, small and large intestine, however, no changes in weight were observed. Infection resulted in changes in histology of the intestinal lumen and

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inflammation was observed with increased goblet cells in the colon. Mice that were infected with the *bfp* mutant also had similar effects to mice infected with WT. Overall, EPEC infection in mice resulted in adherence and colonization (with micro-colony formation) in intestinal epithelial lumen and accumulation of actin at the site of infection leading to increased inflammatory responses (Savkovic et al, 2005).

Shifflett and colleagues (2005) also used 6 to 8- week-old C57BL/6 mice to study the effect of EPEC on tight junctions in *vivo*. EPEC colonized the ileum and colon of infected mice until day 5 post-infection (pi). Barrier function of infected mice was reduced at 5 days pi due to the *EspF* mutant (Shifflett et al, 2005). Zhang and colleagues (2010), also studied changes in tight junction morphology and epithelial barrier function as a result of infection caused by EPEC in C57BL/6 mice pretreated with streptomycin. Mice infected with WT EPEC developed mild inflammation with crypt expansion and increased inflammatory cell infiltration, accompanied by tissue damage in the ileal and colonic mucosa (Zhang et al, 2010).

Mundy et al (2006), compared colonization dynamics of mice infected with EPEC and *C. rodentium* and found that EPEC E2348/69 strain colonized C57BL/6 and C3H/Hej mice for 14 to 28 days. Mice infected with EPEC shed 10^4 CFU g^{-1} stool for approximately 6 days which was found to be lower than 10^8 CFU $g^{-1}$of mice infected with *C. rodentium*. Mice infected with E2348/69Δ*escN* and E2348/69Δ*eae* strains shed for a short period than the WT EPEC indicating the importance of intimin and the T3SS. Overall, EPEC colonized in cecum of mice at low levels and had no disease outcome resulting in commensal interaction with the host (Mundy et al, 2006). Streptomycin-treated mice have been used to study infection caused by EPEC by reducing normal gut microbiota in order to avoid competitive exclusion and facilitate colonization (Royan et al, 2010). Dupont and colleagues (2016), recently developed an EPEC infection model using infant and adult mice. This study showed the importance of virulence factor of the T3SS and adherence factor (bfp). Infection in this model was able to highlight stool shedding, colonization, microcolony formation, increased inflammatory responses and alteration of microbiota

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(Dupont et al, 2016). This study however, was not able to fully mimic human EPEC infection due to mice not developing diarrhoea.

Adult human volunteers were also used to study the roles of *eae, espB*, and bfp during EPEC pathogenesis (Donnenberg et al, 1993; Tacket et al, 2000) and in a re-challenge study (Donnenberg et al, 1998). Intestinal biopsies were extracted from human volunteers infected with WT EPEC in order to determine disruption of the intestinal brush border. All the volunteers developed diarrhoea except one volunteer that was infected with the *espB* mutant (Tacket et al, 2000).

Overall, all these models have provided basic knowledge in understanding the effect of EPEC pathogenesis. Most of the studies mentioned above have reported on colonization, adherence patterns, inflammatory responses as a result of disruption of tight junctions. However, these models have not been able to report on clinical outcomes such as weight changes and diarrhoea. Therefore, there is still a need to develop an affordable, suitable small animal model that mimics clinical outcomes of human EPEC infection.

2.3. ENTEROTOXIGENIC *E. coli*

ETEC commonly causes moderate to severe diarrhoea in children residing in low-income countries (Kotloff et al, 2017), and has been mostly found to cause traveller’s diarrhoea (Gupta et al, 2008). The GEMS and MAL-ED studies have found that ETEC causes diarrhoea in children aged between 0 to 59 months (Kotloff et al, 2013; Platts-Mills et al, 2018) and infected children were reported to have high risk of death (Croxen et al, 2013). ETEC has been estimated to infect approximately 157 000 persons yearly and 9% of these results in deaths (Qadri et al, 2007). Transmission is mainly via faecal-oral route. The infective dose is high with 10^{6}-10^{10} CFU (Farrar et al, 2013). Incubation period is 1-2 days resulting in vomiting, anorexia, abdominal cramps and diarrhoea (Kaper et al, 2004; Farrar et al, 2013).

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2.3.1. ETEC PATHOGENESIS MECHANISM

Upon entry into the intestinal lumen of the host, ETEC requires fimbrial structures, colonization factors (CFs) for adherence on the intestinal epithelial cells (Mooi and De Graaf, 1985; Kharat et al, 2017). About 25 CFs have been identified (Gaastra and Svennerholm, 1996). The CF/I, has been reported extensively and it is made of 100 copies of Cfa-B and Cfa-E molecules (Fleckenstein et al, 2010). Figure 2.3 shows the ETEC pathogenesis model.

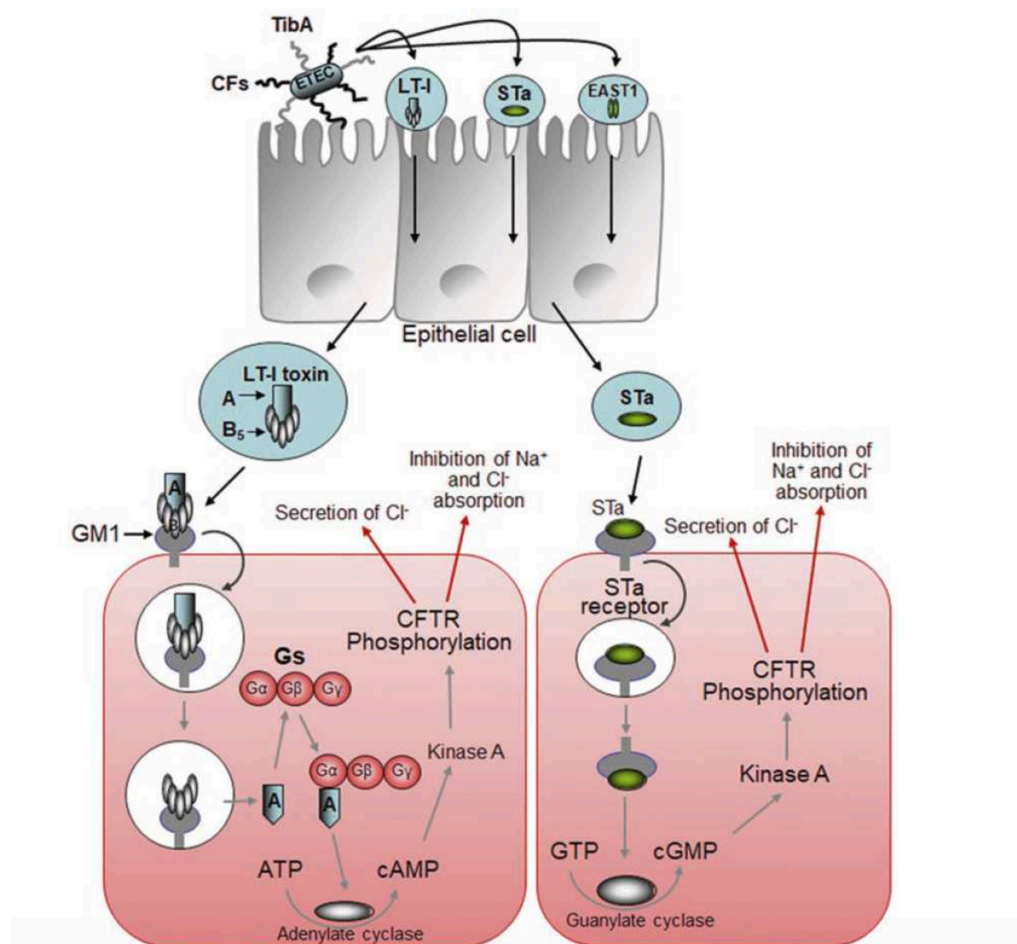

Figure 2.3: ETEC pathogenesis model (Bhunia, 2018). ETEC attaches to cells using colonization factors (CFs) or non-fimbrial adhesion factor, TibA and produces either LT, ST and EAST1 enterotoxins. During LT mechanism, cAMP is increased, and during ST mechanism, cGMP is increased and both toxins are mediated by phosphorylation of cystic fibrosis transmembrane conductance regulator (CFTR) resulting in increased chlorine ion (Cl^-) secretion in crypt cells and reduced absorption of sodium (Na^+) and Cl^- by absorptive cells. Keywords: ATP= adenosine

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triphosphate, cAMP = cyclic adenosine monophosphate, cGMP = cyclic guanosine monophosphate, LT = heat liable toxin, ST heat stable toxin, EAST1 = EAEC heat stable enterotoxin 1.

ETEC produces the heat-liable enterotoxin (LT) (similar to cholera toxin) and heat-stable enterotoxins (ST) (Fleckenstein et al, 2010). During infection, ETEC produces LT and ST enterotoxins and in certain cases produces one of these enterotoxins (Fleckenstein et al, 2010). The LT is a heterohexomic molecule made of a single A subunit and pentameric B subunit (Sixma et al, 1991). The A subunit has two domains that are attached by a disulphide bond. The A1 is the active toxin molecule while A2 is the helical portion of the molecule that attaches to the B pentamer (Merritt et al, 1994). Binding of LT to the GM1 ganglioside causes endocytosis on the cells. The A-subunit is then passed through the membrane and interacts with ADP ribosylating factor (Tsai et al, 1987). Suppression of Gsα Guanosine triphosphatase activity causes activation of adenylate cyclase which in turn stimulates intracellular cAMP in order to activate the chloride channels, cystic fibrosis transmembrane regulator (CFTR) on the intestinal lining (Fleckenstein et al, 2010, Croxen et al, 2013). This activation leads to increased secretion of electrolytes and water into the intestinal lumen causing diarrhoea (Croxen et al, 2013; Bhunia, 2018).

Secretion of LT through the outer membrane to the host is by either N-terminal signal peptides of the subunits during transportation of sec-dependent across the inner membrane to the periplasm where it assembles into in a holotoxin (Hirst and Holmgren, 1987). Secretion of LT across the outer membrane is via the Type 2 secretion system (T2SS) also referred to as the general secretion pathway (Tauschek et al, 2002). The LT produces different effects that are beneficial to the host. The innate immune responses are down-regulated by LT including the defensins, and this enables ETEC to attach and colonize on the small intestinal lumen (Chakraborty et al, 2008; Fleckenstein et al, 2010).

The ST are made up of cysteine-rich peptides that adhere to guanylyl cyclase C on the microvilli. Intracellular catalytic domain is activated by the guanylyl cyclase-C resulting in increased intracellular Cyclic adenosine monophosphate (cGMP) (Weiglmeier et al, 2010). The cGMP-dependent protein kinase is activated by an increase in the cGMP

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resulting in phosphorylation of CFTR (Weiglmeier et al, 2010; Croxen et al, 2013). This leads to increased secretion of chlorine channel accompanied by suppression of sodium chloride absorption resulting in diarrhoea (Figure 2.3) (Chao et al, 1994, Bhunia, 2018).

2.4.2. *IN VITRO* MODELS OF ETEC

Different cell lines have been used to evaluate the adhesion structures (Knutton et al, 1987; Torres et al, 2005), LT and ST enterotoxins (Wang et al, 2012) and genes involved in ETEC pathogenesis (Gaastra and Svennerholm, 1996; Torres et al, 2005). ETEC infection in mucus-secreting HT29 cell lines have been reported. ETEC adherence on these cells was observed on the brush border and not on the goblet cells (Kerneis et al, 1994). Fujiwara and colleagues (2001) studied ETEC adherence on HCT-8 cells and found that protein produced by *B. longum* inhibited ETEC binding to HCT-8 cells in a dose dependent manner and this was due to inhibition of CFA/II antigen (Fujiwara et al, 2001). ETEC infection in HCT-8 cells has also been reported to induce inflammatory responses such as IL-8 (Huang et al, 2004). Genes such as *tib,* are involved in adherence have been reported in HCT-8 cell lines and has been found to act as an adhesin, assisting in ETEC pathogenesis (Lindenthal and Elsinghorst, 2001). ETEC vaccine development has also been studied *in vitro* in order to determine antibody adherence activity against ETEC strains that express CFA/I and various surface *E. coli* surface antigens. The antibodies against CFA were found to be protective by inhibiting adherence and neutralizing activities against the enterotoxins (Ruan et al, 2015).

2.3.4. *IN VIVO* MODELS OF ETEC

ETEC naturally infects humans and animals such as piglets, dogs, calves and sheep resulting in diarrhoea (Dubreuil et al, 2016). Animal models such as piglets (Zhang et al, 2008), rats (Whipp, 1990), rabbits (Spira et al, 1981; Svennerholm et al, 1990), mice (Moon et al,1979; Ren et al, 2014; Luiz et al, 2015; Bolick et al, 2018) and human volunteers (Harro et al, 2011) have been used to study ETEC infections. These models have provided insight on colonization, adhesion factors, immune response, expression of

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ETEC virulence traits and vaccine development (Bertin, 1983; Svennerholm et al, 1990; Ren et al, 2014; Luiz et al, 2015; Norton et al; 2015; Bolick et al, 2018).

ETEC infection in piglets results in clinical outcomes that are similar to humans resulting in diarrhoea and dehydration (Zhang et al, 2008). The porcine and human ETEC strains have similar pathogenesis mechanism, however, these strains have different LT and ST enterotoxins. An isogenic ETEC strain having the ability to express both human and porcine enterotoxins (ST and LT) has been developed (Zhang et al, 2008). All ETEC infected piglets were reported to developed diarrhoea (Zhang et al, 2008). Piglets have therefore been suggested to be good models for studying ETEC infection (Zhang et al, 2008). However, due to production time of these models, the piglets are often not readily available in large quantities and maintenance is costly. A suitable small animal that is produced in a short space of time is therefore, required for studying infections such as ETEC (Luiz et al, 2015). Rats have been used to study the effect of STb. The jejunal loops infected with the STb enterotoxin was found to induce more intestinal secretory responses than in infant mice (Whipp, 1990). The rabbit intestine model has been used to study the role of ETEC surface-associated antigens of CFA/II (Spira et al, 1981; Svennerholm et al, 1990).

Various Infant murine models have been widely used to evaluate adherence and colonization traits of ETEC (Moon et al,1979; Bertin, 1983; Goldhar et al, 1986; Duchet-Suchaux et al, 1990; Bertin, 1992; Grange and Mouricout, 1996). Duchet-Suchaux and colleagues (1990) studied the differences in susceptibility of outbred and inbred infant mice infected with ETEC originating from human, bovine and porcine. The ETEC infected C57BL/6, BALAB/cBy and CBA mice had high mortality rates due to the bovine strains, and some of the porcine strains induced mortality levels with some exhibiting diarrhoea following 3-day infection. However, the human ETEC H10407 infected mice were less susceptible, while the CBA mice showed increased susceptibility with no outcomes of diarrhoea (Duchet-Suchaux et al,1990). A lethal neonatal DBA/2 mouse challenge model infected with lethal dose of ETEC H10407 has been used in testing ETEC adhesin

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vaccine. It was found that the CFA-E provides protective mechanism in vaccinated neonatal mice following ETEC infection (Duchet-Suchaux et al,1990).

Bernal-Reynaga and colleagues (2013) used C57-CD40 ligand deficient mice as a potential model for ETEC infection. Following oral infection with ETEC, colonization was reported up to 14 days in the C57-CD40 mice and for 3 to 7 days in C57BL/6 mice; however, diarrhoea and other clinical outcomes were not observed. The Immunoglobulin (Ig) A levels were found to be lower in the C57-CD40 mice. The presence of LT was found to increase the ability of ETEC to colonize in the intestine (Bernal-Reynaga et al, 2013).

An increase in intestinal innate immune responses caused by ETEC SE470 strain using 6-week-old female ICR mice have been reported (Ren et al, 2014). Mice were pretreated with streptomycin and cimetidine prior infection, and most of the infected mice died within 24-hours pi. Infection resulted in increased innate immune responses such as Toll-like receptor-4, NFκB and mitogen-activated protein kinase pathways (Ren et al, 2014). Recently an ETEC H10407 infection in weaned C57BL/6 mice pretreated with broad-spectrum antibiotic cocktail has been developed (Bolick et al, 2018). This infection resulted in weight loss, diarrhoea and increased inflammatory markers and stool shedding occurred for more than 10 days following infection. The study highlighted the importance of ETEC infection, with the ability to mimic clinical outcomes similar to that of children.

ETEC enterotoxins have been widely studied *in vivo*, as possible vaccine candidates (Fleckenstein et al, 2010; Norton et al, 2015; Fleckenstein and Kuhlmann, 2019). Norton and colleagues (2015) used the LT-A subunit in order to evaluate its immunogen and as an antigen for protection against ETEC using *in vitro* and *in vivo* analyses. This study found that the human sera from adults that were challenged with ETEC H10407 strain after 10 days developed antibodies against LT-A and LT-B. BALB/c mice were intranasally immunized with the LT-A or/and LT-B, and these mice were found to also produce antibodies against the LT enterotoxin (Norton et al, 2015). However, when mice were immunized with both the LT-A and -B toxins, they produced a higher concentration against the LT enterotoxin. Overall the LT subunits resulted in antibody production,

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providing protection against LT-causing diarrhoea and thus acting as possible candidates for vaccine development (Norton et al, 2015). Human volunteer studies in ETEC infection has been reported, with participants developing diarrhoea (Harro et al, 2011). Currently studies are underway, using human volunteers for ETEC vaccine development (Harro et al, 2011; Fleckenstein and Kuhlmann, 2019).

2.4. ENTEROAGGREGATIVE *E. coli*

EAEC infection leads to persistent diarrhoea in young children (Okhuysen and DuPont, 2010), and has been reported as the second leading cause of traveller's diarrhoea (Bamidele et al, 2019). EAEC also infects immunocompromised patients such as HIV infected persons resulting in severe complications (Mathewson et al, 1998). EAEC infections occur sporadically with a dosage of 10^{10} CFU (Nataro et al, 1995). These infections result in watery diarrhoea that can either be acute or persistent (Farrar et al, 2013). Infections can result in growth decrements (Guerrant et al, 2008), increased intestinal inflammation (Steiner et al, 2000, Opintan et al, 2010; Rogawski et al, 2017) and symptoms can occur with or without diarrhoea (Rogawski et al, 2017).

2.4.1 EAEC PATHOGENESIS MECHANISM

EAEC attaches on HEp-2 cells and the intestinal mucosa using fimbrial structures called aggregative adherence fimbriae (AAF) (Vial et al, 1988). EAEC has been characterized by formation of stacked brick-like pattern, also called aggregative adherence (AA) (Figure 2.4) on epithelial cells (Kaper et al, 2004; Pereira et al, 2008). The AAFs of EAEC strains differ in morphology and genetic makeup, and these structures are essential for attachment of EAEC to epithelial cells (Harrington et al, 2006; Boisen et al, 2008; Oloomi et al, 2009). *In vitro*, the expressions of AAF-I, III or IV are required in order to induce polymorphonuclear cell transmigration (Nataro et al, 1992; Czeczulin et al, 1997; Bernier et al, 2002). The AAFs are hydrophobic and can easily agglutinate in an aqueous

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environment. EAEC requires dispersin (aap) in order to colonize and for AAF to adhere to cells (Sheikh et al, 2002).

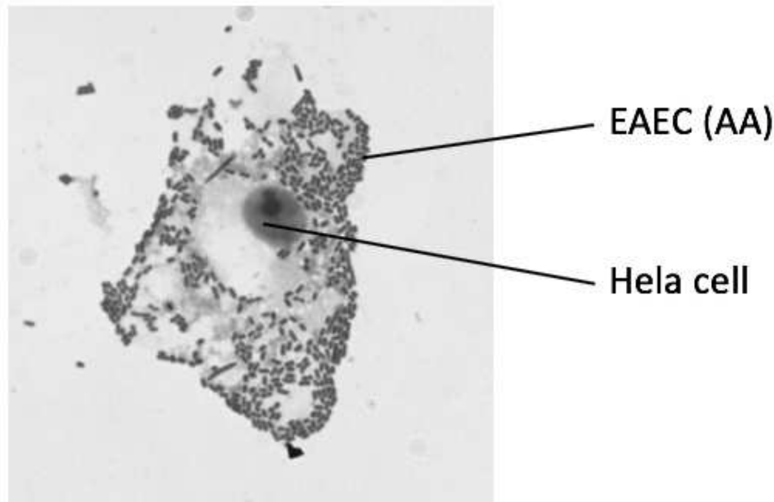

Figure 2.4: EAEC aggregative adherence (AA) showing stacked-brick-like pattern on HeLA cells following 3-hour infection viewed using Zeiss microscope. Magnification X100 (Image by Ledwaba SE)

EAEC colonization is characterized by distinct biofilm formation (Kaper et al, 2004). The biofilm protects EAEC from being recognized in the epithelial cells resulting in increased chronic infection (Mohamed et al, 2007; Telli et al, 2010). Aggregative regulator (AggR) is the main transcriptional regulator of EAEC and is required for activation of virulence genes (Harrington et al, 2006). EAEC also produces enterotoxins during infection namely, EAEC heat-stable enterotoxin-1 (EAST-1), plasmid-encoded toxin and *Shigella* enterotoxin-1 which have cytotoxic effects (Elias and Navarro-Garcia, 2016).

2.4.2. *IN VITRO* EAEC STUDIES

Cell lines such as Caco-2 (Pereira et al, 2008), HEp-2, HeLA (Aslani et al, 2011), HCT-8 (Medeiros et al, 2013) and T84 (Nataro et al, 1996; Pereira et al, 2008) cells have been used to study the characteristics of EAEC infection. EAEC infection in HEp-2 cells results in a distinct stacked-brick pattern due to the presence of fimbria structures (Kaper et al,

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2004). The Caco-2 cells have been used to determine biofilm formation of EAEC during infection (Pereira et al, 2008). The intestinal polarized cells have also made it possible to measure cytokine, IL-8, during infection (Steiner et al, 2000). Polarized T84 cells have also been used to determining adhesion and cytotoxin-mediated tissue damage (Nataro et al, 1996). HeLA cells, on the other hand, have been found to be a good model for determining the adherence pattern (Pereira et al, 2010; Aslani et al, 2011), biofilm formation (Pereira et al, 2010) and cytokine released during infection. These cells have been commonly used in clinical studies to determine the adherence pattern of EAEC strains isolated from human with or without diarrhoea (Steiner et al, 2000; Huang et al, 2004; Pereira et al, 2010; Aslani et al, 2011). Other cell lines such as HT29 from rat jejunum has been used to study the effects of the plasmid encoded toxin produced by EAEC during infection (Navarro-García et al, 1999).

2.4.3. *IN VIVO* EAEC STUDIES

EAEC infections have been studied in animal models such as piglets (Tzipori 1992), *C. elegans* (Hwang et al, 2010), rabbits (Kang et al, 2001; Munera et al, 2014), wax moths (Jønsson et al, 2017) and mice (Roche et al, 2010; Bolick et al, 2013; Ren et al, 2014). Vial and colleagues (1988) studied EAEC infection using ligated intestinal loops in NZB rabbits and Fischer 344 rats, with infection causing lesions in the intestine, limb paralysis and some animals died during infection (Vial et al, 1988).

Tzipori and colleagues (1992) studied EAEC infection in neonatal gnotobiotic piglets, and infection resulted in lesions in intestine, with piglets developing diarrhoea and some died during infection. This model was able to mimic EAEC infection in the colonic mucosa and was found to closely resemble human infection (Tzipori et al, 1992; Philipson et al, 2013).

Roche and colleagues (2010) studied EAEC infection using malnourished 6-day-old C57BL/6 mice. These mice were orally inoculated with EAEC 042 and/or JM221 strains. Shedding was reported to occur for more than 3 weeks and inflammation was observed in the intestinal tissue (Roche et al, 2010). Bolick et al (2013), also studied EAEC infection

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using malnourished C57BL/6 mice. Infection resulted in changes in body weight, persistent EAEC stool shedding until 14 days pi and tissue burden was also recorded until14 days pi (Bolick et al, 2013). Interestingly, administration of nitazoxanide resulted in reduced weight loss and EAEC shedding, thus suggesting that improved nutrition plays an important role in disease outcome (Bolick et al, 2013). 6-week-old BALB/c and C3H/He mice pretreated with streptomycin were used to evaluate the role of aggR involved in regulation during EAEC pathogenesis (Morin et al, 2010). Other animal models were also used to study virulence genes involved during EAEC pathogenesis in order to understand the mechanism involved during infection (Eslava et al, 2002; Harrington et al, 2009; Medeiros et al, 2013). Severe combined immunodeficient mice and xerographs from foetal intestinal tissue has also been used to produce human tissue model for infection (Boll et al, 2012). The AAF polymorphonuclear cell migration in EAEC have been studied using this model (Boll et al, 2012).

2.5 OCCURRENCE OF CO-INFECTIONS

Co-infections or mixed infections refers to occurrence and interaction of multiple pathogens within a host at the same time. The occurrence of co-infections has been reported in both human (Adhikari et al, 1985; Bilenko et al, 2004; Vilchez et al, 2009; Bonkoungou et al, 2012; Ledwaba et al, 2018, Lima et al, 2018) and animal (Wada et al, 1996; da Rocha et al, 2014; Zhao et al, 2016; Ortega et al, 2017) studies. Co-infections have been reported to occur globally in infections related to diseases such as tuberculosis (Madhi et al, 2000), hepatitis (Balogun et al, 2012), HIV (Madhi et al, 2000) and malaria (Epelboin et al, 2012). Studies in respiratory (Cebey-Lopez et al, 2015; Torres, 2015; Bakaletz, 2017) and diarrhoeal (Yatsuyanagi et al, 2002; Bilenko et al, 2004; Mason et al, 2013; Zhang et al, 2016; Ledwaba et al, 2018; Mathew et al, 2019) infections have also reported on co-infecting pathogens. For decades, epidemiological studies have been reporting on the occurrence of co-infections across the world (Adhikari et al, 1985; Vilchez et al, 2009; El Feghaly et al, 2013; Mason et al, 2013; Tobias et al, 2015; Zhang et al,

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2016; Iijima et al, 2017; Shrivastava et al, 2017; Andersson et al, 2018; Broujerdi et al, 2018; Dutta et al, 2018; Ledwaba et al, 2018; Tareen et al, 2019). These natural interactions of pathogenic organisms have raised questions in trying to understand the effect of the pathogens in the host. Most of the epidemiological studies have suggested that the microbe-microbe interactions might result in critical disease outcomes in the infected host (Tobias et al, 2015; Zhang et al, 2016; Ledwaba et al, 2018; Mathew et al, 2019). It is important to further study the interaction of co-infections in a suitable model in order to fully understand the interaction and determine the disease outcome.

The microbe-host interaction is of great importance as it produces a series of different traits in which infection can either be mutualistic or parasitic (Crane et al, 2006; May and Nelson, 2014; Gill and Hecht, 2018). Co-infections on the other hand, involves microbe-microbe interaction within the host, this interaction can also result in either synergistic or antagonistic effects (Crane et al, 2006; Toledo et al, 2011; Bhavnani et al, 2012; May and Nelson, 2014; Bartelt et al, 2017; Long et al, 2019). Studies have suggested that there might be greater competition between different co-infecting pathogens and thus affecting the host (Susi et al, 2015). Co-infections within the host often results in pathogens competing with each other for metabolites (Estrada et al, 2012; Reynolds et al, 2017), and direct interaction might result in increased virulence (Crane et al, 2006; May and Nelson, 2014; Galván-Moroyoqui et al, 2008; Mosser et al, 2015). This can greatly affect the immune system of the host (Galván-Moroyoqui et al, 2008; Bartelt et al, 2017), eventually leading to death (Wada et al, 1996).

2.5.1 MODELS OF CO-INFECTIONS

Different models of co-infections have been used to study the interaction of co-infecting pathogens (Crane et al, 2006; Mosser et al, 2015; Bartelt et al, 2017; Shen et al, 2019). These models have provided insight in understanding the innate immune response and clinical outcomes of the pathogens. *In vitro* (Crane et al, 2006; Galván-Moroyoqui et al, 2008; Manko et al, 2017 Reynolds et al, 2017) and *in vivo* (Toledo et al, 2011; Mosser et al, 2015; Manko et al, 2017; Bartelt et al, 2017; Reynolds et al, 2017; Shen et al, 2019)

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analyses have shown that co-infections can have synergistic (organisms interacting together to enable drastic disease outcome) or antagonistic (organism suppressing the growth or function of another organism resulting in reduced or diminished disease outcome) effects.

Crane and colleagues (2006) reported on the interaction of EPEC and ETEC *in vitro*, with infection resulting in synergistic effects, EPEC infection was enhanced by the presence of ETEC (Crane et al, 2006). Galván-Moroyoqui and colleagues (2008) studied the interaction of *Entamoeba histolytica*, *E. dispar* with EPEC and *Shigella dysenteriae in vitro*. Co-infections resulted in increased cytopathic effects accompanied with increased IL-8 and virulence expression of Gal/GalNAc lectin causing damage to the cells (Galván-Moroyoqui et al, 2008).

Interaction of bacteria-parasite co-infections have been widely reported in murine models (Galván-Moroyoqui et al, 2008; Bartelt et al, 2017; Manko et al, 2017; Reynolds et al, 2017). Reynolds and colleagues (2017) studied intestinal helminths and *Salmonella* co-infections by determining intestinal metabolites interaction using streptomycin-treated mice. This co-infection resulted in synergistic effects, with helminths upregulating the pathogenesis of *Salmonella*, and this led to enhanced bacterial pathogenicity and increased colonization due to alteration of the metabolic profile (Reynolds et al, 2017). Manko et al (2017) studied *Giardia duodenalis* and A/E enteropathogens (EPEC and *C. rodentium*) co-infections using C57BL/6 mice and found that co-infected mice had increased weight loss and MPO levels. EPEC growth was greatly inhibited when co-infected with *G. duodenalis* in Caco-2 cells (Manko et al, 2017).

2.5.1.1. Diarrheagenic *E. coli* co-infection models

The interaction between various DEC pathotypes has been reported by several studies (Wada et al, 1996; Yatsuyanagi et al, 2002; Crane et al, 2006; Toledo et al; 2011; Dutta et al, 2015; Leonard et al; 2016). Wada and colleagues (1996) reported on ETEC and attaching and effacing *E. coli* (EPEC) co-infections in post-weaning pigs developing

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diarrhoea and four of the pigs died following infection. *In vitro*, Crane and colleagues (2006) studied the interaction of EPEC E2348/69 and ETEC H10407 co-infections using T84 cells. Infection with these strains resulted in increased ATP release, and chloride secretion was also enhanced by crude ETEC filtrates. LT was found to have a synergistic effect resulting in formation of vacuoles (Crane et al, 2006). Toledo and colleagues (2011) studied the interaction of EHEC and EPEC using BALB/c mice. Mice that were orally pre-challenged with EPEC followed by EHEC infection had a protective effect, with mice having increased weight similar to the uninfected group and developing mild symptoms. Interestingly, mice that were not pre-challenged developed mild to severe symptoms with increased weight loss (Toledo et al, 2011). The Shiga Toxin producing *E. coli* and ETEC hybrid strains (Leonard et al, 2016), together with the P/T hybrid strains (Dutta et al, 2015) have been reported to cause severe outbreaks. The *E. coli* pathotypes are evolving, thus harbouring virulence genes of other *E. coli* pathotypes resulting in severe disease outcome (Bielaszewska et al, 2007; Bando et al, 2009; Ruiz et al, 2014; Hazen et al, 2017). Further understanding of co-infection mechanisms in the host is greatly needed. More studies are therefore required in order to determine the transmission pathways, the interaction and clinical outcome in a closed-controlled environment.

2.6. INFLAMMATORY RESPONSES

Inflammation is a defensive response that is triggered by the presence of harmful particles such as tissue damage-derived molecules (Medzhitov, 2008; Takeuchi and Akira, 2010). Inflammation is clinically characterized by swelling, redness, pain, heat and loss of tissue function (Takeuchi and Akira, 2010). Upon infection, macrophages and mast cells initially respond to the site of damage. This results in production of inflammatory mediators, chemokines, cytokines, eicosanoids and vasoactive amines (Medzhitov, 2008). The plasma proteins and neutrophils gain access through the post-capillary venules at the site of infection while preventing erythrocytes from exiting the blood vessels. Neutrophils are activated once they reach the site of infection or through secretion of cytokines

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(Medzhitov, 2008). These neutrophils attack the pathogen by producing toxic substances such as reactive oxygen species and reactive nitrogen species (Nathan, 2006; Medzhitov, 2008; Takeuchi and Akira, 2010).

Cytokines play an important role in controlling intestinal inflammation caused by environmental (microbial infection, diet, stress and antibiotics), genetic factors (antimicrobial peptides) and inflammatory bowel diseases (Crohn's disease and ulcerative colitis). Cytokines are small peptide proteins made up of different cell populations including T-helper cells, dendric cells, neutrophils, natural killer cells, regulatory T cells, macrophages and innate lymphoid cells (Zhang and An, 2007; Neurath, 2014). Upon infection or tissue trauma in the intestinal tissue, disruption of the intestinal lumen or barrier function results in activation of chemokines, pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines such as IL-1β, IL-6 and Tumour necrosis factor (TNF-a), IL-12, IL-15, IL-17 and interferon gamma are involved during pathological pain (Zhang and An, 2007; Neurath, 2014). In order to balance inflammation, anti-inflammatory cytokines such as IL-4, IL-10, IL-11, IL-13 are released in order to control the presence of pro-inflammatory cytokines (Zhang and An, 2007; Jaffer et al, 2010).

DEC pathotypes require flagella in order to be motile and adhere to intestinal tissue (Croxen et al, 2013). Adherence of these pathogens results in inflammatory markers being released during infection. During EAEC infection, the binding of the flagellin is recognized by Toll like receptor-5 resulting in secretion of IL-8 by epithelial cells (Steiner et al, 2000; Hayashi et al, 2001). Pathogens such as EAEC (EAST-1) and ETEC (STa) produce enterotoxins resulting in activation of the chloride secretory channels, CFTR, and this leads to induction of IL-8 (Berkes et al, 2003). The LT enterotoxin stimulates inflammation of ETEC through cAMP-dependent Ras-like guanosine triphosphatase (Wang et al, 2012), resulting in increased IL-6 and TNF-α in T84 cells (Chutkan and Kuehn, 2011). EPEC infection has also been reported to disrupt intestinal barrier function through injection of the T3SS into the host cell (Berkes et al, 2003). EPEC infection in murine models has also been reported to cause mild inflammation resulting in histological

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changes (increase in intraepithelial lymphocytes, lamina propria polymorphonucleocytes and goblet cells) in the colon (Savkovic et al, 2005; Zhang et al, 2010).

2.6.1 FAECAL BIOMARKERS OF INFLAMMATION

2.6.1.1. Lipocalin-2 (LCN-2)

LCN-2 is also called neutrophil gelatinase-associated Lipocalin (NGAL) or 24p3 (Abella et al, 2015). It is a secreted glycoprotein that forms part of the small lipophilic (Abella et al, 2015; Prata et al, 2016). LCN-2 has surfaced as a pleiotropic molecule with different physiologic and pathophysiological functions such as metabolic homeostasis, apoptosis, infection and immune response (Abella et al, 2015). Also, LCN-2 has been suggested as a biomarker of cardiovascular diseases, intestinal inflammation, acute kidney injury and lupus nephritis (Abella et al, 2015; Bolick et al, 2018; Medeiros et al, 2019). This biomarker is expressed in different tissues such as the uterus (Huang et al, 1999), liver, spleen, immune cells, kidney, bone marrow, chondrocytes (Owen et al, 2008) and tissues that are infected with microbes (Abella et al, 2015).

During infection pathogens such as *E. coli, Klebsiella* and *Salmonella* species., produce a siderophores called enterobactin (Abella et al, 2015). This enterobactin enables the bacteria to scavenge iron from the host and transport it into the pathogen for growth. LCN-2 inhibits transportation of iron into the pathogen by binding on the enterobactin, thus channelling the iron into the mammalian cells (Abella et al, 2015). Depletion of iron results in growth inhibition of the pathogen (Bao et al, 2010, Abella et al, 2015). Berger and colleagues (2006) infected LCN-2 deficient C57BL/6 mice with a lethal dose of *E. coli,* and these mice died 16 hours following infection. The LCN-2 deficient mice died due to septic shock as a result of increased LPS at the site of infection. Overall LCN-2 was reported to assist in anti-bacterial innate immune response as a result of increased iron concentration in the host at the site of infection (Berger et al, 2006). Recently, Bolick and colleagues (2018) reported on LCN-2 as faecal biomarker of intestinal inflammation as a result of ETEC infection in C57BL/6 mice. It was found that mice pretreated with antibiotic

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cocktail and placed on different diets; infection caused by ETEC resulted in elevated LCN-2 across the different diets when compared to uninfected mice (Bolick et al, 2018). Increase in LCN-2 levels have also been reported in C57BL/6 mice infected with *Shigella flexneri,* with mice developing diarrhoea and weight loss (Medeiros et al, 2019).

2.6.1.2. Myeloperoxidase (MPO)

MPO forms part of the mammalian heme peroxidase enzyme family (Prokopowicz et al, 2012). MPO can break down hydrogen peroxide-mediated oxidation of halide ion in order to produce substances such as hypochlorous acid, tyrosyl radicals and reactive nitrogen intermediates (Prata et al, 2016). MPO has been used as a marker of neutrophil infiltration, and has been suggested to be present during active chronic inflammation and assists in down-regulating inflammation in order to limit tissue damage (Prokopowicz et al, 2012). Saiki (1998) previously reported on patients with inflammatory bowel disease and found that the MPO levels in stools were significantly elevated. The MPO pathway has been explored *in vitro* and *in vivo* using human and animal models (Prokopowicz et al, 2012). MPO-deficient mice infected with *Klebsiella pneumonia* have been reported to be more susceptible to infection (Hirche et al, 2005).

MPO has been studied in the intestinal mucosa, and has been found to have the potential of monitoring disease progression and assist in outcomes of treatment in patients having ulcerative colitis (Wagner et al, 2008). Bolick et al (2018) investigated the role of zinc in C57BL/6 mice as a result of ETEC infection. This study found that zinc-deficient mice infected with ETEC had higher faecal MPO levels than those on protein deficient or standard rodent chow diet (Bolick et al, 2018). Medeiros and colleagues (2019) also investigated the role of zinc in C57BL/6 mice infected with *S. flexneri*. Zinc-deficient mice were also reported to have elevated levels of faecal MPO due to *S. flexneri* infection (Medeiros et al, 2019).

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2.6.2. ACUTE-PHASE SYSTEMIC RESPONSE

Acute phase response is an instantaneous inflammatory reaction in the host following infection, tissue injury and trauma (Uhlar and Whitehead, 1999). Its function is to respond to the site of injury, isolate the pathogen or foreign particle and neutralize it in order to prevent further damage to the host (Uhlar and Whitehead, 1999). This response acts as a survival mechanism, occurring for a short period of time as a result of intestinal inflammation due to bacterial infection, or it can be long term in cases of chronic inflammation resulting in severe clinical outcomes (Uhlar and Whitehead, 1999).

Acute phase inflammation affects both systemic and vascular functions as well as other organs including the liver, depending on the site of invasion (Uhlar and Whitehead, 1999). Some acute-phase proteins released during inflammation include C-Reactive protein (CRP), Serum Amyloid A (SAA) and haptoglobin (Gabay and Kushner, 1999). Activation occurs through monocytes and macrophages at inflammatory site. Cytokines such as IL-1 and TNF-α are released as primary cytokines. Secretion of these cytokines enables secondary cytokines, IL-6 and IL-8, and chemokines to be secreted by stroma cells (Uhlar and Whitehead, 1999). Chemokines enable neutrophil infiltration at the site of inflammation (Cassatella, 1995). Released IL-1, TNF-α and IL-6 act by enabling negative-feedback response by producing corticosteroids which allow synthesis of acute-phase proteins that in turn hinder expression of cytokines (Uhlar and Whitehead, 1999).

2.6.2.1. Serum Amyloid A

SAA is made up of apolipoproteins that are primarily produced in the liver (Uhlar and Whitehead, 1999). SAA is categorized into two sub-types based on its responsiveness to inflammation namely; acute phase-SAA (A-SAA) which acts as a major acute-phase protein that increases more than 1000-fold at the site of inflammation (Hoffman and Benditt, 1982); and constitutive SAA which occurs in humans and mice, it is produced at a lower concentration mostly during normal and acute phase response (Coetzee et al, 1986; Uhlar and Whitehead, 1999). Human and mouse SAA proteins are closely related

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and have been well studied (Uhlar and Whitehead, 1999). The SAA1 and SAA2 are synthesized primarily in the liver and are produced mostly during acute inflammation. SAA3 is synthesized in the macrophages and are produced mostly during chronic inflammation (Meek et al, 1992). SAA4 is a constitutive SAA, causing mild inflammation (Uhlar and Whitehead, 1999).

When there is chronic inflammation or acute-phase response the hepatic cells in the liver, produce high levels of SAA thus reducing the ability of the liver to degrade A-SAA, thereby allowing SAA to circulate in the host and at the inflammatory site (Ulhar and Whitehead, 1999). It has been reported *in vivo* that bacterial organisms that can produce lipopolysaccharide, casein, silver nitrate and tissue damage as a result of surgery, produce cytokines and chemokines resulting in increased levels of A-SAA (Uhlar and Whitehead, 1999). Shah and colleagues (2006) studied the effect of SAA on *E. coli* and *Pseudomonas aeruginosa* on macrophages and found that SAA recognized and adhered to the outer membrane of the gram-negative organisms and assisted in phagocytosis by macrophages. Macrophages infected with *P. aeruginosa* had increased IL-10 and TNF-α as a result of SAA induction (Shah et al, 2006).

2.6.2.2. C-Reactive Protein

CRP was first detected in a patient infected with *Streptococcus pneumoniae* precipitating C-polysaccharide during acute phase response and has been described as a sensitive systemic marker of damage in the tissue and also during inflammation (Pepys and Baltz, 1983). CRP is related to the pentraxin family of the ligand adhering calcium plasma proteins. CRP is constantly being produced in humans at a low concentration of about 0.8 mg/L, and in cases where there is acute phase response, the concentration increases up to 10 000-fold (Pepys and Hirschfield, 2003). Plasma CRP is secreted in the liver and is controlled by the transcriptional levels of IL-6 of which in turn is elevated by IL-1β. The NFκB is also required for increased synthesis of CRP (Pepys and Hirschfield, 2003).

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The CRP adheres to the Ig-receptors that assist in phagocytosis of injured tissue or foreign particles at the site of infection (Black et al, 2004). CRP has the ability to identify the pathogens; upon recognition, recruitment and activation of proteins enable phagocytic cells to release CRP acting as the first line of defence in the host (Black et al, 2004). It has been reported that increased production of CRP leads to the synthesis of binding molecules in the aortic endothelial cells by suppressing nitric oxide synthase production in the cells (Venugopal et al, 2002). CRP expression is also stimulated by IL-8, which in turn promotes plasminogen activator inhibitor-1 production and this leads to increased IL-1, IL-18 and IL-6 synthesis. (Ballou and Lozanski, 1992).

2.7. METABOLOMICS

Metabolomics or metabonomics refers to a discipline aimed at measuring metabolites utilized within the cells, tissue or organism in response to genetic alteration or physiological stimuli (Roberts et al, 2012). Metabolites are small molecules that assist in metabolic reaction and are needed for growth, maintenance and normal function of the cell (Dettmer et al, 2007). This discipline is being used as a potential biomarker in nutritional interventions, prediction of outcomes in health status to a disease state and food consumption marker (Sébédio, 2017). The metabolites can be measured from blood, urine and saliva using analytical tools such as nuclear magnetic resonance (NMR) (Eisenreich and Bacher, 2007), mass spectrometry, and high-performance liquid chromatography coupled with mass spectrometry (LC-MS), and lastly with gas-liquid chromatography coupled with mass spectrometry (GC-MS) (Sébédio, 2017). The high-resolution magic angle spinning (HR-MAS) ^{1}H NMR spectroscopy is widely used in various tissues and biopsies of tumours. This technique is advantageous because metabolome can be determined without prior extraction and is good when working with large samples (Sébédio, 2017).

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The metabolome can be interpreted using statistical methods of unsupervised technique called principal component analysis (PCA). The partial least square discriminant analysis (PLS-DA) and orthogonal PLS-DA unsupervised techniques are also used depending on the known sample (Sébédio, 2017). The metabolome produced via biochemical processes results in endogenous and exogenous mixtures. Exogeneous metabolome are metabolites produced by drugs, pollutants, toxins and dietary compounds. Endogenous metabolome are metabolites synthesized by the organism using biochemical pathways (Sébédio, 2017). Animal models have been used to study metabolites produced during disease outcome (Giallourou et al, 2018; Medeiros et al, 2019).

2.8. SUMMARY OF LITERATURE REVIEW

E. coli consists of commensal and pathogenic strains. The commensal *E. coli* colonizes the intestine of humans and animals after birth (Kaper et al, 2004; Croxen et al, 2013). The pathogenic *E. coli* strains lead to various disease outcomes such as meningitis, bacteraemia, intestinal and urinary-tract infections depending on the site of infection (Kaper et al, 2004). DEC pathotypes such as EAEC, EPEC and ETEC have been widely reported to cause moderate to severe diarrhoea mostly in children less than 2 years of age in low-income countries (Kotloff et al, 2013; Platts-Mills et al, 2015).

Typical EPEC causes infantile diarrhoea, leading to deaths in children under 12 months of age (Kotloff et al, 2013). This pathogen is characterized by distinct LA pattern, formation of microcolonies and A/E lesions with pedestals and actin accumulation at the site of infection (Kaper et al, 2004; Croxen et al, 2013; Bhunia, 2018). From the literature, it was found that EPEC has been well studied using *in vitro* analyses (Scaletsky et al, 1984; Knutton et al, 1989; Philpott et al, 1996; McNamara et al, 2001; Cleary et al, 2004; Leverton and Kaper, 2005; Martinez-Argudo et al, 2007; Nieto-Pelegrin and Martinez-Quiles, 2009; Law et al, 2013; Tapia et al, 2017). These models have reported on the adherence traits, T3SS, proteins and genes involved in EPEC pathogenesis and the

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inflammatory responses produced as a result of infection. On the other hand, EPEC infection using *in vivo* studies have also been reported. The literature has revealed that EPEC infection has been studied using different animal models (Milon et al, 1999; Meyer et al, 1992; Savkovic et al, 2005; Law et al, 2013; Malik et al, 2017), and streptomycin-treated mice were frequently used. (Royan et al, 2010; Zang et al, 2010; Dupont et al, 2016). Infection in these murine models resulted in colonization, increased inflammatory responses, disruption of tight junction and importance of virulence genes involved in adherence, T3SS and translocated proteins. However, these models have not reported on EPEC infection resulting in clinical outcomes that mimic human EPEC infection. Therefore, there is still a need for a suitable small animal model for human EPEC infection that mimics clinical outcomes (diarrhoea, weight changes) and inflammation similar to humans.

The literature revealed that C57BL/6 mice are good models for studying human pathogen infections (Duchet-Suchaux et al, 1990; Shifflett et al, 2005; Mundy et al, 2006; Savkovic et al, 2005; Roche et al, 2010; Zhang et al, 2010; Bolick et al, 2013; Dupont et al, 2016; Bolick et al, 2018; Giallourou et al, 2018; Medeiros et al, 2019). Streptomycin-treated C57BL/6 mice have been used in microbial infections (Royan et al, 2010; Zhang et al, 2010; Dupont et al, 2016). However, infections in these models have not been able to replicate disease outcomes such as diarrhoea and weight decrements as observed in humans (Savkovic et al, 2005; Zhang et al, 2010; Dupont et al, 2016). C57BL/6 mice pretreated with broad-spectrum antibiotic cocktail have been used to study *C. jejuni* (Giallourou et al, 2018), ETEC (Bolick et al, 2018) and *S. flexneri* (Medeiros et al, 2019) pathogens. Infections in these models resulted in clinical outcomes of diarrhoea, weight loss changes and metabolic perturbations. Increase in faecal inflammatory biomarkers, MPO and LCN-2 were also reported in these models (Giallourou et al, 2018; Medeiros et al, 2019). These biomarkers are of great importance because they have also been detected in malnourished children (Guerrant et al, 2016; Prata et al, 2016). The models have been able to mimic clinical outcomes as a result of infection (Bolick et al, 2018; Medeiros et al, 2019).

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Co-infections in clinical settings have been reported globally. It has been suggested co-infections are likely to result in increased severe clinical outcomes (Mathew et al, 2019). *In vitro* and *in vivo* models have been developed, in order to understand the interaction of co-infecting pathogens by analysing the microbe-microbe interaction within a host (Crane et al, 2006; Toledo et al, 2011; Bartelt et al, 2017; Reynolds et al, 2017). Co-infections have been reported to result in upregulation of virulence genes and increased colonization in murine models (Bartelt et al, 2017; Reynolds et al, 2017). Few studies have reported on the interaction of DEC co-infections using *in vitro* (Crane et al, 2006) and *in vivo* (Wada et al, 1996; Toledo et al, 2011) analyses. Infections involving A/P and P/T co-infections have been detected in human and animal studies (Adhikari et al, 1985; Wada et al, 1996; Vilchez et al, 2009; Bonkoungou et al, 2012; Ledwaba et al, 2018). Crane et al (2006) reported on the mutual interaction of P/T co-infections *in vitro* and found that there was a synergistic effect. Toledo et al (2011) studied interaction of EHEC and EPEC co-infections *in vivo* and found that there was a protective effect. Further studies concerning interaction of DEC co-infections are still needed as these co-infections are increasingly being detected in clinical settings (Adhikari et al, 1985; Yatsuyanagi et al, 2002; Vilchez et al, 2009; Dutta et al, 2018; Iijima et al, 2017; Andersson et al, 2018; Broujerdi et al, 2018; Ledwaba et al, 2018; Lima et al, 2018; Mathew et al, 2019). *In vivo* models are considered good models for studying pathogens due to their closed-controlled environment (Law et al, 2013). Understanding the underlying interaction between these pathogens will assist in providing a greater explanation in the pathogenesis mechanism and determining if the interaction results in detrimental disease outcomes.

The aim of this study was to, therefore, develop an EPEC murine model using C56BL/6 mice pretreated with a broad-spectrum antibiotic cocktail in order to determine clinical outcomes, colonization, faecal inflammatory biomarkers (MPO and LCN-2), acute-phase systemic markers (SAA and CRP) and metabolic disturbances as a result of human EPEC infection. The developed EPEC murine model was further used to study the interaction of P/T and A/P co-infections by assessing clinical outcomes and changes in inflammatory responses. The interaction of co-infections was further *assessed in vitro* in order to

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determine adherence traits, inflammatory responses and virulence genes produced during infection.

2.9. REFERENCES

Abella, V., Scotece, M., Conde, J., Gómez, R., Lois, A., Pino, J., Gómez-Reino, J.J., Lago, F., Mobasheri, A. and Gualillo, O., 2015. The potential of lipocalin-2/NGAL as biomarker for inflammatory and metabolic diseases. *Biomarkers*, **20**(8), 565-571.

Adhikari, M., Coovadia, Y. and Hewitt, J., 1985. Enteropathogenic *Escherichia coli* (EPEC) and enterotoxigenic (ETEC) related diarrhoeal disease in a neonatal unit. *Annals of Tropical Paediatrics* **5**(1), 19-22.

An, H., Fairbrother, J.M., Désautels, C., Mabrouk, T., Dugourd, D., Dezfulian, H. and Harel, J., 2000. Presence of the LEE (locus of enterocyte effacement) in pig attaching and effacing *Escherichia coli* and characterization of eae, espA, espB and espD genes of PEPEC (pig EPEC) strain 1390. *Microbial Pathogens*, **28**(5), 291-300.

Andersson, M., Kabayiza, J.C., Elfving, K., Nilsson, S., Msellem, M.I., Mårtensson, A., Björkman, A., Bergström, T. and Lindh, M., 2018. Coinfection with Enteric Pathogens in East African Children with Acute Gastroenteritis—Associations and Interpretations. *American Journal of Tropical Medicine and Hygiene*, **98**(6), 1566-1570.

Anyanful, A., Dolan-Livengood, J.M., Lewis, T., Sheth, S., DeZalia, M.N., Sherman, M.A., Kalman, L.V., Benian, G.M. and Kalman, D., 2005. Paralysis and killing of *Caenorhabditis elegans* by enteropathogenic *Escherichia coli* requires the bacterial tryptophanase gene. *Molecular Microbiology*, **57**(4), 988-1007.

© University of Venda

Aslani, M.M., Alikhani, M.Y., Zavari, A., Yousefi, R. and Zamani, A.R., 2011. Characterization of enteroaggregative *Escherichia coli* (EAEC) clinical isolates and their antibiotic resistance pattern. *International Journal of Infectious Diseases*, **15**(2), e136-e139.

Bakaletz, L.O., 2017. Viral–bacterial co-infections in the respiratory tract. *Current Opinion in Microbiology*, **35**, 30-35.

Ballou, S.P. and Lozanski, G., 1992. Induction of inflammatory cytokine release from cultured human monocytes by C-reactive protein. *Cytokine*, **4**(5), 361-368.

Balogun, T.M., Emmanuel, S. and Ojerinde, E.F., 2012. HIV, Hepatitis B and C viruses' coinfection among patients in a Nigerian tertiary hospital. *Pan African Medical Journal*, **12**(1).

Bamidele, O., Jiang, Z.D. and Dupont, H., 2019. Occurrence of putative virulence-related genes, aatA, aggR and aaiC, of Enteroaggregative *Escherichia coli* (EAEC) among adults with travelers' diarrhea acquired in Guatemala and Mexico. *Microbial Pathogens*, **128**, 97-99.

Bando, S.Y., Andrade, F.B., Guth, B.E., Elias, W.P., Moreira-Filho, C.A. and Pestana de Castro, A.F., 2009. Atypical enteropathogenic *Escherichia coli* genomic background allows the acquisition of non-EPEC virulence factors. *FEMS Microbiology Letters*, **299**(1), 22-30.

Bao, G., Clifton, M., Hoette, T.M., Mori, K., Deng, S.X., Qiu, A., Viltard, M., Williams, D., Paragas, N., Leete, T. and Kulkarni, R., 2010. Iron traffics in circulation bound to a siderocalin (Ngal)–catechol complex. *Nature Chemical Biology***,** **6**(8), 602.

Barba, J., Bustamante, V.H., Flores-Valdez, M.A., Deng, W., Finlay, B.B. and Puente, J.L., 2005. A positive regulatory loop controls expression of the locus of enterocyte

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effacement-encoded regulators Ler and GrlA. *Journal of Bacteriology*, **187**(23), 7918-7930.

Bartelt, L.A., Bolick, D.T., Mayneris-Perxachs, J., Kolling, G.L., Medlock, G.L., Zaenker, E.I., Donowitz, J., Thomas-Beckett, R.V., Rogala, A., Carroll, I.M. and Singer, S.M., 2017. Cross-modulation of pathogen-specific pathways enhances malnutrition during enteric co-infection with *Giardia lamblia* and enteroaggregative *Escherichia coli*. *PLoS Pathogens*, **13**(7).

Baumann, D., Salia, H., Greune, L., Norkowski, S., Körner, B., Uckeley, Z.M., Frankel, G., Guenot, M., Rüter, C. and Schmidt, M.A., 2018. Multitalented EspB of enteropathogenic *Escherichia coli* (EPEC) enters cells autonomously and induces programmed cell death in human monocytic THP-1 cells. *International Journal of Medical Microbiology*, **308**(3), 387-404.

Berger, T., Togawa, A., Duncan, G.S., Elia, A.J., You-Ten, A., Wakeham, A., Fong, H.E., Cheung, C.C. and Mak, T.W., 2006. Lipocalin 2-deficient mice exhibit increased sensitivity to *Escherichia coli* infection but not to ischemia-reperfusion injury. *Proceedings of the National Academy of Science*, **103**(6), 1834-1839.

Berkes, J., Viswanathan, V.K., Savkovic, S.D., Hecht, G., 2003. Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation. *Gut*, **52**(3), 439-51.

Bernal-Reynaga, R., Thompson-Bonilla, R., Lopez-Saucedo, C., Pech-Armenta, M., Estrada-Parra, S. and Estrada-Garcia, T., 2013. C57-CD40 ligand deficient mice: a potential model for enterotoxigenic *Escherichia coli* (H10407) colonization. *Veterinary and Immunology Immunopathology*, **152**(1-2), 50-56.

Bernier, C., Gounon, P. and Le Bouguénec, C., 2002. Identification of an aggregative adhesion fimbria (AAF) type III-encoding operon in enteroaggregative *Escherichia coli* as

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a sensitive probe for detecting the AAF-encoding operon family. *Infection and Immunity*, **70**(8), 4302-4311.

Bertin, A., 1983. Virulence factors of enterotoxigenic *E. coli* studied in the infant mouse model. *Annales de Recherches Vétérinaires*, **14**(2), 169- 182

Bertin, A.N.N.E., 1992. Comparison of susceptibility of inbred and outbred infant mice to *Escherichia coli* heat-stable enterotoxin STa. *Infection and Immunity*, **60**(8), 3117-3121.

Bhavnani, D., Goldstick, J.E., Cevallos, W., Trueba, G. and Eisenberg, J.N., 2012. Synergistic effects between rotavirus and coinfecting pathogens on diarrheal disease: evidence from a community-based study in northwestern Ecuador. *American Journal of Epidemiology*, **176**(5), 387-395.

Bhunia, A.K., 2018. *Foodborne microbial pathogens: mechanisms and pathogenesis*. Springer.

Bielaszewska, M., Prager, R., Köck, R., Mellmann, A., Zhang, W., Tschäpe, H., Tarr, P.I. and Karch, H., 2007. Shiga toxin gene loss and transfer *in vitro* and *in vivo* during enterohemorrhagic *Escherichia coli* O26 infection in humans. *Applied Environmental Microbiology*, **73**(10), 3144-3150.

Bilenko, N., Levy, A., Dagan, R., Deckelbaum, R.J., El-On, Y. and Fraser, D., 2004. Does co-infection with *Giardia lamblia* modulate the clinical characteristics of enteric infections in young children?. *European Journal of Epidemiology*, **19**(9), 877-883.

Black, S., Kushner, I. and Samols, D., 2004. C-reactive protein. *Journal of Biological Chemistry*, **279**(47), 48487-48490.

Boisen, N., Struve, C., Scheutz, F., Krogfelt, K.A. and Nataro, J.P., 2008. New adhesin of enteroaggregative *Escherichia coli* related to the Afa/Dr/AAF family. *Infection and Immunity*, **76**(7), 3281-3292.

© University of Venda

Bolick, D.T., Medeiros, P.H.Q.S., Ledwaba, S.E., Lima, A.A., Nataro, J.P., Barry, E.M. and Guerrant, R.L., 2018. Critical role of zinc in a new murine model of enterotoxigenic *Escherichia coli* diarrhea. *Infection and Immunity*, **86**(7), e00183-18.

Bolick, D.T., Roche, J.K., Hontecillas, R., Bassaganya-Riera, J., Nataro, J.P. and Guerrant, R.L., 2013. Enteroaggregative *Escherichia coli* strain in a novel weaned mouse model: exacerbation by malnutrition, biofilm as a virulence factor and treatment by nitazoxanide. *Journal of Medical Microbiology*, **62**(Pt 6), 896.

Boll, E.J., Struve, C., Sander, A., Demma, Z., Nataro, J.P., McCormick, B.A. and Krogfelt, K.A., 2012. The fimbriae of enteroaggregative *Escherichia coli* induce epithelial inflammation *in vitro* and in a human intestinal xenograft model. *Journal of Infectious Diseases*, **206**(5), 714-722.

Bonkoungou, I.J.O., Lienemann, T., Martikainen, O., Dembelé, R., Sanou, I., Traoré, A.S., Siitonen, A., Barro, N. and Haukka, K., 2012. Diarrhoeagenic *Escherichia coli* detected by 16-plex PCR in children with and without diarrhoea in Burkina Faso. *Clinical Microbiology and Infection*, **18**(9), 901-906.

Broujerdi, S.M., Ardakani, M.R. and Rezatofighi, S.E., 2018. Characterization of diarrheagenic *Escherichia coli* strains associated with diarrhea in children, Khouzestan, Iran. *The Journal of Infection in Developing Countries*, **12**(08), 649-656.

Cassatella, M.A., 1995. The production of cytokines by polymorphonuclear neutrophils. *Immunology Today*, **16**(1), 21-26.

Cebey-Lopez, M., Herberg, J., Pardo-Seco, J., Gomez-Carballa, A., Martinón-Torres, N., Salas, A., Martinón-Sánchez, J.M., Gormley, S., Sumner, E., Fink, C. and Martinon-Torres, F., 2015. Viral co-infections in pediatric patients hospitalized with lower tract acute respiratory infections. *PLoS one*, **10**(9).

© University of Venda

Chakraborty, K., Ghosh, S., Koley, H., Mukhopadhyay, A.K., Ramamurthy, T., Saha, D.R., Mukhopadhyay, D., Roychowdhury, S., Hamabata, T., Takeda, Y. and Das, S., 2008. Bacterial exotoxins downregulate cathelicidin (hCAP-18/LL-37) and human β-defensin 1 (HBD-1) expression in the intestinal epithelial cells. *Cellular Microbiology*, **10**(12), 2520-2537.

Chao, A.C., de Sauvage, F.J., Dong, Y.J., Wagner, J.A., Goeddel, D.V. and Gardner, P., 1994. Activation of intestinal CFTR Cl-channel by heat-stable enterotoxin and guanylin via cAMP-dependent protein kinase. *The EMBO Journal*, **13**(5), 1065-1072.

Chutkan, H. and Kuehn, M.J., 2011. Context-dependent activation kinetics elicited by soluble versus outer membrane vesicle-associated heat-labile enterotoxin. *Infection and Immunity*, **79**(9), 3760-3769.

Clarke, S.C., Haigh, R.D., Freestone, P.P.E. and Williams, P.H., 2003. Virulence of enteropathogenic *Escherichia coli*, a global pathogen. *Clinical Microbiology Reviews*, **16**(3), 365-378.

Cleary, J., Lai, L.C., Shaw, R.K., Straatman-Iwanowska, A., Donnenberg, M.S., Frankel, G. and Knutton, S., 2004. Enteropathogenic *Escherichia coli* (EPEC) adhesion to intestinal epithelial cells: role of bundle-forming pili (BFP), EspA filaments and intimin. *Microbiology*, **150**(3), 527-538.

Coetzee, G.A., Strachan, A.F., Van Der Westhuyzen, D.R., Hoppe, H.C., Jeenah, M.S. and De Beer, F.C., 1986. Serum amyloid A-containing human high density lipoprotein 3. Density, size, and apolipoprotein composition. *Journal of Biological Chemistry*, **261**(21), 9644-9651.

Crane, J.K., Choudhari, S.S., Naeher, T.M. and Duffey, M.E., 2006. Mutual enhancement of virulence by enterotoxigenic and enteropathogenic *Escherichia coli*. *Infection and Immunity*, **74**(3), 1505-1515.

© University of Venda

Croxen, M.A., Law, R.J., Scholz, R., Keeney, K.M., Wlodarska, M. and Finlay, B.B., 2013. Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clinical Microbiology Reviews*, **26**(4), 822-880.

Czeczulin, J.R., Balepur, S., Hicks, S., Phillips, A., Hall, R., Kothary, M.H., Navarro-Garcia, F. and Nataro, J.P., 1997. Aggregative adherence fimbria II, a second fimbrial antigen mediating aggregative adherence in enteroaggregative *Escherichia coli*. *Infection and Immunity*, **65**(10), 4135-4145.

da Rocha Gizzi, A.B., Oliveira, S.T., Leutenegger, C.M., Estrada, M., Kozemjakin, D.A., Stedile, R., Marcondes, M. and Biondo, A.W., 2014. Presence of infectious agents and co-infections in diarrheic dogs determined with a real-time polymerase chain reaction-based panel. *BMC Veterinary Research*, **10**(1), 23.

Dean, P. and Kenny, B., 2004. Intestinal barrier dysfunction by enteropathogenic *Escherichia coli* is mediated by two effector molecules and a bacterial surface protein. *Molecular Microbiology*, **54**(3), 665-675.

Dean, P. and Kenny, B., 2009. The effector repertoire of enteropathogenic *E. coli*: ganging up on the host cell. *Current Opinion in Microbiology*, **12**(1), 101-109.

Dean, P., Maresca, M. and Kenny, B., 2005. EPEC's weapons of mass subversion. *Current Opinion in Microbiology*, **8**(1), 28-34.

Dettmer, K., Aronov, P.A. and Hammock, B.D., 2007. Mass spectrometry-based metabolomics. *Mass Spectrometry Reviews*, **26**(1), 51-78.

Donnenberg, M.S., Tacket, C.O., James, S.P., Losonsky, G., Nataro, J.P., Wasserman, S.S., Kaper, J.B. and Levine, M.M., 1993. Role of the eaeA gene in experimental enteropathogenic *Escherichia coli* infection. *Journal of Clinical Investigation*, **92**(3), 1412-1417.

© University of Venda

Donnenberg, M.S., Tacket, C.O., Losonsky, G., Frankel, G., Nataro, J.P., Dougan, G. and Levine, M.M., 1998. Effect of Prior Experimental Human Enteropathogenic *Escherichia coli* Infection on Illness following Homologous and Heterologous Rechallenge. *Infection and Immunity*, **66**(1), 52-58.

Dubreuil, J.D., Isaacson, R.E. and Schifferli, D.M., 2016. Animal enterotoxigenic *Escherichia coli*. *EcoSal Plus*, **7**(1).

Duchet-Suchaux, M.A.R.I.O.N., Le Maitre, C.A.R.O.L.E. and Bertin, A., 1990. Differences in susceptibility of inbred and outbred infant mice to enterotoxigenic *Escherichia coli* of bovine, porcine and human origin. *Journal of Medical Microbiology*, **31**(3), 185-190.

Dupont, A., Sommer, F., Zhang, K., Repnik, U., Basic, M., Bleich, A., Kühnel, M., Bäckhed, F., Litvak, Y., Fulde, M. and Rosenshine, I., 2016. Age-dependent susceptibility to enteropathogenic *Escherichia coli* (EPEC) infection in mice. *PLoS Pathogens* **12**(5).

Dutta, K., Pal, B., Mandal, R., Mullick, S. and Mandal, P., 2018. Circulating rotavirus types and drug-resistant diarrheagenic *Escherichia coli* causing enteric infection in under-five children in rural west Bengal, India. *Journal of Bioengineering and Biomedical Sciences*, **8**(255), 2.

Dutta, S., Pazhani, G.P., Nataro, J.P. and Ramamurthy, T., 2015. Heterogenic virulence in a diarrheagenic *Escherichia coli*: evidence for an EPEC expressing heat-labile toxin of ETEC. *International Journal Medical Microbiol*ogy, **305**(1), 47-54.

Eisenreich, W. and Bacher, A., 2007. Advances of high-resolution NMR techniques in the structural and metabolic analysis of plant biochemistry. *Phytochemistry*, **68**(22-24), 2799-2815.

El Feghaly, R.E., Stauber, J.L., Tarr, P.I. and Haslam, D.B., 2013. Viral co-infections are common and are associated with higher bacterial burden in children with *C. difficile* infection. *Journal of Pediatric Gastroenterology and Nutrition*, **57**(6), 813.

© University of Venda

Elias, W.P. and Navarro-Garcia, F., 2016. Enteroaggregative *Escherichia coli* (EAEC). In *Escherichia coli in the Americas* (27-57). Springer, Cham.

Epelboin, L., Hanf, M., Dussart, P., Ouar-Epelboin, S., Djossou, F., Nacher, M. and Carme, B., 2012. Is dengue and malaria co-infection more severe than single infections? A retrospective matched-pair study in French Guiana. *Malaria Journal*, **11**(1), 142.

Eslava, C., Sainz, T., Perez, J., Fresan, M.C., Flores, V., Jimenez, L., Hernandez, U. and Herrera, I., 2002. Histological Alterations and Immune Response Induced by Pet Toxin During Colonization with Enteroaggregative *Escherichia coli* (EAEC) in a mouse model infection. *Journal of Microbiology*, **40**(2), 91-97.

Estrada, A.E.R., Jonkers, W., Kistler, H.C. and May, G., 2012. Interactions between Fusarium verticillioides, Ustilago maydis, and Zea mays: an endophyte, a pathogen, and their shared plant host. *Fungal Genetic and Biology*, **49**(7), 578-587.

Farrar, J., Hotez, P.J., Junghanss, T., Kang, G., Lalloo, D. and White, N.J., 2013. *Manson's Tropical Diseases E-Book*. Elsevier Health Sciences.

Fleckenstein, J.M. and Kuhlmann, F.M., 2019. Enterotoxigenic *Escherichia coli* Infections. *Current Infectious Disease Reports*, **21**(3), 9.

Fleckenstein, J.M., Hardwidge, P.R., Munson, G.P., Rasko, D.A., Sommerfelt, H. and Steinsland, H., 2010. Molecular mechanisms of enterotoxigenic *Escherichia coli* infection. *Microbes and Infection*, **12**(2), 89-98.

Frankel, G., Phillips, A.D., Rosenshine, I., Dougan, G., Kaper, J.B. and Knutton, S., 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Molecular Microbiology*, **30**(5), 911-921.

© University of Venda

Fröhlicher, E., Krause, G., Zweifel, C., Beutin, L. and Stephan, R., 2008. Characterization of attaching and effacing *Escherichia coli* (AEEC) isolated from pigs and sheep. *BMC Microbiology*, **8**(1), 144.

Fujiwara, S., Hashiba, H., Hirota, T. and Forstner, J.F., 2001. Inhibition of the binding of enterotoxigenic *Escherichia coli* Pb176 to human intestinal epithelial cell line HCT-8 by an extracellular protein fraction containing BIF of *Bifidobacterium longum* SBT2928: suggestive evidence of blocking of the binding receptor gangliotetraosylceramide on the cell surface. *International Journal of Food Microbiology*, **67**(1-2), 97-106.

Gaastra, W. and Svennerholm, A.M., 1996. Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). *Trends in Microbiology*, **4**(11), 444-452.

Gabay, C. and Kushner, I., 1999. Acute-phase proteins and other systemic responses to inflammation. *New England Journal of Medicine*, **340**(6), 448-454.

Galván-Moroyoqui, J.M., Del Carmen Domínguez-Robles, M., Franco, E. and Meza, I., 2008. The interplay between Entamoeba and enteropathogenic bacteria modulates epithelial cell damage. *PLoS Neglected Tropical Diseases*, **2**(7).

Garcia, B.G., Tadasuke Ooka, Yasuhiro Gotoh, Mônica AM Vieira, Denise Yamamoto, Yoshitoshi Ogura, Dennys M. Girão et al., 2016. Genetic relatedness and virulence properties of enteropathogenic *Escherichia coli* strains of serotype O119: H6 expressing localized adherence or localized and aggregative adherence-like patterns on HeLA cells. *International Journal Medical Microbiol*ogy*,* **306**(3), 152-164.

Gärtner, J.F. and Schmidt, M.A., 2004. Comparative analysis of locus of enterocyte effacement pathogenicity islands of atypical enteropathogenic *Escherichia coli*. *Infection and Immunity*, **72**(11), 6722-6728.

© University of Venda

Giallourou, N., Medlock, G.L., Bolick, D.T., Medeiros, P.H., Ledwaba, S.E., Kolling, G.L., Tung, K., Guerry, P., Swann, J.R. and Guerrant, R.L., 2018. A novel mouse model of *Campylobacter jejuni* enteropathy and diarrhea. *PLoS Pathogens*, **14**(3), e1007083.

Gill, R.K. and Hecht, G.A., 2018. Host-pathogen interactions in pathophysiology of diarrheal disorders. In *Physiology of the Gastrointestinal Tract* (1547-1577). Academic Press.

Giron, J. A., Ho, A. S. and Schoolnik, G. K., 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. *Science,* **254**, 710-713.

Goldhar, J., Zilberberg, A. and Ofek, I., 1986. Infant mouse model of adherence and colonization of intestinal tissues by enterotoxigenic strains of *Escherichia coli* isolated from humans. *Infection and Immunity*, **52**(1), 205-208.

Gomes, T.A., Elias, W.P., Scaletsky, I.C., Guth, B.E., Rodrigues, J.F., Piazza, R.M., Ferreira, L.C. and Martinez, M.B., 2016. Diarrheagenic *Escherichia coli*. *Brazilian Journal of Microbiology*, **47**, 3-30.

Grange, P.A. and Mouricout, M., 1996. Susceptibility of infant mice to F5 (K99) *E. coli* infection: Differences in glycosyltransferase activities in intestinal mucosa of inbred CBA and DBA/2 strains. *Glycoconjugate Journal*, **13**(1), 45-52.

Guerrant, R.L., Leite, A.M., Pinkerton, R., Medeiros, P.H., Cavalcante, P.A., DeBoer, M., Kosek, M., Duggan, C., Gewirtz, A., Kagan, J.C. and Gauthier, A.E., 2016. Biomarkers of environmental enteropathy, inflammation, stunting, and impaired growth in children in northeast Brazil. *PLoS One*, **11**(9), e0158772.

Guerrant, R.L., Oriá, R.B., Moore, S.R., Oriá, M.O. and Lima, A.A., 2008. Malnutrition as an enteric infectious disease with long-term effects on child development. *Nature Reviews*, **66**(9), 487-505.

© University of Venda

Guerrant, R.L., Walker, D.H. and Weller, P.F., 2011. In *Trop Infect Dis: Principles, Pathogens and Practice E-Book*. Elsevier Health Sciences.

Gupta, S.K., Keck, J., Ram, P.K., Crump, J.A., Miller, M.A. and Mintz, E.D., 2008. Part III. Analysis of data gaps pertaining to enterotoxigenic *Escherichia coli* infections in low and medium human development index countries, 1984–2005. *Epidemiology and Infection*, **136**(6), 721-738.

Hammerum, A.M. and Heuer, O.E., 2009. Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. *Clinical Infectious Diseases*, **48**(7), 916-921.

Harrington, S.M., Dudley, E.G. and Nataro, J.P., 2006. Pathogenesis of enteroaggregative *Escherichia coli* infection. *FEMS Microbiology Letters*, **254**(1), 12-18.

Harrington, S.M., Sheikh, J., Henderson, I.R., Ruiz-Perez, F., Cohen, P.S. and Nataro, J.P., 2009. The Pic protease of enteroaggregative *Escherichia coli* promotes intestinal colonization and growth in the presence of mucin. *Infection and Immunity*, **77**(6), 2465-2473.

Harro, C., Chakraborty, S., Feller, A., DeNearing, B., Cage, A., Ram, M., Lundgren, A., Svennerholm, A.M., Bourgeois, A.L., Walker, R.I. and Sack, D.A., 2011. Refinement of a human challenge model for evaluation of enterotoxigenic *Escherichia coli* vaccines. *Clinical and Vaccine Immunology*, **18**(10), 1719-1727.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Eugene, C.Y., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M. and Aderem, A., 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*, **410**(6832), 1099-1103.

Hazen, T.H., Michalski, J., Luo, Q., Shetty, A.C., Daugherty, S.C., Fleckenstein, J.M. and Rasko, D.A., 2017. Comparative genomics and transcriptomics of *Escherichia coli* isolates carrying virulence factors of both enteropathogenic and enterotoxigenic *E. coli*. *Scientific Reports*, **7**(1), 1-17.

© University of Venda

Hirche, T.O., Gaut, J.P., Heinecke, J.W. and Belaaouaj, A., 2005. Myeloperoxidase plays critical roles in killing *Klebsiella pneumoniae* and inactivating neutrophil elastase: effects on host defense. *Journal of Immunology*, **174**(3), 1557-1565.

Hirst, T.R. and Holmgren, J., 1987. Conformation of protein secreted across bacterial outer membranes: a study of enterotoxin translocation from *Vibrio cholerae*. *Proceedings of the National Academy of Science*, **84**(21), 7418-7422.

Hoffman, J.S. and Benditt, E.P., 1982. Changes in high density lipoprotein content following endotoxin administration in the mouse. Formation of serum amyloid protein-rich subfractions. *Journal of Biological Chemistry*, **257**(17), 10510-10517.

Huang, D.B., DuPont, H.L., Jiang, Z.D., Carlin, L. and Okhuysen, P.C., 2004. Interleukin-8 response in an intestinal HCT-8 cell line infected with enteroaggregative and enterotoxigenic *Escherichia coli*. *Clinical and Diagnostic Laboratory Immunology*, **11**(3), 548-551.

Huang, H.L., Chu, S.T. and Chen, Y.H., 1999. Ovarian steroids regulate 24p3 expression in mouse uterus during the natural estrous cycle and the preimplantation period. *Journal of Endocrinology*, **162**(1), 11-19.

Hwang, J., Mattei, L.M., VanArendonk, L.G., Meneely, P.M. and Okeke, I.N., 2010. A pathoadaptive deletion in an enteroaggregative *Escherichia coli* outbreak strain enhances virulence in a *Caenorhabditis elegans* model. *Infection and Immunity*, **78**(9), 4068-4076.

Iijima, Y., Oundo, J.O., Hibino, T., Saidi, S.M., Hinenoya, A., Osawa, K., Shirakawa, T., Osawa, R. and Yamasaki, S., 2017. High prevalence of diarrheagenic *Escherichia coli* among children with diarrhea in Kenya. *Japan Journal of Infectious Diseases*, **70**, 80-83.

Jaffer, U., Wade, R.G. and Gourlay, T., 2010. Cytokines in the systemic inflammatory response syndrome: a review. *HSR Proceedings in Intensive Care and Cardiovascular Anesthesia*, **2**(3), 161.

© University of Venda

Jønsson, R., Struve, C., Jenssen, H. and Krogfelt, K.A., 2017. The wax moth *Galleria mellonella* as a novel model system to study Enteroaggregative *Escherichia coli* pathogenesis. *Virulence*, **8**(8), 1894-1899.

Kang, G., Pulimood, A.B., Mathan, M.M. and Mathan, V.I., 2001. Enteroaggregative *Escherichia coli* infection in a rabbit model. *Pathology*, **33**(3), 341-346.

Kaper, J.B., Nataro, J.P. and Mobley, H.L., 2004. Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*, **2**(2), 123.

Kerneis, S., Bernet, M.F., Coconnier, M.H. and Servin, A.L., 1994. Adhesion of human enterotoxigenic *Escherichia coli* to human mucus secreting HT-29 cell subpopulations in culture. *Gut*, **35**(10), 1449-1454.

Kharat, V.B., Ahmed, M., Jiang, Z.D., Riddle, M.S. and DuPont, H.L., 2017. Colonization factors in enterotoxigenic *Escherichia coli* strains in travelers to Mexico, Guatemala, and India compared with children in Houston, Texas. *American Journal of Tropical Medicine and Hygiene*, **96**(1), 83-87.

Knutton, S., Baldwin, T., Williams, P.H. and McNeish, A.S., 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infection and Immunity*, **57**(4), 1290-1298.

Knutton, S., Lloyd, D.R. and McNeish, A.S., 1987. Identification of a new fimbrial structure in enterotoxigenic *Escherichia coli* (ETEC) serotype O148: H28 which adheres to human intestinal mucosa: a potentially new human ETEC colonization factor. *Infection and Immunity*, **55**(1), 86-92.

Kotloff, K.L., Nataro, J.P., Blackwelder, W.C., Nasrin, D., Farag, T.H., Panchalingam, S., Wu, Y., Sow, S.O., Sur, D., Breiman, R.F. and Faruque, A.S., 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global

© University of Venda

Enteric Multicenter Study, GEMS): a prospective, case-control study. *The Lancet*, **382**(9888), 209-222.

Kotloff, K.L., Platts-Mills, J.A., Nasrin, D., Roose, A., Blackwelder, W.C. and Levine, M.M., 2017. Global burden of diarrheal diseases among children in developing countries: Incidence, etiology, and insights from new molecular diagnostic techniques. *Vaccine*, **35**(49), 6783-6789.

Law, R.J., Gur-Arie, L., Rosenshine, I. and Finlay, B.B., 2013. *In vitro* and *in vivo* model systems for studying enteropathogenic *Escherichia coli* infections. *Cold Spring Harbor Perspectives in Medicine*, **3**(3), a009977.

Ledwaba, S.E., Kabue, J.P., Barnard, T.G., Traore, A.N. and Potgieter, N., 2018. Enteric pathogen co-infections in the paediatric population from rural communities in the Vhembe District, South Africa. *South African Journal of Child Health*, **12**(4), 170-174.

Leonard, S.R., Mammel, M.K., Rasko, D.A. and Lacher, D.W., 2016. Hybrid Shiga toxin-producing and enterotoxigenic *Escherichia* sp. cryptic lineage 1 strain 7v harbors a hybrid plasmid. *Applied Environmental Microbiology*, **82**(14), 4309-4319.

Leverton, L.Q. and Kaper, J.B., 2005. Temporal expression of enteropathogenic *Escherichia coli* virulence genes in an *in vitro* model of infection. *Infection and Immunity*, **73**(2), 1034-1043.

Lima, A.A., Soares, A.M., José Filho, Q.S., Havt, A., Lima, I.F., Lima, N.L., Abreu, C.B., Junior, F.S., Mota, R., Pan, W.K.Y. and Troeger, C., 2018. Enteroaggregative *Escherichia coli* subclinical infection and coinfections and impaired child growth in the MAL-ED Cohort Study. *Journal of Pediatric Gastroenterology and Nutrition*, **66**(2), 325-333.

Lindenthal, C. and Elsinghorst, E.A., 2001. Enterotoxigenic *Escherichia coli* TibA glycoprotein adheres to human intestine epithelial cells. *Infection and Immunity*, **69**(1), 52-57.

© University of Venda

Long, A., Garver, K.A. and Jones, S.R., 2019. Synergistic osmoregulatory dysfunction during salmon lice (*Lepeophtheirus salmonis*) and infectious hematopoietic necrosis virus co-infection in sockeye salmon (*Oncorhynchus nerka*) smolts. *Journal of Fish Diseases*, **42**(6), 869-882.

Luiz, W.B., Rodrigues, J.F., Crabb, J.H., Savarino, S.J. and Ferreira, L.C., 2015. Maternal vaccination with a fimbrial tip adhesin and passive protection of neonatal mice against lethal human enterotoxigenic *Escherichia coli* challenge. *Infection and Immunity,* **83**(12), 4555-4564.

Madhi, S.A., Huebner, R.E., Doedens, L., Aduc, T., Wesley, D. and Cooper, P.A., 2000. HIV-1 co-infection in children hospitalised with tuberculosis in South Africa. *International Journal of Tuberculosis and Lung Disease*, **4**(5), 448-454.

Malik, A., Nagy, B., Kugler, R. and Szmolka, A., 2017. Pathogenic potential and virulence genotypes of intestinal and faecal isolates of porcine post-weaning *enteropathogenic Escherichia coli*. *Research in Veterinary Science*, **115**, 102-108.

Manko, A., Motta, J.P., Cotton, J.A., Feener, T., Oyeyemi, A., Vallance, B.A., Wallace, J.L. and Buret, A.G., 2017. Giardia co-infection promotes the secretion of antimicrobial peptides beta-defensin 2 and trefoil factor 3 and attenuates attaching and effacing bacteria-induced intestinal disease. *PLoS One*, **12**(6).

Manthey, C.F., Autran, C.A., Eckmann, L. and Bode, L. 2014. Human milk oligosaccharides protect against enteropathogenic *Escherichia coli* attachment in vitro and EPEC colonization in suckling mice. *Journal of Pediatric Gastroenterology and Nutrition* **58**(2), 165-168.

Martinez-Argudo, I., Sands, C. and Jepson, M.A., 2007. Translocation of enteropathogenic *Escherichia coli* across an *in vitro* M cell model is regulated by its type III secretion system. *Cellular Microbiology*, **9**(6), 1538-1546.

© University of Venda

Mason, J., Iturriza-Gomara, M., O'Brien, S.J., Ngwira, B.M., Dove, W., Maiden, M.C. and Cunliffe, N.A., 2013. Campylobacter infection in children in Malawi is common and is frequently associated with enteric virus co-infections. *PLoS One*, **8**(3), e59663.

Mathew, S., Smatti, M.K., Al Ansari, K., Nasrallah, G.K., Al Thani, A.A. and Yassine, H.M., 2019. Mixed viral-bacterial infections and their effects on gut microbiota and clinical Illnesses in children. *Scientific Reports*, **9**(1), 1-12.

Mathewson, J.J., Salameh, B.M., DuPont, H.L., Jiang, Z.D., Nelson, A.C., Arduino, R., Smith, M.A. and Masozera, N., 1998. HEp-2 cell-adherent *Escherichia coli* and intestinal secretory immune response to human immunodeficiency virus (HIV) in outpatients with HIV-associated diarrhea. *Clinical and Diagnostic Laboratory Immunology*, **5**(1), 87-90.

May, G. and Nelson, P., 2014. Defensive mutualisms: do microbial interactions within hosts drive the evolution of defensive traits?. *Functional Ecology*, 356-363.

McNamara, B.P., Koutsouris, A., O'Connell, C.B., Nougayréde, J.P., Donnenberg, M.S. and Hecht, G., 2001. Translocated EspF protein from enteropathogenic *Escherichia coli* disrupts host intestinal barrier function. *Journal of Clinical Investigation*, **107**(5).621-629.

Medeiros, P., Bolick, D.T., Roche, J.K., Noronha, F., Pinheiro, C., Kolling, G.L., Lima, A. and Guerrant, R.L., 2013. The micronutrient zinc inhibits EAEC strain 042 adherence, biofilm formation, virulence gene expression, and epithelial cytokine responses benefiting the infected host. *Virulence,* **4**(7), 624-633.

Medeiros, P.H. Q.S., Ledwaba, S.E., Bolick, D.T., Giallourou, N., Yum, L.K., Costa, D.V., Oriá, R.B., Barry, E.M., Swann, J.R., Lima, A.Â.M. and Agaisse, H., 2019. A murine model of diarrhea, growth impairment and metabolic disturbances with *Shigella flexneri* infection and the role of zinc deficiency. *Gut microbes*, **10**(5), 615-630.

Medzhitov, R., 2008. Origin and physiological roles of inflammation. *Nature*, **454**(7203), 428-435.

© University of Venda

Meek, R.L., Eriksen, N. and Benditt, E.P., 1992. Murine serum amyloid A3 is a high density apolipoprotein and is secreted by macrophages. *Proceedings of the National Academy of Science*, **89**(17), 7949-7952.

Mellies, J.L., Barron, A.M., Haack, K.R., Korson, A.S. and Oldridge, D.A., 2006. The global regulator Ler is necessary for enteropathogenic *Escherichia coli* colonization of *Caenorhabditis elegans*. *Infection and Immunity,* **74**(1), 64-72.

Merritt, E.A., Pronk, S.E., Sixma, T.K., Kalk, K.H., van Zanten, B.A. and Hol, W.G., 1994. Structure of partially-activated *E. coli* heat-labile enterotoxin (LT) at 2.6 Å resolution. *FEBS letters*, **337**(1), 88-92.

Meyer, A., Corboz, L., Straumann-Kunz, U. and Pospischil, A., 1992. Infections with enteropathogenic *Escherichia coli* (EPEC) in cattle. 1. Comparison of different animal models and a cell culture system for the establishment of a detection system for" attaching and effacing"(AE) lesions. *Zentralblatt fur Veterinarmedizin. Reihe B. Journal of Veterinary Medicine. Series B*, **39**(8), 575-584.

Milon, A., Oswald, E. and De Rycke, J., 1999. Rabbit EPEC: a model for the study of enteropathogenic *Escherichia coli*. *Veterinary Research, BioMed Central,* **30**(2-3), hal-00902566

Mohamed, F.M., El Hendy, A.H. and El Shehedi, M.A., 2019. Weanling rabbit mortalities caused by enteropathogenic bacteria: Bacteriological and pathological investigation. *Journal of Applied Veterinary Science*, **4**(1), 18-29.

Mohamed, J.A., Huang, D.B., Jiang, Z.D., DuPont, H.L., Nataro, J.P., Belkind-Gerson, J. and Okhuysen, P.C., 2007. Association of putative enteroaggregative *Escherichia coli* virulence genes and biofilm production in isolates from travelers to developing countries. *Journal of Clinical Microbiology*, **45**(1), 121-126.

© University of Venda

Mooi, F.R. and De Graaf, F.K., 1985. Molecular biology of fimbriae of enterotoxigenic *Escherichia coli.* In *Genetic Approaches to Microbial Pathogenicity*, 119-138

Moon, H.W., Fung, P.Y., Isaacson, R.E. and Booth, G.D., 1979. Effects of age, ambient temperature, and heat-stable *Escherichia coli* enterotoxin on intestinal transit in infant mice. *Infection and Immunity*, **25**(1), 127-132.

Mora, A., Blanco, M., Yamamoto, D., Dahbi, G., Blanco, J.E., López, C., Alonso, M.P., Vieira, M.A., Hernandes, R.T., Abe, C.M. and Piazza, R.M., 2009. HeLA-cell adherence patterns and actin aggregation of enteropathogenic *Escherichia coli* (EPEC) and Shiga-toxin-producing *E. coli* (STEC) strains carrying different *eae* and *tir* alleles. *International Microbiology*, **12**, 243-251

Morampudi, V., Graef, F.A., Stahl, M., Dalwadi, U., Conlin, V.S., Huang, T., Vallance, B.A., Hong, B.Y. and Jacobson, K., 2017. Tricellular tight junction protein tricellulin is targeted by the enteropathogenic *Escherichia coli* effector EspG1, leading to epithelial barrier disruption. *Infection and Immunity*, **85**(1), e00700-16.

Morin, N., Tirling, C., Ivison, S.M., Kaur, A.P., Nataro, J.P. and Steiner, T.S., 2010. Autoactivation of the AggR regulator of enteroaggregative *Escherichia coli in vitro* and *in vivo*. *FEMS Immunology and Medical Microbiology*, **58**(3), 344-355.

Mosser, T., Talagrand-Reboul, E., Colston, S.M., Graf, J., Figueras, M.J., Jumas-Bilak, E. and Lamy, B., 2015. Exposure to pairs of Aeromonas strains enhances virulence in the *Caenorhabditis elegans* infection model. *Frontiers in Microbiology*, **6**, 1218.

Mundy, R., Girard, F., FitzGerald, A.J. and Frankel, G., 2006. Comparison of colonization dynamics and pathology of mice infected with enteropathogenic *Escherichia coli*, enterohaemorrhagic *E. coli* and *Citrobacter rodentium*. *FEMS Microbiology Letters*, **265**(1), 126-132.

© University of Venda

Mundy, R., MacDonald, T.T., Dougan, G., Frankel, G. and Wiles, S., 2005. *Citrobacter rodentium* of mice and man. *Cellular Microbiology*, **7**(12), 1697-1706.

Munera, D., Ritchie, J.M., Hatzios, S.K., Bronson, R., Fang, G., Schadt, E.E., Davis, B.M. and Waldor, M.K., 2014. Autotransporters but not pAA are critical for rabbit colonization by Shiga toxin-producing *Escherichia coli* O104: H4. *Nature Communications*, **5**, 3080.

Mylonakis, E. and Aballay, A., 2005. Worms and flies as genetically tractable animal models to study host-pathogen interactions. *Infection and Immunity*, **73**(7), 3833-3841.

Nataro, J.P., Deng, Y.I.K.A.N.G., Maneval, D.R., German, A.L., Martin, W.C. and Levine, M.M., 1992. Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. *Infection and Immunity*, **60**(6), 2297-2304.

Nataro, J.P., Hicks, S., Phillips, A.D., Vial, P.A. and Sears, C.L., 1996. T84 cells in culture as a model for enteroaggregative *Escherichia coli* pathogenesis. *Infection and Immunity*, **64**(11), 4761-4768.

Nataro, J.P., Yikang, D., Cookson, S., Cravioto, A., Savarino, S.J., Guers, L.D., Levine, M.M. and Tacket, C.O., 1995. Heterogeneity of enteroaggregative *Escherichia Coli* virulence demonstrated. *Journal of Infectious Diseases*, **171**(2), 465-468.

Nathan, C., 2006. Neutrophils and immunity: challenges and opportunities. *Nature Reviews Immunology*, **6**(3), 173-182.

Navarro-García, F., Sears, C., Eslava, C., Cravioto, A. and Nataro, J.P., 1999. Cytoskeletal effects induced by Pet, the serine protease enterotoxin of enteroaggregative *Escherichia coli*. *Infection and Immunity*, **67**(5), 2184-2192.

Neurath, M.F., 2014. Cytokines in inflammatory bowel disease. *Nature Reviews Immunology*, **14**(5), 329-342.

© University of Venda

Nieto-Pelegrin, E. and Martinez-Quiles, N., 2009. Distinct phosphorylation requirements regulate cortactin activation by Tir EPEC and its binding to N-WASP. *Cell Communication and Signalling*, **7**(1), 11.

Nisa, S., Scanlon, K.M. and Donnenberg, M.S., 2013. Enteropathogenic *Escherichia coli*. In *Escherichia coli,* 75-119. Academic Press.

Norton, E.B., Branco, L.M. and Clements, J.D., 2015. Evaluating the A-subunit of the heat-labile toxin (LT) as an immunogen and a protective antigen against enterotoxigenic *Escherichia coli* (ETEC). *PLoS One*, **10**(8), e0136302.

Okhuysen, P.C. and DuPont, H.L., 2010. Enteroaggregative *Escherichia coli* (EAEC): a cause of acute and persistent diarrhea of worldwide importance. *Journal of Infectious Diseases,* **202**(4), 503-505.

Oloomi, M., Bouzari, S. and Emami, S., 2009. A recombinant hybrid peptide composed of AAF adhesin of enteroaggregative *Escherichia coli* and Shiga toxin B subunit elicits protective immune response in mice. *European Journal of Clinical Microbiology and Infectious Diseases*, **28**(11), 1311-1316.

Opintan, J.A., Newman, M.J., Ayeh-Kumi, P.F., Affrim, R., Gepi-Attee, R., Sevilleja, J.E., Roche, J.K., Nataro, J.P., Warren, C.A. and Guerrant, R.L., 2010. Pediatric diarrhea in southern Ghana: etiology and association with intestinal inflammation and malnutrition. *American Journal of Tropical Medicine and Hygiene*, **83**(4), 936-943.

Ortega, A.F., Martínez-Castañeda, J.S., Bautista-Gómez, L.G., Muñoz, R.F. and Hernández, I.Q., 2017. Identification of co-infection by rotavirus and parvovirus in dogs with gastroenteritis in Mexico. *Brazilian Journal of Microbiology*, **48**(4), 769-773.

Owen, H.C., Roberts, S.J., Ahmed, S.F. and Farquharson, C., 2008. Dexamethasone-induced expression of the glucocorticoid response gene lipocalin 2 in chondrocytes. *American Journal of Physiology-Endocrinology and Metabolism*, **294**(6), E1023-E1034.

© University of Venda

Pelayo, J.S., Scaletsky, I.C.A., Pedroso, M.Z., Sperandio, V., Giron, J.A., Frankel, G. and Trabulsi, L.R., 1999. Virulence properties of atypical EPEC strains. *Journal of Medical Microbiology*, **48**(1), 41-49.

Pepys, M.B. and Baltz, M.L., 1983. Acute phase proteins with special reference to C-reactive protein and related proteins (pentaxins) and serum amyloid A protein. *Advanced Immunology,* **34**, 141-212. Academic Press.

Pepys, M.B. and Hirschfield, G.M., 2003. C-reactive protein: a critical update. *Journal of Clinical Investigation*, **111**(12), 1805-1812.

Pereira, A.C.M., Britto-Filho, J.D., de Carvalho, J.J., de Luna, M.D.G. and Rosa, A.C.P., 2008. Enteroaggregative *Escherichia coli* (EAEC) strains enter and survive within cultured intestinal epithelial cells. *Microbial Pathogens*, **45**(5-6), 310-314.

Pereira, A.L., Silva, T.N., Gomes, A.C., Araújo, A.C. and Giugliano, L.G., 2010. Diarrhea-associated biofilm formed by enteroaggregative *Escherichia coli* and aggregative *Citrobacter freundii*: a consortium mediated by putative F pili. *BMC Microbiology*, **10**(1), 57.

Philipson, C.W., Bassaganya-Riera, J. and Hontecillas, R., 2013. Animal models of enteroaggregative *Escherichia coli* infection. *Gut Microbes*, **4**(4), 281-291.

Philpott, D.J., McKay, D.M., Sherman, P.M. and Perdue, M.H., 1996. Infection of T84 cells with enteropathogenic *Escherichia coli* alters barrier and transport functions. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, **270**(4), G634-G645.

Platts-Mills, J.A., Babji, S., Bodhidatta, L., Gratz, J., Haque, R., Havt, A., McCormick, B.J., McGrath, M., Olortegui, M.P., Samie, A. and Shakoor, S., 2015. Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). *The Lancet Global health*, **3**(9), e564-e575.

© University of Venda

Platts-Mills, J.A., Liu, J., Rogawski, E.T., Kabir, F., Lertsethtakarn, P., Siguas, M., Khan, S.S., Praharaj, I., Murei, A., Nshama, R. and Mujaga, B., 2018. Use of quantitative molecular diagnostic methods to assess the aetiology, burden, and clinical characteristics of diarrhoea in children in low-resource settings: a reanalysis of the MAL-ED cohort study. *The Lancet Global Health*, **6**(12), e1309-e1318.

Prata, M.D.M.G., Havt, A., Bolick, D.T., Pinkerton, R., Lima, A.A.M. and Guerrant, R.L., 2016. Comparisons between myeloperoxidase, lactoferrin, calprotectin and lipocalin-2, as fecal biomarkers of intestinal inflammation in malnourished children. *Journal of Translational Science*, **2**(2), 134.

Prokopowicz, Z., Marcinkiewicz, J., Katz, D.R. and Chain, B.M., 2012. Neutrophil myeloperoxidase: soldier and statesman *Archivum immunologiae et therapiae experimentalis*, **60**(1), 43-54.

Puthenedam, M., Williams, P.H., Lakshmi, B.S. and Balakrishnan, A., 2007. Modulation of tight junction barrier function by outer membrane proteins of enteropathogenic *Escherichia coli*: Role of F-actin and junctional adhesion molecule-1. *Cell Biology International*, **31**(8), 836-844.

Qadri, F., Saha, A., Ahmed, T., Al Tarique, A., Begum, Y.A. and Svennerholm, A.M., 2007. Disease burden due to enterotoxigenic *Escherichia coli* in the first 2 years of life in an urban community in Bangladesh. *Infection and Immunity*, **75**(8), 3961-3968.

Ren, W., Yin, J., Duan, J., Liu, G., Zhu, X., Chen, S., Li, T., Wang, S., Tang, Y. and Hardwidge, P.R., 2014. Mouse intestinal innate immune responses altered by enterotoxigenic *Escherichia coli* (ETEC) infection. *Microbes and Infection*, **16**(11), 954-961.

Reynolds, L.A., Redpath, S.A., Yurist-Doutsch, S., Gill, N., Brown, E.M., van der Heijden, J., Brosschot, T.P., Han, J., Marshall, N.C., Woodward, S.E. and Valdez, Y., 2017. Enteric

© University of Venda

helminths promote Salmonella coinfection by altering the intestinal metabolome. *Journal of Infectious Diseases,* **215**(8), 1245-1254.

Rhee, K.J., Cheng, H., Harris, A., Morin, C., Kaper, J.B. and Hecht, G., 2011. Determination of spatial and temporal colonization of enteropathogenic *E. coli* and enterohemorrhagic *E. coli* in mice using bioluminescent *in vivo* imaging. *Gut Microbes*, **2**(1), 34-41.

Roberts, L.D., Souza, A.L., Gerszten, R.E. and Clish, C.B., 2012. Targeted metabolomics. *Current Protocols in Molecular Biology*, **98**(1), 30-2.

Roche, J.K., Cabel, A., Sevilleja, J., Nataro, J. and Guerrant, R.L., 2010. Enteroaggregative *Escherichia coli* (EAEC) impairs growth while malnutrition worsens EAEC infection: a novel murine model of the infection malnutrition cycle. *Journal of Infectious Diseases*, **202**(4), 506-514.

Rogawski, E.T., Guerrant, R.L., Havt, A., Lima, I.F., Medeiros, P.H., Seidman, J.C., McCormick, B.J., Babji, S., Hariraju, D., Bodhidatta, L. and Shrestha, J., 2017. Epidemiology of enteroaggregative *Escherichia coli* infections and associated outcomes in the MAL-ED birth cohort. *PLoS Neglected Tropical Diseases*, **11**(7), e0005798.

Royan, S.V., Jones, R.M., Koutsouris, A., Roxas, J.L., Falzari, K., Weflen, A.W., Kim, A., Bellmeyer, A., Turner, J.R., Neish, A.S. and Rhee, K.J., 2010. Enteropathogenic *E. coli* non-LEE encoded effectors NleH1 and NleH2 attenuate NF-κB activation. *Molecular Microbiology*, **78**(5), 1232-1245.

Ruan, X., Sack, D.A. and Zhang, W., 2015. Genetic fusions of a CFA/I/II/IV MEFA (multiepitope fusion antigen) and a toxoid fusion of heat-stable toxin (STa) and heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC) retain broad anti-CFA and antitoxin antigenicity. *PLoS One*, **10**(3).

© University of Venda

Ruchaud-Sparagano, M.H., Maresca, M. and Kenny, B., 2007. Enteropathogenic *Escherichia coli* (EPEC) inactivate innate immune responses prior to compromising epithelial barrier function. *Cellular Microbiology*, **9**(8), 1909-1921.

Ruiz, R.C., Melo, K., Rossato, S.S., Barbosa, C.M., Corrêa, L.M., Elias, W.P. and Piazza, R.M., 2014. Atypical enteropathogenic *Escherichia coli* secretes plasmid encoded toxin. *BioMed Research International*, **2014**.

Saiki, T., 1998. Myeloperoxidase concentrations in the stool as a new parameter of inflammatory bowel disease. *Kurume Medical Journal*, **45**(1), 69-73.

Sanchez-Villamil, J., Tapia-Pastrana, G. and Navarro-Garcia, F., 2016. Pathogenic lifestyles of *E. coli* pathotypes in a standardized epithelial cell model influence inflammatory signaling pathways and cytokines secretion. *Frontiers in Cellular and Infection Microbiology*, **6**, 120.

Sannes, M.R., Kuskowski, M.A., Owens, K., Gajewski, A. and Johnson, J.R., 2004. Virulence factor profiles and phylogenetic background of *Escherichia coli* isolates from veterans with bacteremia and uninfected control subjects. *Journal of Infectious Diseases*, **190**(12), 2121-2128.

Savkovic, S.D., Villanueva, J., Turner, J.R., Matkowskyj, K.A. and Hecht, G., 2005. Mouse model of enteropathogenic *Escherichia coli* infection. *Infection and Immunity*, **73**(2), 1161-1170.

Scaletsky, I.C., Silva, M.L. and Trabulsi, L.R., 1984. Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLA cells. *Infection and Immunity*, **45**(2), 534-536.

Scaletsky, I.C.A., 2019. Enteropathogenic *Escherichia coli*. In *The Universe of Escherichia coli*. IntechOpen.

© University of Venda

Sébédio, J.L., 2017. Metabolomics, nutrition, and potential biomarkers of food quality, intake, and health status. In *Advances in Food and Nutrition Research* **82**, 83-116. Academic Press.

Shah, C., Hari-Dass, R. and Raynes, J.G., 2006. Serum amyloid A is an innate immune opsonin for gram-negative bacteria. *Blood*, **108**(5), 1751-1757.

Sheikh, J., Czeczulin, J.R., Harrington, S., Hicks, S., Henderson, I.R., Le Bouguénec, C., Gounon, P., Phillips, A. and Nataro, J.P., 2002. A novel dispersin protein in enteroaggregative *Escherichia coli*. *Journal of Clinical Investigation*, **110**(9), 1329-1337.

Shen, S.S., Qu, X.Y., Zhang, W.Z., Li, J. and Lv, Z.Y., 2019. Infection against infection: parasite antagonism against parasites, viruses and bacteria. *Infectious Disease of Poverty*, **8**(1), 49.

Shifflett, D. E., Clayburgh, D. R., Koutsouris, A., Turner, J. R. and Hecht, G. A., 2005. Enteropathogenic *E. coli* disrupts tight junction barrier function and structure *in vivo*. *Laboratory Investigation*, **85**(1), 1308-1324

Shrivastava, A.K., Kumar, S., Mohakud, N.K., Suar, M. and Sahu, P.S., 2017. Multiple etiologies of infectious diarrhea and concurrent infections in a pediatric outpatient-based screening study in Odisha, India. *Gut Pathogens*, **9**(1), 16.

Sithara, M.S., George, A.J., Vijayan, N., Manomohan, C.B. and Mani, B.K., 2017. *In vivo* mouse pathogenicity study of *E. coli* isolated from porcine gastrointestinal tract. *Indian Journal of Veterinary Sciences & Biotechnology*, **12**(3).

Sixma, T.K., Pronk, S.E., Kalk, K.H., Wartna, E.S., van Zanten, B.A., Witholt, B. and Hoi, W.G., 1991. Crystal structure of a cholera toxin-related heat-labile enterotoxin from *E. coli*. *Nature*, **351**(6325), 371.

© University of Venda

Spira, W.M., Sack, R.B. and Froehlich, J.L., 1981. Simple adult rabbit model for *Vibrio cholerae* and enterotoxigenic *Escherichia coli* diarrhea. *Infection and Immunity*, **32**(2), 739-747.

Steiner, T.S., Nataro, J.P., Poteet-Smith, C.E., Smith, J.A. and Guerrant, R.L., 2000. Enteroaggregative *Escherichia coli* expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells. *Journal of Clinical Investigation*, **105**(12), 1769-1777.

Susi, H., Barrès, B., Vale, P.F. and Laine, A.L., 2015. Co-infection alters population dynamics of infectious disease. *Nature Communications*, **6**(1), 1-8.

Svennerholm, A.M., Wennerås, C., Holmgren, J., McConnell, M.M. and Rowe, B., 1990. Roles of different coli surface antigens of colonization factor antigen II in colonization by and protective immunogenicity of enterotoxigenic *Escherichia coli* in rabbits. *Infection and Immunity*, **58**(2), 341-346.

Tacket, C.O., Sztein, M.B., Losonsky, G., Abe, A., Finlay, B.B., McNamara, B.P., Fantry, G.T., James, S.P., Nataro, J.P., Levine, M.M. and Donnenberg, M.S., 2000. Role of EspB in Experimental human enteropathogenic *Escherichia coli* infection. *Infection and Immunity*, **68**(6), 3689-3695.

Takeuchi, O. and Akira, S., 2010. Pattern recognition receptors and inflammation. *Cell*, **140**(6), 805-820.

Tapia, R., Kralicek, S.E. and Hecht, G.A., 2017. EPEC effector EspF promotes Crumbs3 endocytosis and disrupts epithelial cell polarity. *Cellular Microbiology*, **19**(11), e12757.

Tareen, A.M., Ullah, K., Asmat, T.M., Samad, A., Iqbal, A., Mustafa, M.Z. and Ahmad, I., 2019. Incidence of Diarrheagenic *Escherichia coli* pathotypes in children suffering from diarrhea in tertiary care hospitals, Quetta, Pakistan. *Pakistan Journal of Zoology*, **51**(6).

© University of Venda

Tauschek, M., Gorrell, R.J., Strugnell, R.A. and Robins-Browne, R.M., 2002. Identification of a protein secretory pathway for the secretion of heat-labile enterotoxin by an enterotoxigenic strain of *Escherichia coli*. *Proceedings of the National Academy of Science*, **99**(10), 7066-7071.

Telli, M., Guiral, E., Martínez, J.A., Almela, M., Bosch, J., Vila, J. and Soto, S.M., 2010. Prevalence of enterotoxins among *Escherichia coli* isolates causing bacteraemia. *FEMS Microbiology Letters*, **306**(2), 117-121.

Tobias, J., Kassem, E., Rubinstein, U., Bialik, A., Vutukuru, S.R., Navaro, A., Rokney, A., Valinsky, L., Ephros, M., Cohen, D. and Muhsen, K., 2015. Involvement of main diarrheagenic *Escherichia coli*, with emphasis on enteroaggregative *E. coli*, in severe non-epidemic pediatric diarrhea in a high-income country. *BMC Infectious Diseases*, **15**(1), 79.

Toledo, C.C., Arvidsson, I. and Karpman, D., 2011. Cross-reactive protection against enterohemorrhagic *Escherichia coli* infection by enteropathogenic *E. coli* in a mouse model. *Infection and Immunity*, **79**(6), 2224-2233.

Torres, F., 2015. Viral co-infections in pediatric patients hospitalized with lower tract acute respiratory infections. *PLoS One*, **10**(9).

Torres, A.G., Zhou, X. and Kaper, J.B., 2005. Adherence of diarrheagenic *Escherichia coli* strains to epithelial cells. *Infection and Immunity*, **73**(1), 18-29.

Trabulsi, L.R., Keller, R. and Gomes, T.A.T., 2002. Typical and atypical enteropathogenic *Escherichia coli*. *Emerging Infectious Diseases*, **8**(5), 508.

Tsai, S.C., Noda, M., Adamik, R., Moss, J. and Vaughan, M., 1987. Enhancement of choleragen ADP-ribosyltransferase activities by guanyl nucleotides and a 19-kDa membrane protein. *Proceedings of the National Academy of Science*, **84**(15), 5139-5142.

© University of Venda

Tzipori, S., Montanaro, J., Robins-Browne, R.M., Vial, P., Gibson, R. and Levine, M.M., 1992. Studies with enteroaggregative *Escherichia coli* in the gnotobiotic piglet gastroenteritis model. *Infection and Immunity*, **60**(12), 5302-5306.

Uhlar, C.M. and Whitehead, A.S., 1999. Serum amyloid A, the major vertebrate acute-phase reactant. *European Journal of Biochemistry*, **265**(2), 501-523.

Vallance, B.A. and Finlay, B.B., 2000. Exploitation of host cells by enteropathogenic Escherichia coli. *Proceedings of the National Academy of Sciences*, **97**(16), 8799-8806.

Venugopal, S.K., Devaraj, S., Yuhanna, I., Shaul, P. and Jialal, I., 2002. Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. *Circulation*, **106**(12), 1439-1441.

Vial, P.A., Robins-Browne, R., Lior, H., Prado, V., Kaper, J.B., Nataro, J.P., Maneval, D., Elsayed, A.E.D. and Levine, M.M., 1988. Characterization of enteroadherent-aggregative *Escherichia coli*, a putative agent of diarrheal disease. *Journal of Infectious Diseases*, **158**(1), 70-79.

Vieira, M.A., Dias, R.C., dos Santos, L.F., Rall, V.L., Gomes, T.A. and Hernandes, R.T., 2019. Diversity of strategies used by atypical enteropathogenic *Escherichia coli* to induce attaching and effacing lesion in epithelial cells. *Journal of Medical Microbiology*, **68**(6), 940-951.

Vilchez, S., Reyes, D., Paniagua, M., Bucardo, F., Möllby, R. and Weintraub, A., 2009. Prevalence of diarrhoeagenic *Escherichia coli* in children from Leon, Nicaragua. *Journal of Medical Microbiology*, **58**(5), 630-637.

Wada, Y., Nakaoka, Y., Kondo, H., Nakazawa, M. and Kubo, M., 1996. Dual infection with attaching and effacing *Escherichia coli* and enterotoxigenic *Escherichia coli* in post-weaning pigs. *Journal of Comparative Pathology*, **114**(1), 93-99.

© University of Venda

Wagner, M., Peterson, C.G., Ridefelt, P., Sangfelt, P. and Carlson, M., 2008. Fecal markers of inflammation used as surrogate markers for treatment outcome in relapsing inflammatory bowel disease. *World Journal of Gastroenterology*, **14**(36), 5584.

Wang, X., Gao, X. and Hardwidge, P.R., 2012. Heat-labile enterotoxin-induced activation of NF-κB and MAPK pathways in intestinal epithelial cells impacts enterotoxigenic *Escherichia coli* (ETEC) adherence. *Cellular Microbiology*, **14**(8), 1231-1241.

Weiglmeier, P.R., Rösch, P. and Berkner, H., 2010. Cure and curse: *E. coli* heat-stable enterotoxin and its receptor guanylyl cyclase C. *Toxins*, **2**(9), 2213-2229.

Whipp, S.C., 1990. Assay for enterotoxigenic *Escherichia coli* heat-stable toxin b in rats and mice. *Infection and Immunity*, **58**(4), 930-934.

Yatsuyanagi, J., Saito, S., Sato, H., Miyajima, Y., Amano, K.I. and Enomoto, K., 2002. Characterization of enteropathogenic and enteroaggregative *Escherichia coli* isolated from diarrheal outbreaks. *Journal of Clinical Microbiology*, **40**(1), 294-297.

Zhang, J.M. and An, J., 2007. Cytokines, inflammation and pain. *International Anaesthesiology Clinics*, **45**(2), 27.

Zhang, Q., Li, Q., Wang, C., Liu, X., Li, N. and Li, J. 2010. Enteropathogenic *Escherichia coli* changes distribution of occludin and ZO-1 in tight junction membrane microdomains in vivo. *Microbial Pathogens,* **48**, 28-34.

Zhang, S.X., Zhou, Y.M., Xu, W., Tian, L.G., Chen, J.X., Chen, S.H., Dang, Z.S., Gu, W.P., Yin, J.W., Serrano, E. and Zhou, X.N., 2016. Impact of co-infections with enteric pathogens on children suffering from acute diarrhea in southwest China. *Infectious Disease of Poverty*, **5**(1), 64.

Zhang, W., Robertson, D.C., Zhang, C., Bai, W., Zhao, M. and Francis, D.H., 2008. *Escherichia coli* constructs expressing human or porcine enterotoxins induce identical

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diarrheal diseases in a piglet infection model. *Applied Environmental Microbiology*, **74**(18), 5832-5837.

Zhao, Z.P., Yang, Z., Lin, W.D., Wang, W.Y., Yang, J., Jin, W.J. and Qin, A.J., 2016. The rate of co-infection for piglet diarrhea viruses in China and the genetic characterization of porcine epidemic diarrhea virus and porcine kobuvirus. *Acta Virologica*, **60**(1), 55-61.

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CHAPTER 3

OBJECTIVE 1: **TO DEVELOP AN INFECTIOUS EPEC MURINE MODEL USING C57BL/6 MICE TREATED WITH ANTIBIOTIC COCKTAIL**

3.1. ABSTRACT

EPEC are recognized as one of the leading bacterial causes of infantile diarrhoea worldwide. Weaned C57BL/6 mice pretreated with antibiotics were challenged orally with wild type EPEC, *bfpA* and *escN* mutants (lacking bundle forming pili or type 3 secretion system) to determine colonization, inflammatory responses and clinical outcomes during infection. Antibiotic disruption of intestinal microbiota led to efficient colonization by wild type EPEC and the *bfpA* mutant, resulting in weight loss. Increases in inflammatory biomarkers were observed in intestinal tissues. The *escN* mutant colonized the mice, however, no weight changes or inflammatory biomarkers were observed. Metabolome changes were also observed in EPEC infected mice with changes in TCA cycle intermediates, increased creatine excretion and shifts in gut microbial metabolite levels. In conclusion, a murine infection model treated with antibiotics has been developed to partially mimic clinical outcomes as seen in children with EPEC infection and to examine potential roles of selected virulence traits. This model can help in further understanding mechanisms involved in the pathogenesis of EPEC infections and thus assist in the development of potential preventive or therapeutic interventions.

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3.2. INTRODUCTION

Gastroenteritis remains a major cause of morbidity and mortality in young children especially in developing countries (Liu et al, 2015). EPEC has been recognized by the GEMS and MAL-ED studies as one of the major causes of moderate to severe diarrhoea in children (Kotloff et al, 2013; Platts-Mills et al, 2015). Infection results in acute watery diarrhoea accompanied by fever, vomiting and dehydration (Kaper et al, 2004; Guerrant et al, 2011).

EPEC contains the *Ler* gene, a major transcriptional activator of LEE open reading frames (Frankel et al, 1998; Friedberg et al, 1999). EPEC virulence is mediated by the T3SS and is characterized by formation of A/E lesions (Frankel et al, 1998) and consists of esc and esp which assist in virulence. In addition, *escN* is the main driving force assisting in the ATPase response to enable activation of the T3SS, for efficient transportation of effector proteins into the enterocytes of the host (Andrade et al, 2007). During infection, EPEC attaches to epithelial cells via bfp (Giron et al, 1991) followed by intimate adherence with the aid of the *tir* and *eae*, which results in actin accumulation and formation of pedestal structures at the site of infection (Knutton et al, 1989; Guerrant et al, 2011). EPEC is characterized by the presence (typical EPEC) or absence (atypical EPEC) of *bfp*. Typical EPEC are characterized by LA pattern *in vitro* (Kaper et al, 2004) and also reported to cause severe diarrhoea in children under 12 months of age and in certain cases results in death (Kotloff et al, 2013; Platts-Mills et al, 2015). Atypical EPEC is characterized by LA-like (Scaletsky et al, 2010), AA or DA patterns *in vitro* (Pelayo et al, 1999; Mora et al, 2009) and are increasingly being detected in children worldwide (Abe et al, 2009; Hu and Torres, 2015).

Pathogens such as EPEC and EHEC compete with the resident microbiota for nutrients in order to colonize the intestinal environment. According to Freter's nutrient niche, in order for microbes to be successful, it must have the capacity to grow fast in the intestine compared to its competitors (Freter et al, 1983). These pathogens require the same

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carbon pathways which commensal *E. coli* uses, such as mannose and galactose *in vivo* (Fabich et al, 2008).

EPEC have been studied extensively *in vitro,* and are considered good models for studying LA traits, A/E formation and expression of the T3SS effector proteins (Giron et al, 1991; Knotton et al, 1999; Leverton and Kaper, 2005). The *in vivo* studies have shown that a complete intestinal environment is key in determining the specific roles of EPEC traits and infections in animals and humans (Law et al, 2013). Animal models such as *C. elegans*, rabbits, pigs, and cattle have been used to study EPEC infections (Moon et al, 1983; Larsen et al, 1995; Dean-Nystrom et al, 2002; Misyurina et al, 2010). Infections induced by EPEC in C57BL/6 mouse models have also been reported (Savkovic et al, 2005; Royan et al, 2010; Zhang et al, 2010; Rhee et al, 2011; Manthey et al, 2014; Dupont et al, 2016), showing colonization of EPEC in the intestinal epithelial microvilli (Savkovic et al, 2005), changes in tight junction morphology and epithelial barrier function accompanied by inflammatory responses (Zhang et al, 2010). These animal models have provided insights in the understanding of potential pathogenetic mechanisms of EPEC infection in humans. However, these models have not been able to replicate clinical outcomes observed in humans. This section describes a murine model in which the microbiota have been disrupted via broad-spectrum antibiotic cocktail to enable efficient colonization and clinical outcomes of EPEC infection in weaned mice.

3.3. MATERIALS AND METHODS

3.3.1. ANIMAL HUSBANDRY

The mice used in the study have been handled with strict accordance with the approvals from the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010) of the National Institutes of Health. This project has been registered and approved by the University of Venda higher degree committee. The protocol has been accepted by

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the Committee on the Ethics of Animal Experiments of the University of Virginia (Protocol Number: 3315). All efforts were taken in order to reduce animal distress. This is also in accordance with the Institutional Animal Care and Use Committee policies of the University of Virginia. The University of Virginia is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). 22-day old, male C57BL/6 mice strains were ordered from Jackson Laboratories (Jackson Laboratories, Bar Harbor, Maine, USA). Upon arrival, mice weight was recorded at approximately 10 grams and were co-housed in groups of up to 4 animals per cage. The temperature of the vivarium was kept between 20-23°C with a 14-hour light and 10-hour dark cycle.

3.3.2. RODENT DIET AND ANTIBIOTICS

Mice were allowed to acclimate for 3 days upon arrival. Mice were fed standard rodent House Chow diet upon arrival and throughout the infection challenge. Four days prior to challenge with EPEC, mice were given an antibiotic cocktail of gentamicin (35 mg/L), vancomycin (45 mg/L), metronidazole (215 mg/L), and colistin (850 U/mL) in drinking water for 3 days in order to disrupt resident microbiota, followed by 1 day on normal water in order to clear antibiotics (Bolick et al, 2018).

3.3.3. PREPARATION OF EPEC FOR MOUSE INFECTION

Bacterial strains used included: WT EPEC E2348/69 (O127:H6) (Levine et al, 1985), EPEC E2348/69 *bfpA*- UMD901(Zhang and Donnenberg, 1996) and EPEC E2348/69 ∆*escN* CVD425 (Jarvis et al, 1995). Bacterial cultures were prepared from glycerol stocks maintained at -80 °C. Cultures were grown in 20 mL Dulbecco's modified Eagle's medium (DMEM) containing phenol red glucose (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 37 °C in a shaking incubator until cultures turned orange indicating optimal growth. Cultures were centrifuged at 3500 *x g* for 10 min at 4 °C. The bacterial pellet was resuspended in DMEM high glucose in order to obtain 10^{10} CFU/mL. The mice were administered 100 µL of 10^{10} CFU/mL (each mouse received 10^9 CFU in a

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total volume of 10^{10} CFU/mL) bacterial culture in DMEM high glucose orally using 22-gauge feeding needles. Uninfected control mice were administered only 100µl DMEM high glucose.

During infection, all mice were weighed and stools were collected daily. The study duration ranged between 3- and 8-days post infection (pi). For intestinal tissue burden, some mice were euthanized at day 3 and 8 pi. Figure 3.1 shows the schematic presentation summarizing the experimental procedure. At day 3 pi, blood was immediately collected via cardiac puncture and placed in 1.5 mL eppendorf tube. The blood was then centrifuged at 8000 x g for 5 min at 4 °C. The serum was collected and placed at -80 °C. At days 3 and 8 pi, the intestinal tissues (duodenum, jejunum, ileum and colon) were immediately collected after euthanasia and placed in cryotubes. These samples were then flash frozen in liquid nitrogen and stored at -80 °C until further analysis.

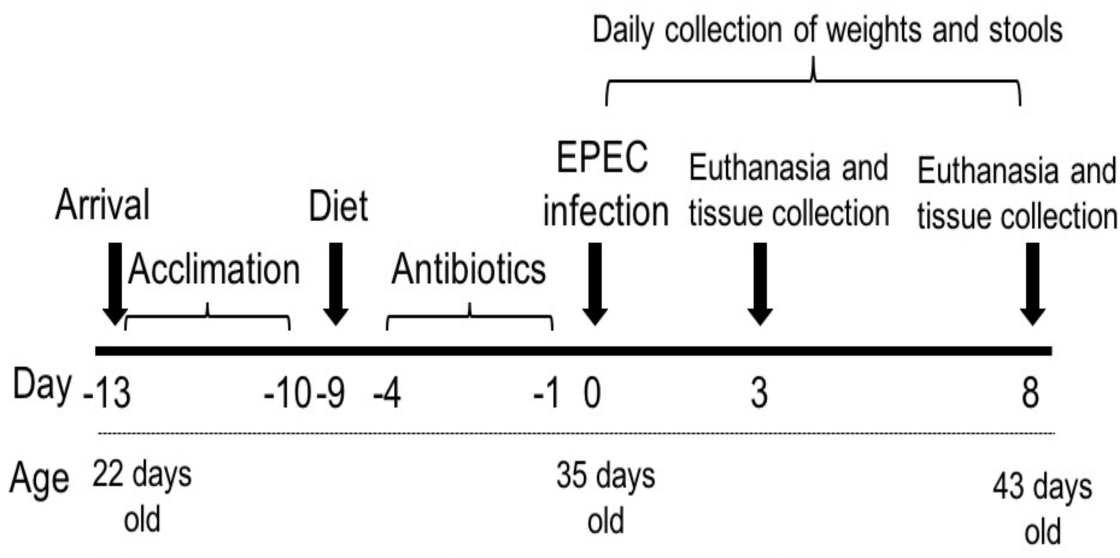

Figure 3.1: Schematic presentation of the animal experimental procedure with the age of mice, and the infection cycle. 22-day-old C57BL/6 mice were placed on standard rodent chow diet. Mice were administered drinking water containing antibiotic cocktail 4 days prior infection, and clean water was replaced at 1-day prior infection. Mice were orally infected with either WT EPEC, *bfpA*-

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or Δ*escN* strains and followed daily for changes in weights and stool collection. Mice were euthanised at days 3 and 8 pi.

3.3.4. STOOL SHEDDING AND TISSUE BURDEN

For stool shedding, DNA extraction from stool specimen was performed using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For tissue burden, tissues containing tissue lysis buffer, ATL buffer (Qiagen, Hilden, Germany) were homogenized using the beat-beater and DNA was extracted using DNeasy blood and tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The *eae* primer was used as a specific target for detecting EPEC in stools and tissues. Primer sequences included *eae* 5'-CCCGAATTCGGCACAAGCATAAGC-3' (sense) and 5'-CCCGGATCCGTCTCGCCAGTATTCG-3' (antisense) (Zhang et al, 2002). Real-time PCR was performed using Bio-Rad CFX connect real-time PCR (Bio-Rad, Hercules, California, USA) under the following conditions: 95 °C for 3 min, followed by 40 cycles of 15 sec at 95 °C 60 sec at 55 °C and lastly 20 sec at 72 °C.

3.3.5. EPEC ADHERENCE ON THE INTESTINE

The Ileal and colonic tissue segments were fixed in 4% formalin (Sigma-Aldrich, St. Louis, Missouri, USA) embedded in paraffin, the slides were stained with rabbit anti-intimin at the University of Virginia Histology core, and viewed using light microscope.

For Transmission Electron Microscopy (TEM), the Ileal tissue was fixed with 4% glutaraldehyde (Sigma-Aldrich, St. Louis, Missouri, USA). The sample was washed with 1 X cacodylate buffer (Sigma-Aldrich, St. Louis, Missouri, USA) for 10 min and placed in 2% osmium tetroxide (Sigma-Aldrich, St. Louis, Missouri, USA) for 1 hour. The sample was then washed for 10 min with cacodylate buffer and then with distilled water. Followed by dehydration with 30% ethanol for 10 min and in concentrations of 50%, 70%, 95% and

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100% ethanol all for 10 min each. About 1:1 ethanol/propylene oxide (PO) (Fisher Scientific, Waltham, Massachusetts, USA) was used for 10 min, followed by 100% PO for 10 min. The sample was then placed in 1:1 PO/epoxy resin (EPON) overnight, followed by 1:2 PO/EPON for 2 hours, then 1:4 PO/EPON for 4 hours and lastly 100% EPON overnight. The sample was then embedded in fresh 100% EPON and allowed to bake at 65 °C in an oven. Ultra-thin sections were cut at 75 nm and picked up on 200 mesh copper grids. Sections were stained with 0.25% lead citrate (Sigma-Aldrich, St. Louis, Missouri, USA) and 2% uranyl acetate (Polysciences, Niles, Illinois, USA). The slides were viewed using JEOL 1230 microscope, with 4k x 4k CCD camera (JOEL ltd, Akishima, Tokyo).

3.3.6. INTESTINAL INFLAMMATORY RESPONSE

Protein lysates were extracted from the stools, ileum, colon and cecal contents using radioimmunoprecipitation assay (RIPA) buffer (150 mM sodium chloride, 20 mM Tris [pH 7.5], 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA) containing protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitors (1 mM sodium orthovanadate, 5 mM sodium fluoride, 1 mM microcystin LR, and 5 mM β-glycerophosphate). Lysates were centrifuged at 8000 *x g* for 5 min. The supernatant was used to perform protein assay using the bicinchoninic acid assay using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). LCN-2 was measured using mouse lipocalin-2/NGAL DuoSet ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) and MPO was measured using mouse myeloperoxidase DuoSet ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer instructions. IL-6 was measured using mouse magnetic Luminex assay, premixed multiplex and Luminex machine (Bio-Rad, Hercules, California, USA).LCN-2, MPO and IL-6 levels were measured as picograms per milligram (pg/mg) of protein.

3.3.7. SYSTEMIC INFLAMMATION

The collected serum was used to measure SAA and CRP. The concentrations of SAA were measured using mouse Serum Amyloid A DuoSet ELISA kit (R&D Systems,

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Minneapolis, Minnesota, USA) according to the manufacturer's instructions. The results were measured as picograms per millilitre (pg/mL). Protein lysates from the ileum and colon were used to measure systemic marker CRP. Protein concentrations were measured using Pierce BCA Protein Assay Kit according to manufacturer instructions. CRP levels were measured using mouse magnetic Luminex assay, premixed multiplex and Luminex machine (Bio-Rad, Hercules, California, USA). CRP levels were measured as picograms per milligram (pg/mg) of protein.

3.3.8. ^{1}H NMR SPECTROSCOPY BASED METABOLIC PROFILING

Urine specimens were collected daily in a sterile 1.5 mL eppendorf tube and placed at -80 °C until further analysis. The metabolic profiling was performed on all urine samples using ^{1}H NMR spectroscopy (Bruker, Billerica, Massachusetts, USA). A 30 µL urine aliquot was combined with 30 µL of phosphate buffer (pH 7.4, 100% D_2O, 0.2 M Na_2HPO_4/NaH_2PO_4) containing 1 mM of the internal standard, 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]-propionic acid and 2 mM sodium azide as a bactericide. Samples were then vortexed and centrifuged at 13000 *x g* for 10 min and 50 µL of the supernatant was then transferred to 1.7 mm NMR tubes (Bruker, Billerica, Massachusetts, USA). Spectroscopic analysis was performed on a 600-megahertz Bruker Avance™ NMR spectrometer at 300 K using a Bruker BBI probe and an automated SampleJet for tube handling (Bruker, Billerica, Massachusetts, USA). ^{1}H NMR spectra of the urine samples were obtained using a standard one-dimensional pulse sequence [recycle delay -90°-t_1–90°-t_m-90°-acquire free induction decay]. The water signal was suppressed through irradiation during the recycled delay of 4 sec and a mixing time (t_m) of 100 ms was used. For each spectrum, 64 scans were obtained into 64 K data points using a spectral width of 12.001 parts per million. The NMR spectra were calibrated to the time-stretched pulse resonance at 0 parts per million using TopSpin 3.5 NMR software (Bruker, Billerica, Massachusetts, USA) and imported into MATLAB (R2018a, Mathworks Inc, Natick, Massachusetts, USA) using in-house scripts. Regions containing the TSP, water and urea resonances were removed from the urinary spectra. ^{1}H NMR spectra were manually aligned and normalized to the unit area.

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3.3.9. STATISTICAL ANALYSIS

Data was recorded and analysed using GraphPad Prism 7 software (GraphPad, San Diego, California, USA). The raw data was analysed using one-way ANOVA and Tukey's *post hoc* and multiple *t-test*. Differences were considered significant when $p<0.05$. The data is represented as $\pm$standard error of the mean (SEM). Experiments were repeated at least two times. For metabolomics data analysis multivariate statistical modelling was performed using PCA and the Imperial Metabolic Profiling and Chemometrics Toolbox (https://github.com/csmsoftware/IMPaCTS) in MATLAB (Version 2018a) (Mathworks Inc, Natick, Massachusetts, USA) and unsupervised hierarchical clustering analysis to unveil metabolic differences between EPEC infected and control mice. Unsupervised clustering for all specimens was prepared using the normalized abundance of metabolites that were identified through the PCA models. Hierarchical clusters were produced using an average-linkage method by means of the pdist and linkage functions in the MATLAB bioinformatics toolbox. Heat maps and dendrograms after the hierarchical clustering analysis were produced using the MATLAB imagesc and dendrogram functions. Pathway analysis was done using the MetaboAnalyst 4.0 platform (https://www.metaboanalyst.ca/).

3.4. RESULTS

3.4.1. EPEC INFECTION LEADS TO WEIGHT LOSS AND DIARRHOEA

C57BL/6 mice pretreated with antibiotic cocktail were infected with WT EPEC, *bfpA*- (site-directed mutant that renders the bfp protein non-functional) and Δ*escN* (lacking T3SS function) strains (Figure 3.1). Weaned mice infected with *bfpA*- had significant weight loss at day 1 pi ($p<0.005$) when compared to controls (Figure 3.2.A). EPEC infected mice also

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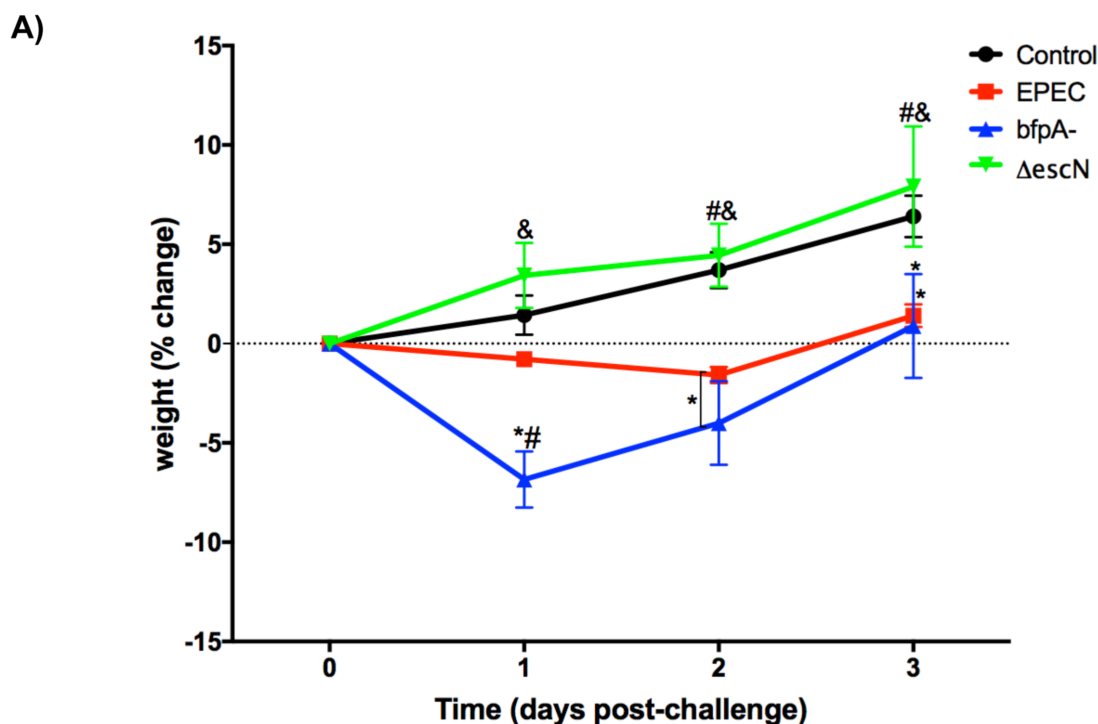
had a significant weight change at day 2 and day 3 pi when compared to Δ*escN* infected mice ($p<0.005$). The Δ*escN* infected mice did not exhibit any weight decrements and gained weight at a similar rate as the control mice (Figure 3.2.A). By day 4 pi all mice had recovered from the infection (Appendix A1). The infection was also assessed based on the consistency and appearance of stools (Figure 3.2.B). Nine out of 12 mice infected with WT EPEC developed watery mucoid stools from day 1 to 3 pi; 8 out of 12 *bfpA*-infected mice developed bloody stools at day 1 and 2 pi and later developed watery stools, while 4 out of 12 had watery diarrhoea. All Δ*escN* infected mice (12 out of 12) had well-formed stools similar to uninfected mice throughout the infection period. At day 4 pi, faecal specimens of all groups were tested for the presence of blood using a haemoglobin assay kit. Although not significant, there was an increase in haemoglobin levels in the stools of mice infected with the *bfpA*- strain, following the appearance of bloody stools that were observed at days 1 and 2 pi (Appendix A2).

A)



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B **i)** **ii)** **iii)**

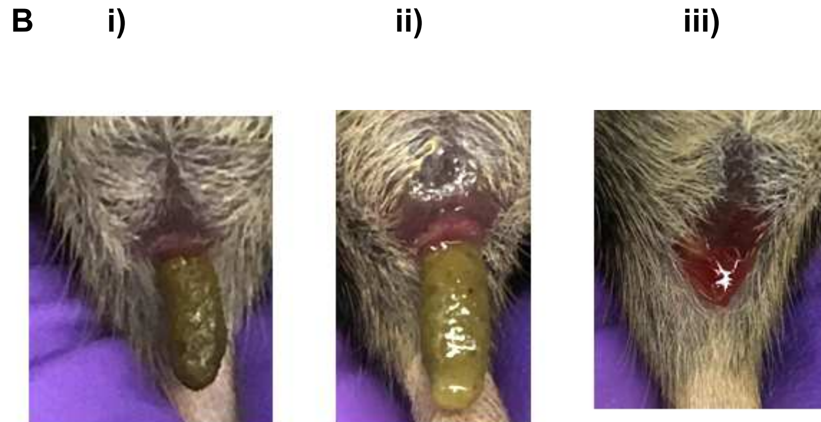

Figure 3.2: Change in weight and clinical outcomes of EPEC infection in C57BL/6 mice. **A)** Weaned mice treated with antibiotic cocktail infected with WT EPEC, *bfpA*- and ∆*escN* were monitored daily for changes in weight (n = 10-12/group). *p<0.005 vs control group, #p<0.005 vs EPEC, &p<0.005 vs *bfpA*-. Data analysed using two-way ANOVA and Tukey's *post hoc* test. **B)** Appearance of faecal specimens collected at day 1pi**. i)** Control uninfected mice had hard formed stools. **ii)** Mice infected with WT EPEC had watery mucoid stools. **iii)** *bfpA*- infected mice had bloody stools.

3.4.2. SHEDDING AND TISSUE BURDEN IN WEANED MICE INFECTED WITH EPEC

During infection, stool specimens were collected and analysed for EPEC shedding using qPCR. As seen in Figure 3.3, EPEC WT shedding was higher at day 1 pi ($\geq 10^9$ organisms/10mg stool) when compared to *bfpA*- ($<10^8$ organisms/10mg stool) infected mice (p<0.0001). WT EPEC shedding was also higher at day 1 pi when compared to ∆*escN* ($\leq 10^7$ organisms/10mg stool) infected mice (p=0.0001). Faecal shedding thereafter was similar up to day 8 pi with WT and *bfp*- infections ($\sim 10^7$ organisms/10mg stool), while ∆*escN* was shedding at least 1 log lower by day 8 pi (Figure 3.3).

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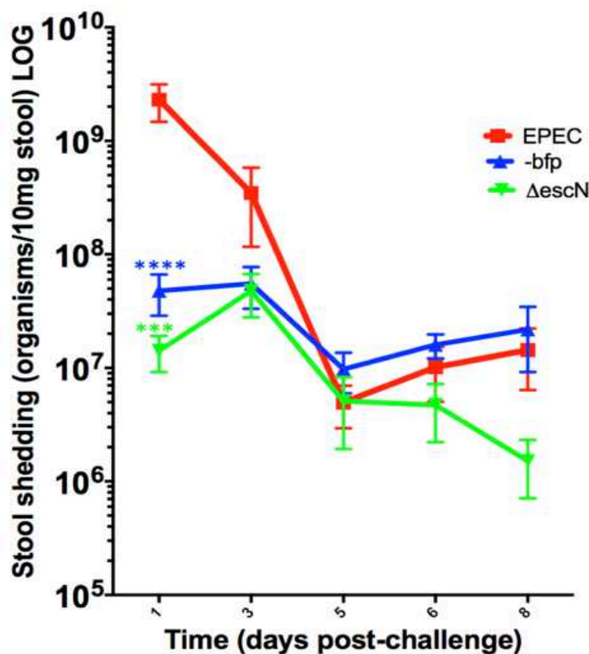

Figure 3.3: Stool shedding in EPEC infected mice. Weaned C57BL/6 mice were challenged with WT EPEC, *bfpA*- or Δ*escN*. DNA was extracted from collected stools and qPCR was used to determine shedding (n=10-12/group). Data analysed using two-way ANOVA and Tukey's *post hoc* test. ***p=0.0001, ****p<0.0001

Tissue burdens were assessed across all tissue sections of infected groups (Figure 3.4**)**. One set of the mice from each group were euthanized at day 3 pi and the remaining group were euthanized at day 8 pi. EPEC have been reported to colonize the ileum and colon. In this study, WT EPEC tissue burdens were observed across all tissue sections at day 3 pi; however, higher bacterial concentrations were observed in the ileum (~10^6 organisms/10mg tissue) and colon (~10^7 organisms/10mg tissue). At day 8 pi, WT EPEC colonized mostly in the ileal and the colonic tissue (~10^4 and ~10^6 organisms/10mg tissue). The *bfpA*- tissue burden was also detected in all tissue sections at day 3 pi with high colonization in the colon (about 7 logs organisms/10mg tissue). At day 8 pi the *bfpA*- colonized in the duodenum (≤10^5 organisms/10mg tissue), ileum (≤10^9 organisms/10mg tissue) and colon (≤10^6 organisms/10mg tissue). A significant difference was observed in the colon of Δ*escN* infected mice between day 3 and 8 pi ($p<0.05$). TEM of mice infected with WT EPEC was observed at day 3 pi with disruption of the microvilli when compared

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to uninfected control mice (Figure 3.5A and Appendix A3). Figure 3.5B indicates blunted ileal sections of mice infected with WT EPEC stained with rabbit anti-intimin.

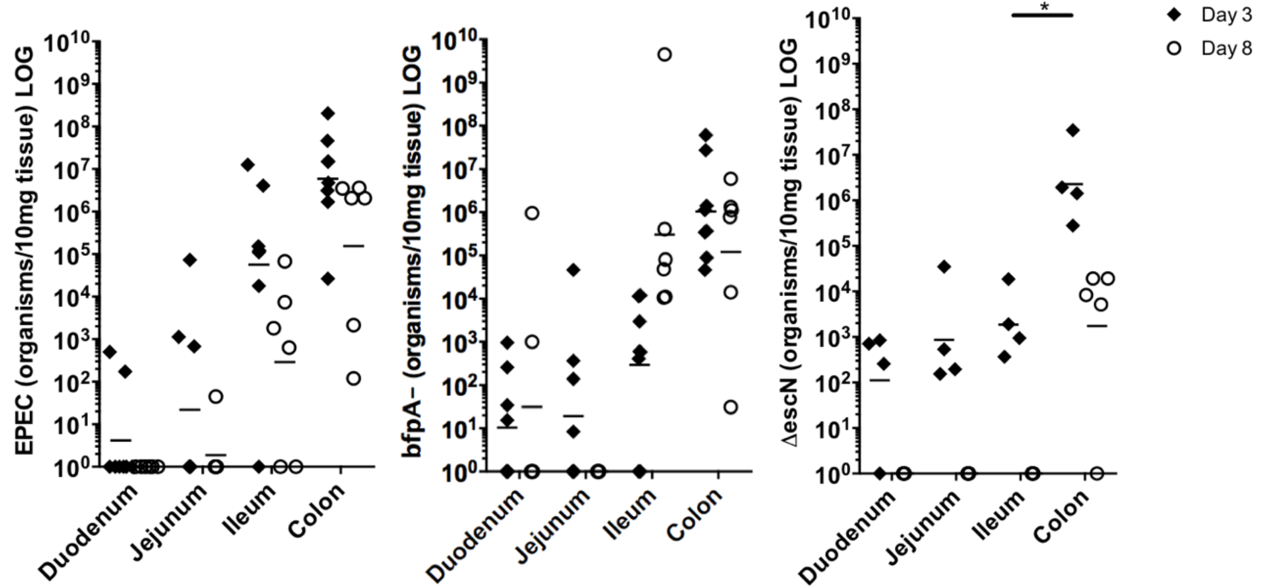
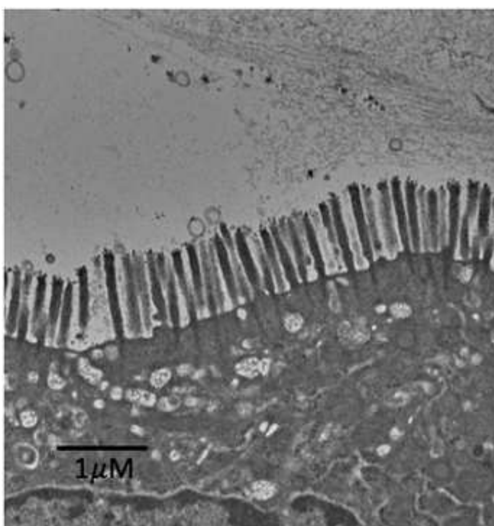
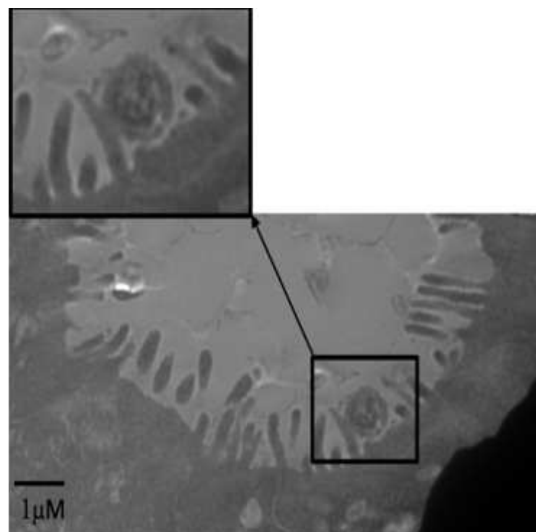


Figure 3.4: Tissue burden in EPEC infected mice. Weaned C57BL/6 mice were challenged with WT EPEC, *bfpA-* or Δ*escN*. Tissue sections of infected mice were collected at day 3 and 8 pi, intestinal tissues were homogenized, DNA was extracted and qPCR was used to determine WT EPEC, *bfpA-* and Δ*escN* (n = 5-8/group) tissue burden. *$p<0.05$.

A) i)



ii)



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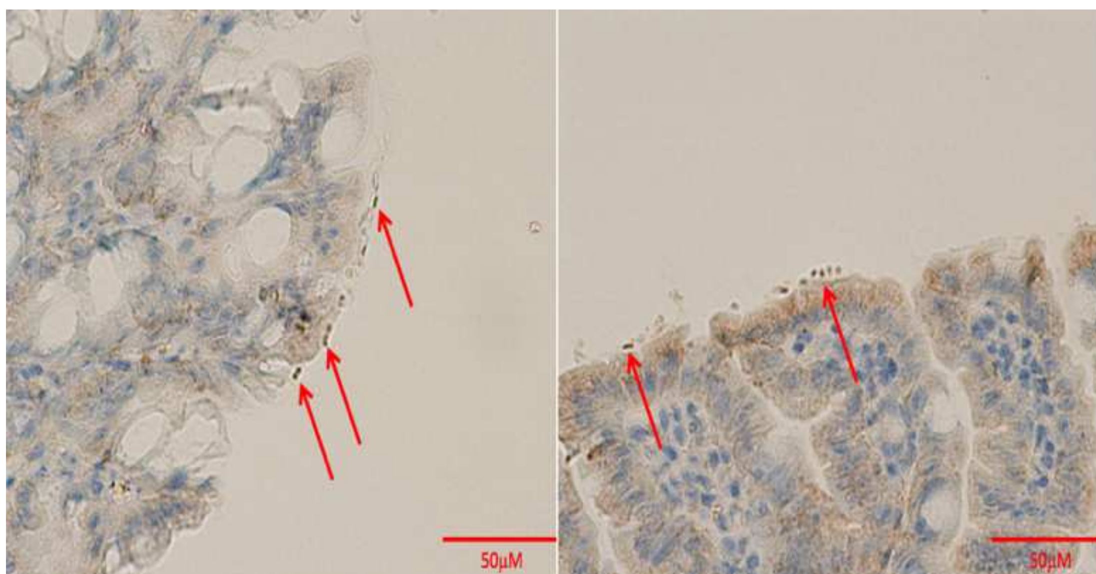

Figure 3.5: Adherence on intestinal microvilli. A) Transmission electron microscope at day 3 pi **i)** Uninfected ileum **ii)** Ileum infected with WT EPEC showing disruption of the microvilli. **B)** Ileal section stained with rabbit anti-intimin, shows adherence of WT EPEC at day 3 pi.

3.4.3. INFECTION LEADS TO DISRUPTION OF EPITHELIAL FUNCTION AND ACUTE SYSTEMIC RESPONSE

Intestinal inflammatory MPO and LCN-2 biomarkers were used to measure neutrophil infiltration during infection (Moschen et al, 2017). Stool specimens were analysed for MPO and LCN-2 biomarkers. At day 2 pi MPO and LCN-2 levels were higher in all the infected groups, except ∆*escN* infected mice. MPO levels of mice infected with WT and *bfpA-* strains were significantly different when compared to uninfected controls ($p<0.001$) and ∆*escN* ($p<0.001$) infected mice (Figure 3.6A). The same trend was also observed at day 7 pi in which there was a significant increase in LCN-2 levels of EPEC ($p<0.05$) and *bfpA-* ($p<0.001$) when compared to ∆*escN* infected mice. MPO levels were also increased in both EPEC and *bfpA-* infected mice with no significant difference (Figure 3.6.B). In the cecal contents, MPO levels were significantly higher in *bfpA-* infected mice when compared to uninfected controls and ∆*escN* infected mice ($p<0.05$). LCN-2 levels were significantly increased in the cecal contents of mice infected with EPEC when compared to uninfected controls and ∆*escN* infected mice ($p<0.05$) (Figure 3.6C). Histological

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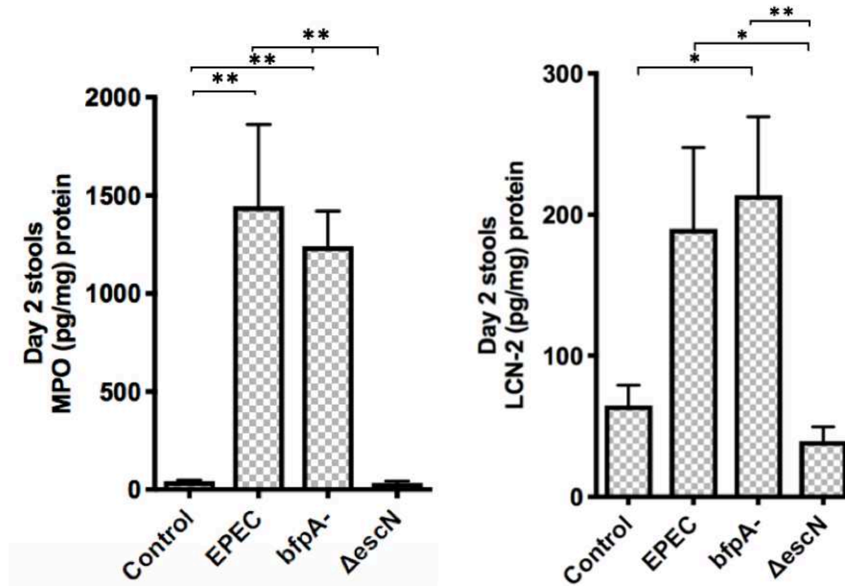
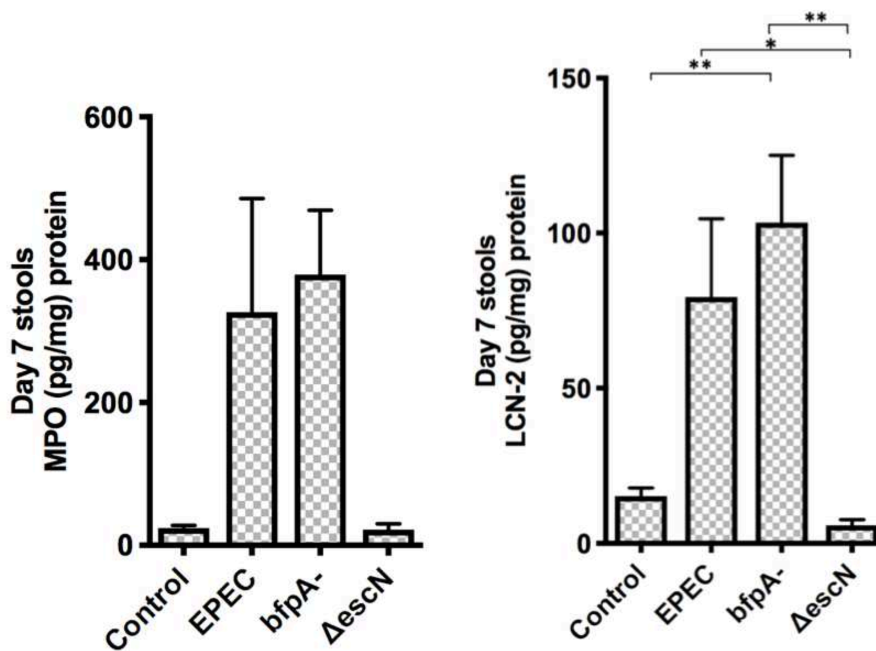
changes in the colonic tissue of mice infected with WT EPEC and *bfpA-* were observed (Figure 3.6.D), showing thinning of the smooth muscle layer as a result of epithelial damage to the in the intestinal lumen.

A)



B)



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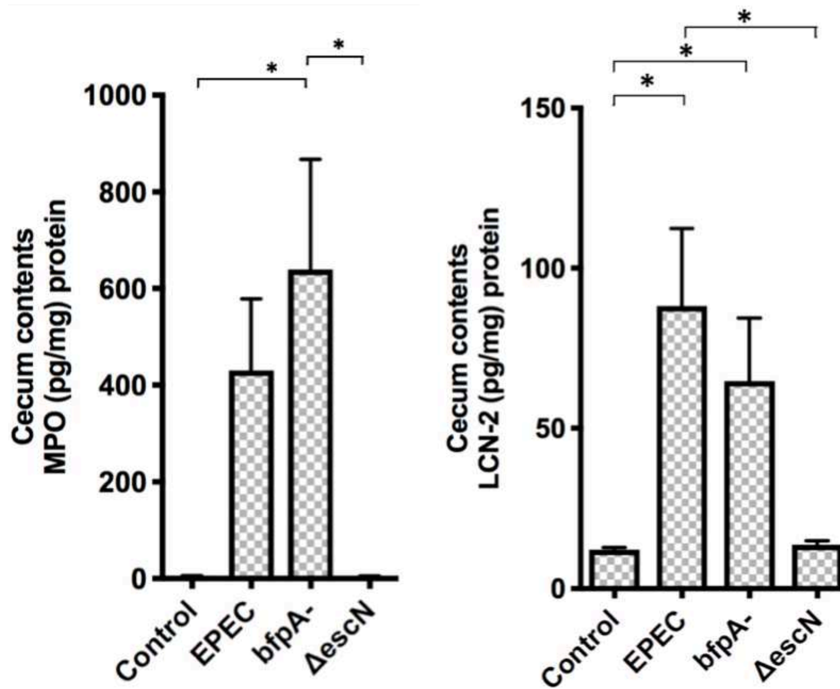
C)



D)

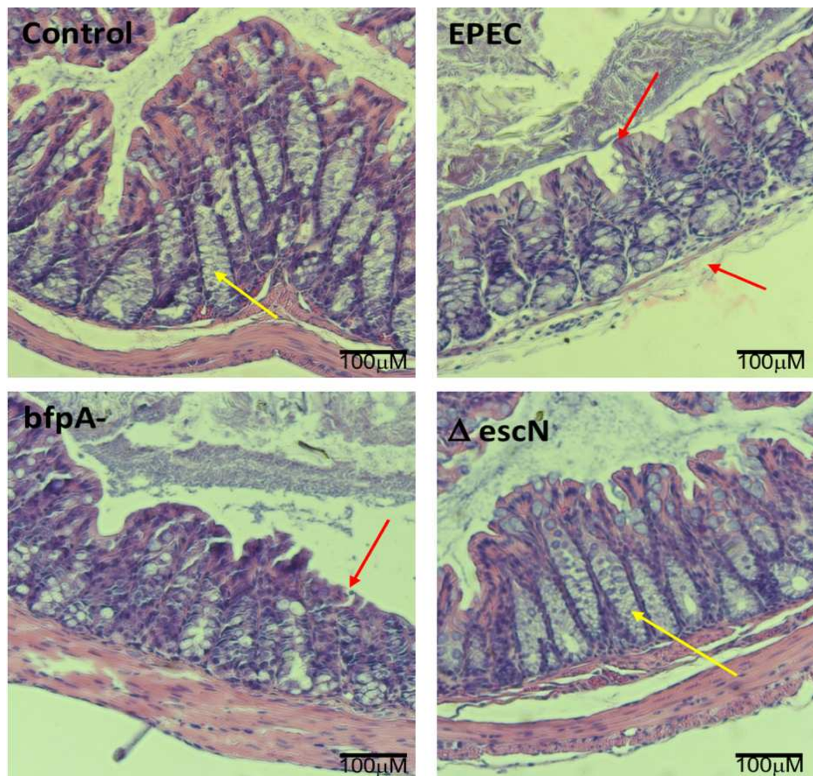


Figure 3.6: Biomarkers of intestinal epithelial damage in stools and cecum contents of EPEC infected mice. **A)** Inflammatory markers MPO (n = 5-9/group) LCN-2 and (n = 5-8/group) measured in stool specimens collected at day 2 pi. **B)** Inflammatory markers, MPO (n = 3-5/group) and LCN-2 (n = 6-8/group) measured in stool specimens collected at day 7 pi. **C)** Mice euthanized

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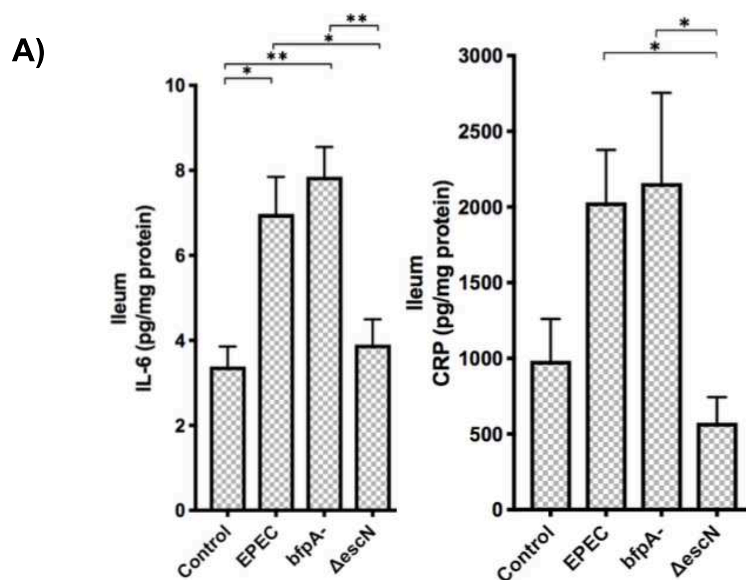
at day 3 pi, cecal contents were collected and lysates were extracted using RIPA buffer, MPO (n = 4-8/group) and LCN-2 (n = 4/group) were measured. Data analysed using one-way ANOVA and Tukey's *post hoc* test, * p<0.05, **p<0.001. **D**) Histology of mice infected with WT EPEC, *bfpA*- and ∆*escN* at day 3 pi (n= 4/group), colonic tissue sections were stained for H&E. Yellow arrow indicates healthy colon with intact crypts. Red arrow indicates thinning of smooth muscle layer and flattening of the colon.

Increase in pro-inflammatory cytokine, IL-6, was detected in ileal (Figure 3.7.A) and colonic (Figure 3.7.B) sections of mice infected with WT EPEC and *bfpA*- strains at day 3 pi. This observation was associated with increased acute inflammatory markers CRP (Figure 3.7.A and B) and SAA (Figure 3.7.C) in mice infected with WT EPEC and *bfpA*- strains.

A)



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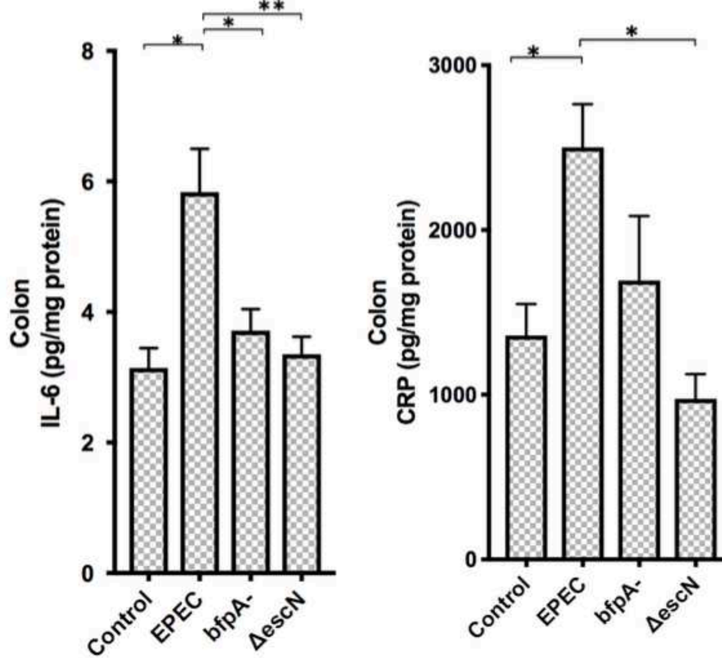
B)



C)

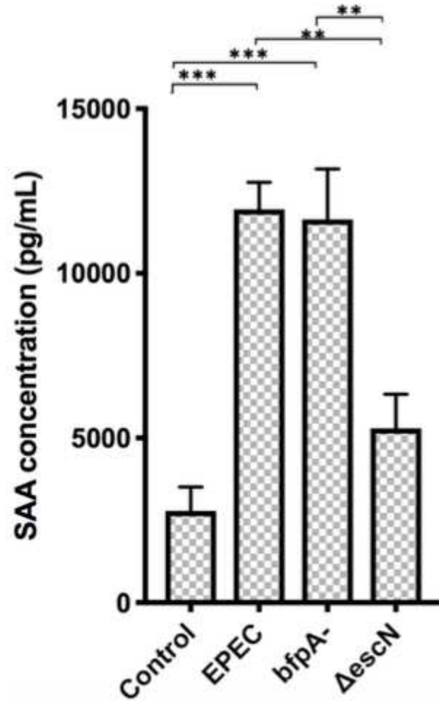


Figure 3.7: Acute phase inflammatory responses. Protein lysates from ileum and colon extracted using RIPA buffer were used to measure IL-6 and CRP using ELISA. **A**) IL-6 and CRP measured in the ileum at day 3 pi (n = 4-6/group). **B**) IL-6 and CRP measured in the colon at day 3 pi (n = 4-9/group). **C**) Serum collected at day 3 pi was used to measure SAA using ELISA (n = 5-8/group).

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Data analysed using one-way ANOVA and Tukey's *post hoc* test. *$p<0.05$, **$p<0.001$, ***$p=0.0001$

3.4.4. EPEC DERIVED METABOLIC PERTURBATIONS

Metabolic perturbations following EPEC infection were further analysed using OPLS-DA. Urinary metabolic profiles of each of the mice infected with EPEC were compared to the age-matched uninfected mice at days 1 and 3 pi. No differences were seen between the controls and EPEC infected mice on the first day of infection (Figure 3.8.A). Infection-driven metabolic variation was observed at day 3 pi (OPLS-DA model: $Q^2Y = 0.57$, $P = 0.027$ (1000 permutations). EPEC infection resulted in reduced excretion of tricarboxylic acid (TCA) cycle metabolites (succinate, cis-aconitate, citrate, 2-oxoglutarate and fumarate) and choline related metabolites [dimethylglycine (DMG) and trimethylamine (TMA)] (Figure 3.8.B). Lower urinary excretion of the tryptophan catabolite N-methylnicotinic acid (NMNA), the creatinine precursor guanidinoacetic acid (GAA) and the amino acid catabolites 2-oxoisocaproate, 2-oxoadipate were also observed. Isethionate, formate, pantothenate, and sucrose were also excreted in lower concentrations following EPEC infection. Increases in the excretion of gut microbial-derived metabolites [acetate, phenylacetylglycine (PAG), m-hydroxyphenylpropionylsulfate (m-HPPS)], were observed in infected mice. Urinary excretion of taurine, creatine and b-oxidation product hexanoylglycine, were also elevated at day 3 pi (Figure 3.8.B). Pathway analysis revealed that the TCA cycle was the biochemical pathway most influenced by the infection (Impact: 0.26, p-value: 2.5E-6, FDR adjusted p-value: 2.1E-4) (Figure 3.8.C).

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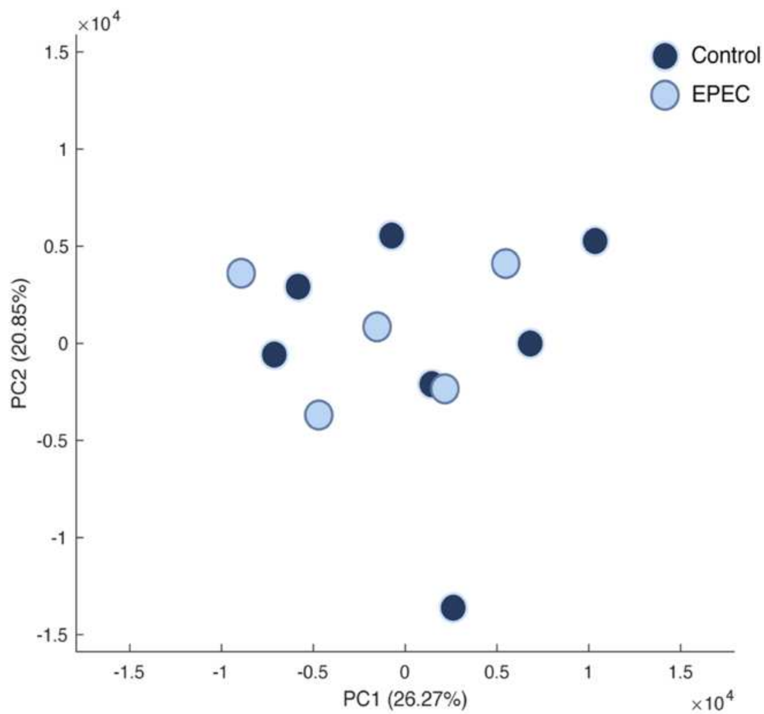
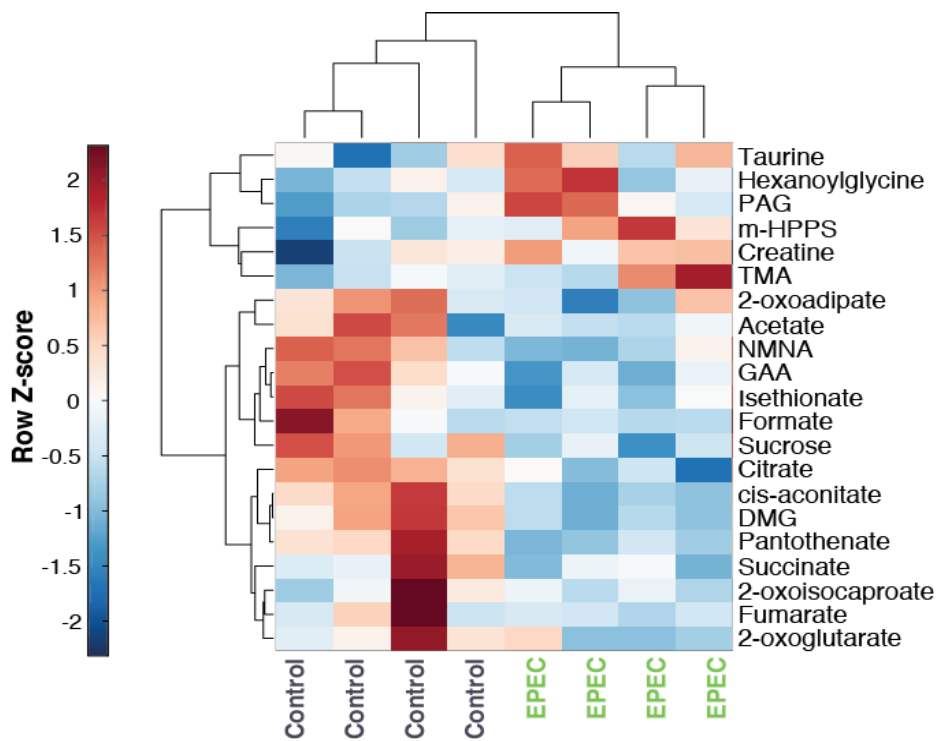
A)



B)



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C)

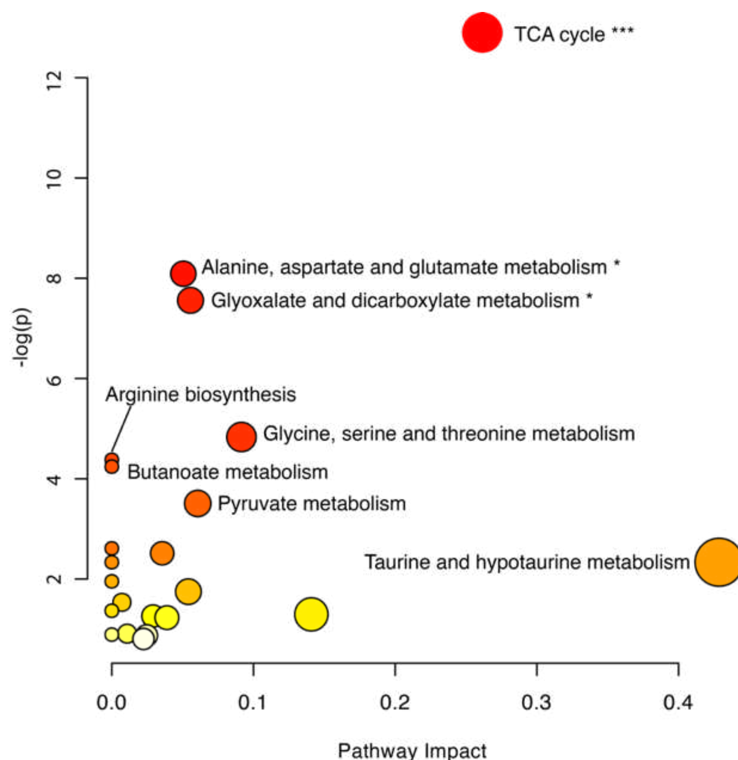

Figure 3.8: Metabolomic analysis of urinary specimens collected from age-matched, control and EPEC infected mice. **A)** Principal component analysis **(**PCA) scores plot at day 1 pi showing no difference between infected and uninfected mice (control n = 7, EPEC n = 5). **B)** Unsupervised hierarchical clustering and heat-map of urinary metabolites from control and EPEC infected mice at day 3 pi (n=4/group). The rows represent a metabolite and each of the columns represent a mouse sample. The row Z-score (scaled expression value) of each metabolite is plotted in red-blue colour. The metabolites indicated in red are high in abundance and metabolites in blue indicate low in abundance. DMG, dimethylglycine; GAA, guanidinoacetic acid; m-HPPS, m-hydroxyphenylpropionylsulfate; NMNA, N-methyl-nicotinic acid; PAG, phenylacetylglycine; TMA, trimethylamine. **C)** Pathway analysis plot generated using MetaboAnalyst 4.0 showing alterations of metabolic pathways induced by EPEC. Each pathway is represented as a circle. Darker colours indicate more significant changes of metabolites annotated on the corresponding pathway. The x-axis indicates the pathway impact value computed from pathway topological analysis, and the y-axis is the-log of the p-value obtained from pathway enrichment analysis. The high-log(p) value and high impact value (top right region) were used to characterize pathways that were significantly changed. FDR adjusted p-values, *** p = 0.0001.

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3.5. DISCUSSION

Typical EPEC infections have been suggested to be associated with inflammatory enteropathy and/or diarrhoea in resource-limited populations (Platts-Mills et al, 2014; Platts-Mills et al, 2018). Infections caused by EPEC have been widely reported *in vitro* studies to demonstrate the effects of adherence traits (Knutton et al, 1999) and T3SS (Andrade et al, 2007). However, there is still a need for a suitable *in vivo* model that clearly shows the effects of human EPEC infection in an intestinal environment. *C. rodentium* is a natural mouse pathogen that has been used to study human EPEC and EHEC due to their genetic similarities; and has also been shown to cause A/E lesions, with the formation of pedestal structures and polarized actin accumulation at the site of infection (Luperchio and Schauer, 2001; Mundy et al, 2006).

Efforts of developing an EPEC model that mimics clinical outcomes, such as weight loss and diarrhoea, as observed in humans, has been a challenge for *in vivo* studies (Savkovic et al, 2005; Law et al, 2013; Vulcano et al, 2014; Dupont et al, 2016). Disruption of intestinal microbiota using streptomycin in mice has been widely reported (Shifflett et al, 2005; Mundy et al, 2006; Zhang et al, 2010; Rhee et al, 2011; Dupont et al, 2016). Savkovic and colleagues (2005) reported on an EPEC murine model using C57BL/6 mice in which infection resulted in colonization and mild inflammatory responses (Savkovic et al, 2005). The study however, did not report on clinical outcomes as a result of EPEC infection. DuPont and colleagues (2016) recently reported on alterations of resident intestinal microbiota in adult mice as a result of antibiotic treatment to enable colonization of EPEC in the ileal and colonic tissue (Dupont et al, 2016). This has provided a pathway in understanding the effects of disrupted microbiota to enable colonization. Previously, disruption of intestinal microbiota using broad-spectrum antibiotic cocktail has been reported to enable colonization of bacterial pathogens such as ETEC (Bolick et al, 2018), *Campylobacter jejuni* (Giallourou et al, 2018) and *S. flexneri* (Medeiros et al, 2019) in C57BL/6 mice. In this study, weaned C57BL/6 mice were also pretreated with antibiotics to enable efficient colonization of EPEC. In addition, oral infection of weaned mice

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resulted in outcomes of changes in stool appearance, transient weight loss and inflammatory responses.

In this study, it was interesting to note that both WT and bfp- infected mice had transient weight loss up to day 4 pi. Even though the mice had fully recovered their body weight, shedding was detected up to day 8 pi. Welkos reported on *C. jejuni* infection in one-day-old chicks that developed enteric symptoms between days 2 and 5 pi and these chicks continued to shed *C. jejuni* up to two weeks with no signs of symptoms (Welkos, 1984). Chen and colleagues (2008) reported on vancomycin-treated mice infected with *C. difficile* developing diarrhoea and by day 9 pi the mice had recovered their body weight by day 9 pi (Chen et al, 2008). Another study, Buffie et al (2012), reported on clindamycin-treated mice infected with *C. difficile* spores with infection resulting in diarrhoea and weight loss occurring up to day 5 pi. The infected *C. difficile* infected mice also recovered their weight with the pathogen being detected in the intestinal tissue up to day 8 pi (Buffie et al, 2012).

In humans, EPEC infection promotes watery diarrhoea and dehydration (Guerrant et al, 2011). EPEC infection in infant C57BL/6 mice has been previously reported to colonize the small intestine and colon for 3 days as a result of human milk oligosaccharides administration (Manthey et al, 2014). Vulcano and colleagues (2014) reported on EPEC infection in 4-week-old AIRmin mice showing histological changes with no outcomes of diarrhoea and weight loss (Vulcano et al, 2014). On the other hand, Savkovic and colleagues reported on C57BL/6 mice infected with EPEC developing semi-solid stools in the proximal colon with no diarrhoea (Savkovic et al, 2005).

Particularly impressive in this model is the striking inflammatory enteropathy (as evidenced by faecal MPO with acute infection with WT EPEC infected mice developing watery mucoid stools. Furthermore, weaned mice infected with the *bfpA* mutant developed similar striking intestinal inflammation and even transient bloody stools and showed systemic inflammation, demonstrating the potential importance of an EPEC strain lacking a functional *bfpA* gene. The *bfpA* mutant used in this study was derived from the

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same typical EPEC E2348/69 strain and has not acquired other virulence traits that are different from the WT. The roles of other virulence strategies in a *bfpA*- background deserve further characterization. It is interesting to also compare the *bfpA*- to other bacteria that are reported to have clinical relevance, such as EHEC O157 strain that causes bloody diarrhoea associated with hemolytic uremic syndrome (Kaper et al, 2004). Atypical EPEC pathogenicity is complex, and these strains are often reported to express virulence genes of other DEC pathotypes (Bando et al, 2009; Ruiz et al, 2014). Atypical EPEC O26 strains have been reported to be descendants of EHEC O26 which may have lost the shiga toxin; however, this EPEC strain has also been associated with bloody diarrhoea (Bielaszewska et al, 2007; Hu and Torres, 2015). Ruiz and colleagues (2014) also reported on an atypical EPEC strain expressing Pet, which has been exclusively reported only in EAEC to cause cell damage by disrupting the cytoskeletal structure of the cells (Ruiz et al, 2014). Finally, in careful analyses of qPCR molecular diagnostics for diarrhoea in 6625 cases among 1715 children age 0-2years old across all 8 MAL-ED sites, both typical and atypical (ie bfp-) EPEC were significantly associated with diarrhoea (Platts-Mills et al, 2018).

In this model, it is hypothesized that the *bfpA* mutant colonized avidly in multiple intestinal regions, as seen in all the intestinal tissue sections during the first 3 days of infection and caused increased systemic inflammation and transient bloody diarrhoea. In addition, the results suggest that EPEC infection in mice does not require a functional *bfpA* for increased virulence. Although there are limitations of comparing animal studies to humans due to differences in the microbiota. Colonization of the *bfpA* mutant strain in all intestinal tissue sections, suggests that lacking *bfpA* may favour broader colonization and this could potentially help explain the high prevalence of atypical EPEC strains in clinical settings (Trabulsi et al, 2002; Afset et al, 2003; Ochoa and Contreras, 2011; Hu and Torres, 2015).

The T3SS is essential for EPEC pathogenesis and requires an effective ATPase energizer, *escN* (Gauthier et al, 2003; Andrade et al, 2007; Zarivach et al, 2007). In this study, weaned mice infected with *escN* mutant resulted in diminished weight loss and no

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inflammatory outcomes. Even without an effective T3SS, *escN* mutant was able to colonize all sections of the intestinal tissue, albeit at much lower levels, until day 8 pi, as shown by the results. These findings reinforce the importance of adherence factors such as *bfp* (Knutton et al, 1989; Giron et al, 1991) and functional T3SS are required in activating the EPEC secreting proteins (Frankel et al, 1998; Andrade et al, 2007) in order to induce detectable disease.

EPEC infection has been reported to impair the tight junction barrier function of ileal and colonic mucosa (McNamara et al, 2001; Mundy et al, 2006; Ugalde-Silva et al, 2016). LCN-2 and MPO were used as biomarkers of acute-phase response, while SAA and CRP were used as markers of systemic levels of inflammation during infection. MPO (Guerrant et al, 2016; McCormick et al, 2017) and SAA (Guerrant et al, 2016) have been used as biomarkers of enteropathy in clinical studies also exhibiting inflammation, growth and development decrements in children infected with different enteropathogens in low-income countries. Although intestinal barrier disruption has not been assessed directly, the upregulation of SAA and CRP show triggering of systemic responses. Furthermore, an increase in MPO and LCN-2 also suggest that there was a functional disruption of the intestinal barrier in this model. The proposed human EPEC infection model in C57BL/6 mice pretreated with an antibiotic cocktail is shown in Figure 3.9.

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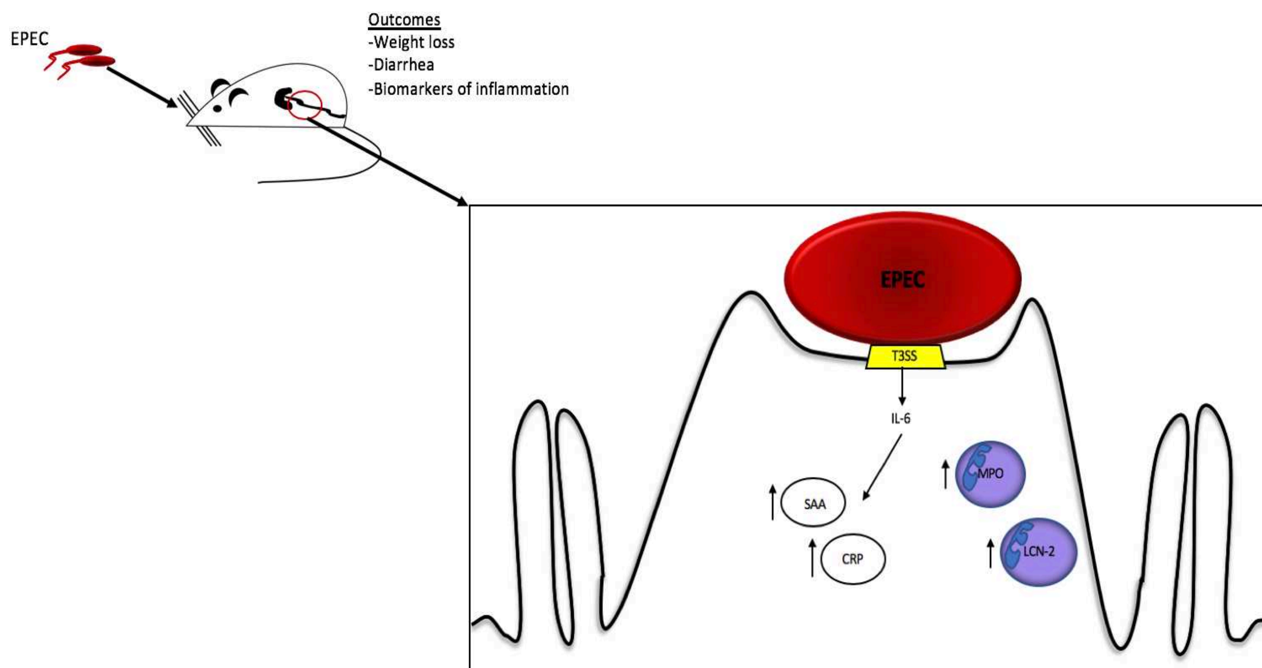

Figure 3.9: Proposed model of EPEC infection in weaned C57BL/6 mice. Antibiotic-treated mice orally infected with WT and/or bfpA- EPEC resulted in transient weight loss and increased inflammatory responses. EPEC colonized in the intestinal tissues with or without bfpA. The presence of bfpA led to mucoid stools while the absence of bfp resulted in bloody stools. Intimate adherence resulted in activation of the T3SS enabling secretion of secretory proteins (with *escN* acting as an ATPase energizer) which in turn caused villus blunting. Disruption of the lumen led to increased neutrophil infiltration that resulted in secretion of inflammatory biomarkers, MPO and LCN-2. Pro-inflammatory cytokine IL-6 including SAA and CRP were released as an indication of acute-phase systemic inflammation.

In this study, the biochemical shifts induced by EPEC were also investigated using ^{1}H NMR based metabolomics. EPEC infection resulted in perturbations of multiple biochemical pathways, but the TCA cycle intermediates appeared to be the most sensitive to the infection. The TCA cycle in *E. coli* is linked to energy metabolism in which concomitant CO_2 is oxidized from pyruvate leading to the production of NADH and $FADH_2$ (Alteri and Mobley, 2012). In this study, the TCA cycle metabolites were excreted in lower quantities following EPEC infection suggesting that energy production was negatively influenced in the infected host. A shut-down of the TCA cycle during infection suggests that the energy requirements of the host were not met, potentially explaining the

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significant weight loss in the infected mice. *C. jejuni* infection in zinc-deficient mice have also been reported to perturb the TCA cycle, affecting amino acid and muscle catabolism as a result of increased creatine excretion (Giallourou et al, 2018). Pantothenate is the key precursor of the fundamental TCA cycle cofactor, coenzyme A (Leonardi and Jackowski, 2007). Reduced pantothenate excretion following EPEC colonization further adds to the TCA cycle disruption by infection. Interestingly, excretion of creatine which is a source for energy production in the form of ATP was also increased during infection. Sugiharto and colleagues (2014) reported on post-weaning pigs infected with *E. coli* F18 and found that there was a reduction of creatine and betaine levels in the plasma, which was due to inhibition of antioxidant system that resulted in piglets developing diarrhoea (Sugiharto et al, 2014).

Taurine has been shown to possess antioxidant properties and its concentrations are elevated in inflamed tissues where oxidants are abundant (Jeon et al, 2009; Oliveira et al, 2010). In this study, EPEC infection was characterized by elevated urinary taurine excretion. As previously observed (Swann et al, 2011), treating rodents with antibiotics suppresses the bacterial metabolism of taurine thus increasing taurine bioavailability and uptake in the host reflected by greater urinary taurine excretion. Guanidinoacetic acid (GAA), the creatine precursor, was also reduced in infected mice suggesting a greater turnover of GAA in favour of increased creatine availability. Metabolites derived from bacteria in the gut were excreted in greater amounts following infection suggesting gut microbial metabolism was altered by EPEC infection. These findings help to understand host metabolism during infection, suggesting potential pathways to be further explored and targeted in future studies.

In conclusion, the data in this study showed weight loss, clinical and inflammatory outcomes as a result of both WT and *bfpA*- EPEC infections in weaned antibiotic pretreated mice. These effects were also dependent on EPEC T3SS. In addition, metabolic perturbations were observed in mice with EPEC infection, suggesting relevant biochemical pathways involved. Further, the findings presented here suggest that EPEC infections can increase intestinal and systemic inflammatory responses, transient overt

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diarrhoea and weight decrements, as is often seen in children with EPEC infections. This model can help further explore mechanisms involved in EPEC pathogenesis and perhaps facilitate in the development of vaccines or therapeutic interventions.

3.6. REFERENCES

Abe, C.M., Trabulsi, L.R., Blanco, J., Blanco, M., Dahbi, G., Blanco, J.E., Mora, A., Franzolin, M.R., Taddei, C.R., Martinez, M.B. and Piazza, R.M., 2009. Virulence features of atypical enteropathogenic *Escherichia coli* identified by the eae+ EAF-negative stx− genetic profile. *Diagnostics Microbiology and Infectious Disease*, **64**(4), 357-365.

Afset, J.E., Bergh, K. and Bevanger, L., 2003. High prevalence of atypical enteropathogenic *Escherichia coli* (EPEC) in Norwegian children with diarrhoea. *Journal of Medical Microbiology*, **52**(11), 1015-1019.

Alteri, C.J. and Mobley, H.L., 2012. *Escherichia coli* physiology and metabolism dictates adaptation to diverse host microenvironments. *Current Opinion in Microbiology*, **15**(1), 3-9.

Andrade, A., Pardo, J. P., Espinosa, N., Perez-Hernandez, G. and Gonzalez-Pedrajo, B., 2007. Enzymatic characterization of the enteropathogenic *Escherichia coli* type III secretion ATPase EscN. *Archives in Biochemistry and Biophysics,* **468**, 121-127

Bando, S.Y., Andrade, F.B., Guth, B.E., Elias, W.P., Moreira-Filho, C.A. and Pestana de Castro, A.F., 2009. Atypical enteropathogenic *Escherichia coli* genomic background allows the acquisition of non-EPEC virulence factors. *FEMS Microbiology Letters*, **299**(1), 22-30.

Bielaszewska, M., Prager, R., Köck, R., Mellmann, A., Zhang, W., Tschäpe, H., Tarr, P.I. and Karch, H., 2007. Shiga toxin gene loss and transfer *in vitro* and *in vivo* during

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enterohemorrhagic *Escherichia coli* O26 infection in humans. *Applied Environmental Microbiology*, **73**(10), 3144-3150.

Bolick, D. T. Medeiros, P.H.Q.S., Ledwaba, S.E., Lima, A.A., Nataro, J.P., Barry, E.M. and Guerrant, R.L., 2018. The Critical Role of Zinc in a New Murine Model of Enterotoxigenic *E. coli* (ETEC) Diarrhea. *Infection and Immunity*, **86**(7), e00183-0018

Buffie, C.G., Jarchum, I., Equinda, M., Lipuma, L., Gobourne, A., Viale, A., Ubeda, C., Xavier, J. and Pamer, E.G., 2012. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to *Clostridium difficile*-induced colitis. *Infection and Immunity*, **80**(1), 62-73.

Chen, X., Katchar, K., Goldsmith, J.D., Nanthakumar, N., Cheknis, A., Gerding, D.N. and Kelly, C.P., 2008. A mouse model of *Clostridium difficile*–associated disease. *Gastroenterology*, **135**(6),1984-1992.

Dean-Nystrom, E. A., Gansheroff, L. J., Mills, M., Moon, H. W. and O'Brien, A. D., 2002. Vaccination of pregnant dams with intimin(O157) protects suckling piglets from *Escherichia coli* O157:H7 infection. *Infection and Immunity*, **70**, 2414-2418.

Dupont, A. Sommer, F., Zhang, K., Repnik, U., Basic, M., Bleich, A., Kühnel, M., Bäckhed, F., Litvak, Y., Fulde, M. and Rosenshine, I., 2016. Age-Dependent Susceptibility to Enteropathogenic *Escherichia coli* (EPEC) Infection in Mice. *PLoS Pathogens,* **12**, e1005616

Fabich, A.J., Jones, S.A., Chowdhury, F.Z., Cernosek, A., Anderson, A., Smalley, D., McHargue, J.W., Hightower, G.A., Smith, J.T., Autieri, S.M. and Leatham, M.P., 2008. Comparison of carbon nutrition for pathogenic and commensal *Escherichia coli* strains in the mouse intestine. *Infection and Immunity*, **76**(3), 1143-1152.

© University of Venda

Frankel, G. Phillips, A.D., Rosenshine, I., Dougan, G., Kaper, J.B. and Knutton, S., 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Molecular Microbiology* **30**, 911-921.

Freter, R., Brickner, H., Botney, M., Cleven, D. and Aranki, A., 1983. Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. *Infection and Immunity*, **39**(2), 676-685.

Friedberg, D., Umanski, T., Fang, Y. and Rosenshine, I., 1999. Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic *Escherichia coli. Molecular Microbiology* **34**, 941-952.

Gauthier, A., Puente, J. L. and Finlay, B. B., 2003. Secretin of the enteropathogenic *Escherichia coli* type III secretion system requires components of the type III apparatus for assembly and localization. *Infection and Immunity* **71**, 3310-3319.

Giallourou, N., Medlock, G.L., Bolick, D.T., Medeiros, P.H., Ledwaba, S.E., Kolling, G.L., Tung, K., Guerry, P., Swann, J.R. and Guerrant, R.L., 2018. A novel mouse model of *Campylobacter jejuni* enteropathy and diarrhea. *PLoS Pathogens*, **14**(3), e1007083.

Giron, J. A., Ho, A. S. & Schoolnik, G. K., 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. *Science* **254**, 710-713.

Guerrant, R.L., Walker, D.H. and Weller, P.F., 2011. *Trop Infect Dis: Principles, Pathogens and Practice E-Book*. Elsevier Health Sciences.

Guerrant, R.L., Leite, A.M., Pinkerton, R., Medeiros, P.H., Cavalcante, P.A., DeBoer, M., Kosek, M., Duggan, C., Gewirtz, A., Kagan, J.C. and Gauthier, A.E., 2016. Biomarkers of environmental enteropathy, inflammation, stunting, and impaired growth in children in northeast Brazil. *PLoS One*, **11**(9), e0158772

© University of Venda

Hu, J. and Torres, A.G., 2015. Enteropathogenic *Escherichia coli*: foe or innocent bystander?. *Clinical Microbiology and Infection*, **21**(8), 729-734.

Jarvis, K. G. Giron, J.A., Jerse, A.E., McDaniel, T.K., Donnenberg, M.S. and Kaper, J.B., 1995. Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proceedings of the National Academy of Science,* **92**, 7996-8000.

Jeon, S. H. Lee, M.Y., Rahman, M.M., Kim, S.J., Kim, G.B., Park, S.Y., Hong, C.U., Kim, S.Z., Kim, J.S. and Kang, H.S., 2009. The antioxidant, taurine reduced lipopolysaccharide (LPS)-induced generation of ROS, and activation of MAPKs and Bax in cultured pneumocytes. *Pulmonary Pharmacology and Therapeutics,* **22**, 562-566.

Kaper, J. B., Nataro, J. P. and Mobley, H. L., 2004. Pathogenic *Escherichia coli*. *Nature Reviews Microbiology* **2**, 123-140.

Knutton, S., Baldwin, T., Williams, P. H. and McNeish, A. S., 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infection and Immunity* **57**, 1290-1298.

Knutton, S., Shaw, R. K., Anantha, R. P., Donnenberg, M. S. and Zorgani, A. A., 1999. The type IV bundle-forming pilus of enteropathogenic *Escherichia coli* undergoes dramatic alterations in structure associated with bacterial adherence, aggregation and dispersal. *Molecular Microbiology,* **33**, 499-509.

Kotloff, K. L. et al., 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *The Lancet* **382**, 209-222

Larsen, P. L., Albert, P. S. and Riddle, D. L., 1995. Genes that regulate both development and longevity in *Caenorhabditis elegans*. *Genetics* **139**, 1567-1583.

© University of Venda

Law, R. J., Gur-Arie, L., Rosenshine, I. and Finlay, B. B., 2013. *In vitro* and *in vivo* model systems for studying enteropathogenic *Escherichia coli* infections. *Cold Spring Harbor Perspectives in Medicine* **3**.

Leonardi, R. and Jackowski, S., 2007. Biosynthesis of pantothenic acid and coenzyme A. *EcoSal Plus*, **2**(2).

Leverton, L. Q. and Kaper, J. B., 2005. Temporal expression of enteropathogenic *Escherichia coli* virulence genes in an *in vitro* model of infection. *Infection and Immunity,* **73**, 1034-1043.

Levine, M.M., Nataro, J.P., Karch, H., Baldini, M.M., Kaper, J.B., Black, R.E., Clements, M.L. and O'Brien, A.D., 1985. The diarrheal response of humans to some classic serotypes of enteteropathogenic *Escherichia coli* is dependent on a plasmid encoding the enteroadhesiveness factor. *Journal of Infectious Diseases,* **152**.

Liu, L., Oza, S., Hogan, D., Perin, J., Rudan, I., Lawn, J.E., Cousens, S., Mathers, C. and Black, R.E., 2015. Global, regional, and national causes of child mortality in 2000-13, with projections to inform post-2015 priorities: an updated systematic analysis. *The Lancet* **385**, 430-440.

Luperchio, S. A. and Schauer, D. B., 2001. Molecular pathogenesis of *Citrobacter rodentium* and transmissible murine colonic hyperplasia. *Microbes and Infection* **3**, 333-340.

Manthey, C.F., Autran, C.A., Eckmann, L. and Bode, L., 2014. Human milk oligosaccharides protect against enteropathogenic *Escherichia coli* attachment *in vitro* and EPEC colonization in suckling mice. *Journal of Paediatric Gastroenterology and Nutrition*, **58**(2), 165-168.

McCormick, B.J., Lee, G.O., Seidman, J.C., Haque, R., Mondal, D., Quetz, J., Lima, A.A., Babji, S., Kang, G., Shrestha, S.K. and Mason, C.J., 2017. Dynamics and trends in fecal

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biomarkers of gut function in children from 1–24 months in the MAL-ED study. *American Journal of Tropical Medicine and Hygiene*, **96**(2), 465-472.

McNamara, B. P. Koutsouris, A., O'Connell, C.B., Nougayréde, J.P., Donnenberg, M.S. and Hecht, G., 2001. Translocated EspF protein from enteropathogenic *Escherichia coli* disrupts host intestinal barrier function. *Journal of Clinical Investigation,* **107**, 621-629.

Medeiros, P.H. Q.S, Ledwaba, S.E., Bolick, D.T., Giallourou, N., Yum, L.K., Costa, D.V., Oriá, R.B., Barry, E.M., Swann, J.R., Lima, A.Â.M. and Agaisse, H., 2019. A murine model of diarrhea, growth impairment and metabolic disturbances with *Shigella flexneri* infection and the role of zinc deficiency. *Gut microbes*,1-16.

Misyurina, O. Asper, D.J., Deng, W., Finlay, B.B., Rogan, D. and Potter, A.A., 2010. The role of Tir, EspA, and NleB in the colonization of cattle by Shiga toxin producing *Escherichia coli* O26:H11. *Canadian Journal of Microbiology,* **56**, 739-747.

Moon, H. W., Whipp, S. C., Argenzio, R. A., Levine, M. M. and Giannella, R. A., 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. *Infection and Immunity,* **41**, 1340-1351.

Mora, A., Blanco, M., Yamamoto, D., Dahbi, G., Blanco, J.E., López, C., Alonso, M.P., Vieira, M.A., Hernandes, R.T., Abe, C.M. and Piazza, R.M., 2009. HeLA-cell adherence patterns and actin aggregation of enteropathogenic *Escherichia coli* (EPEC) and Shiga-toxin-producing *E. coli* (STEC) strains carrying different eae and tir alleles. *International Microbiology,* **12**, 243-251.

Moschen, A. R., Adolph, T. E., Gerner, R. R., Wieser, V. and Tilg, H., 2017. Lipocalin-2: A Master Mediator of Intestinal and Metabolic Inflammation. *Trends in Endocrinology and Metabolism* **28**, 388-397.

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Mundy, R., Girard, F., FitzGerald, A. J. and Frankel, G., 2006. Comparison of colonization dynamics and pathology of mice infected with enteropathogenic *Escherichia coli*, enterohaemorrhagic *E. coli* and *Citrobacter rodentium*. *FEMS Microbiology Letters* **265**.

National Research Council, 2010. *Guide for the care and use of laboratory animals*. National Academies Press.

Ochoa, T. J. and Contreras, C. A., 2011. Enteropathogenic *Escherichia coli* infection in children. *Current Opinion in Infectious Diseases* **24**, 478-483.

Oliveira, M. W., Minotto, J.B., de Oliveira, M.R., Zanotto-Filho, A., Behr, G.A., Rocha, R.F., Moreira, J.C. and Klamt, F., 2010. Scavenging and antioxidant potential of physiological taurine concentrations against different reactive oxygen/nitrogen species. *Pharmacological Reports,* **62**, 185-193.

Pelayo, J.S., Scaletsky, I.C.A., Pedroso, M.Z., Sperandio, V., Giron, J.A., Frankel, G. and Trabulsi, L.R., 1999. Virulence properties of atypical EPEC strains. *Journal of Medical Microbiology*, **48**(1),41-49.

Platts-Mills, J. A., Gratz, J., Mduma, E., Svensen, E., Amour, C., Liu, J., Maro, A., Saidi, Q., Swai, N., Kumburu, H. and McCormick, B.J., 2014. Association between stool enteropathogen quantity and disease in Tanzanian children using TaqMan array cards: a nested case-control study. *American Journal of Tropical Medicine and Hygiene,* **90**, 133-138.

Platts-Mills, J. A., Babji, S., Bodhidatta, L., Gratz, J., Haque, R., Havt, A., McCormick, B.J., McGrath, M., Olortegui, M.P., Samie, A. and Shakoor, S., 2015. Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). *Lancet Global Health,* **3**, e564-575.

Platts-Mills, J.A., Liu, J., Rogawski, E.T., et. al., 2018. Use of quantitative molecular diagnostic methods to assess the aetiology, burden, and clinical characteristics of

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diarrhea in children in low-resource settings: a reanalysis of the MAL-ED cohort study. *Lancet Global Health,* **6**(12), e1309-e1318.

Rhee, K. J., Cheng, H., Harris, A., Morin, C., Kaper, J.B. and Hecht, G., 2011. Determination of spatial and temporal colonization of enteropathogenic *E. coli* and enterohemorrhagic *E. coli* in mice using bioluminescent *in vivo* imaging. *Gut Microbes* **2**, 34-41.

Royan, S. V., Jones, R.M., Koutsouris, A., Roxas, J.L., Falzari, K., Weflen, A.W., Kim, A., Bellmeyer, A., Turner, J.R., Neish, A.S. and Rhee, K.J., 2010. Enteropathogenic *E. coli* non-LEE encoded effectors NleH1 and NleH2 attenuate NF-kappaB activation. *Molecular Microbiology* **78**, 1232-1245.

Ruiz, R.C., Melo, K., Rossato, S.S., Barbosa, C.M., Corrêa, L.M., Elias, W.P. and Piazza, R.M., 2014. Atypical enteropathogenic *Escherichia coli* secretes plasmid encoded toxin. *BioMed Research International*, **2014.**

Savkovic, S. D., Villanueva, J., Turner, J. R., Matkowskyj, K. A. and Hecht, G., 2005. Mouse model of enteropathogenic *Escherichia coli* infection. *Infection and Immunity* **73**, 1161-1170.

Shifflett, D. E., Clayburgh, D. R., Koutsouris, A., Turner, J. R. and Hecht, G. A., 2005. Enteropathogenic *E. coli* disrupts tight junction barrier function and structure *in vivo*. *Laboratory Investigation,* **85**, 1308-1324.

Swann, J. R., Tuohy, K.M., Lindfors, P., Brown, D.T., Gibson, G.R., Wilson, I.D., Sidaway, J., Nicholson, J.K. and Holmes, E., 2011. Variation in antibiotic-induced microbial recolonization impacts on the host metabolic phenotypes of rats. *Journal of Proteome Research,* **10**, 3590-3603.

© University of Venda

Scaletsky, I.C., Aranda, K.R., Souza, T.B. and Silva, N.P., 2010. Adherence factors in atypical enteropathogenic *Escherichia coli* strains expressing the localized adherence-like pattern in HEp-2 cells. *Journal of Clinical Microbiology*, **48**(1), 302-306.

Sugiharto, S., Hedemann, M.S. and Lauridsen, C., 2014. Plasma metabolomic profiles and immune responses of piglets after weaning and challenge with *E. coli*. *Journal of Animal Science and Biotechnology*, **5**(1), 17.

Trabulsi, L.R., Keller, R. and Gomes, T.A.T., 2002. Typical and Atypical Enteropathogenic *Escherichia coli*. *Emerging Infectious Diseases*, **8**(5), 508.

Ugalde-Silva, P., Gonzalez-Lugo, O. and Navarro-Garcia, F., 2016. Tight Junction Disruption Induced by Type 3 Secretion System Effectors Injected by Enteropathogenic and Enterohemorrhagic *Escherichia coli. Frontiers in Cellular and Infection Microbiology* **6**, 87.

Vulcano, A.B., Tino-De-Franco, M., Amaral, J.A., Ribeiro, O.G., Cabrera, W.H.K., Bordenalli, M.A., Carbonare, C.B., Álvares, E.P. and Carbonare, S.B., 2014. Oral infection with enteropathogenic *Escherichia coli* triggers immune response and intestinal histological alterations in mice selected for their minimal acute inflammatory responses. *Microbiology and Immunology*, **58**(6), 352-359.

Welkos, S.L., 1984. Experimental gastroenteritis in newly-hatched chicks infected with *Campylobacter jejuni*. *Journal of Medical Microbiology*, **18**(2), 233-248.

Zarivach, R., Vuckovic, M., Deng, W., Finlay, B. B. and Strynadka, N. C., 2007. Structural analysis of a prototypical ATPase from the type III secretion system. *Nature Sructural and Molecular Biology,* **14**, 131-137.

Zhang, H. Z. and Donnenberg, M. S., 1996. DsbA is required for stability of the type IV pilin of enteropathogenic *Escherichia coli*. *Molecular Microbiology* 21, 787-797.

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Zhang, Q., Li, Q., Wang, C., Liu, X., Li, N. and Li, J., 2010. Enteropathogenic *Escherichia coli* changes distribution of occludin and ZO-1 in tight junction membrane microdomains *in vivo*. *Microbial Pathogenesis,* **48**, 28-34.

Zhang, W.L., Köhler, B., Oswald, E., Beutin, L., Karch, H., Morabito, S., Caprioli, A., Suerbaum, S. and Schmidt, H., 2002. Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. *Journal of Clinical Microbiology*, **40**(12), 4486-4492.

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CHAPTER 4

OBJECTIVE 2: **DETERMINE THE INTERACTION BETWEEN EPEC AND ETEC CO-INFECTIONS**

The objective was to determine the interaction between EPEC and ETEC co-infections *in vitro,* using HCT-8 cells to determine adherence traits, inflammatory responses and expression of virulence genes. To also determine interaction between EPEC and ETEC co-infections *in vivo* using C57BL/6 mice by assessing clinical outcomes, inflammatory responses and virulence gene expressions.

3.1. ABSTRACT

Enteropathogenic *E. coli* and Enterotoxigenic *E. coli* have been reported to cause moderate to severe diarrhoea in young children. Co-infections with these pathogens have been increasingly detected in children and animals. HCT-8 cells were infected with EPEC, ETEC and both pathogens at the same time to determine adherence, inflammation and virulence gene expression. Weaned C57BL/6 mice were also infected with EPEC, ETEC and both the pathogens and followed for 3 days to determine clinical outcomes, adherence, inflammatory responses and expression of virulence genes. *In vitro*, EPEC adherence was higher than ETEC adherence and the cells infected with EPEC/ETEC (P/T) had increased IL-8 levels. Expression of virulent gene, *bfp* was higher in the P/T co-infections. *In vivo*, the mice infected with P/T had increased weight loss up to day 3 post infection. Faecal inflammatory biomarkers LCN-2 and MPO were higher in the co-infected mice at day 2 post infection, with increased systemic markers, IL-6 and SAA at day 3 post infection. Expression of virulent gene, *eltA* involved in expression of LT was reduced in the colon of mice infected with P/T co-infections. In conclusion, Interaction between P/T co-infections have synergistic effects, leading to severe disease outcome.

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4.2. INTRODUCTION

The EPEC and ETEC pathotypes descend from the same *E. coli* family with the difference in colonization traits and disease outcome (Kaper et al, 2004; Croxen et al, 2013). These pathogens contribute significantly to diarrhoeal diseases worldwide and have been reported to mostly infect children less than 5 years of age (Kotloff et al, 2013; Platts-Mills et al, 2015; Ledwaba et al, 2018). Single infections with these pathotypes have been extensively reported for many years (Nataro et al, 1987; Adhikari et al, 1985; Vargas et al, 1998; Estrada-Garcia et al, 2009; Kotloff et al, 2013; Platts-Mills et al, 2015; Ledwaba et al, 2018; Mathew et al, 2019). Typical EPEC infection in children causes infantile diarrhoea with severe disease outcome that can result in deaths in young children less than 2 years of age (Kotloff et al, 2013). ETEC alone is reported to cause traveller's diarrhoea with production of ST and/or LT enterotoxins leading to watery diarrhoea (Croxen et al, 2013).

The frequent detection of EPEC and ETEC infections in humans and animals have led to these pathogens occurring together in one host, as co-infections (Adhikari et al, 1985; Wada et al, 1996; Albert et al, 1999; Vilchez et al, 2009; Bonkoungou et al, 2012; Andersson et al, 2018; Ledwaba et al, 2018). Co-infections in this study, were referred to as the occurrence of infection in a host with more than 1 pathogen at the same time. Some studies have reported these pathogens to occur in combination (that is typical EPEC and ETEC in a single patient) (Adhikari et al, 1985; Albert et al, 1999; Vilchez et al, 2009; Bonkoungou et al, 2012; Iijima et al, 2017; Dutta et al, 2018; Ledwaba et al, 2018). These epidemiological studies have also suggested that patients infected with these co-infections, are likely to develop more severe disease outcomes than single infections (Adhikari et al, 1985; Kinnula et al, 2018; Ledwaba et al, 2018; Mathew et al, 2019).

In vitro and *in vivo* models have provided an insight in understanding in the pathogenesis mechanism of human pathogens (Croxen et al, 2013; Law et al, 2013; Bolick et al, 2018). Different DEC murine models highlighting the EAEC (Bolick et al, 2013; Boisen et al,

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2019), ETEC (Bernal-Reynaga et al, 2013; Bolick et al, 2018), EPEC (Savkovic et al, 2005;Law et al, 2013; Dupont et al, 2016) and EHEC (Ritchie, 2015) infections have been developed. These models are of great importance as they have paved a way in understanding the pathogenesis mechanism of different *E. coli* pathotypes.

Bacteria-bacteria interactions have been reported (Crane et al, 2006; Toledo et al, 2011). Interaction of EPEC and ETEC co-infections *in vitro* has been reported to have synergistic effects resulting in increased disease outcome (Crane et al, 2006). Bacteria-parasitic interactions have also been studied (Galván-Moroyoqui et al; 2008; Bartelt et al, 2017; Manko et al, 2017). EPEC and *E. histolytica* co-infections were reported to have increased virulence gene expression *in vitro* (Galván-Moroyoqui et al, 2008). The EPEC infectious murine model that was developed in Objective 1 was used to further understand the clinical outcomes, inflammatory responses and virulence gene expression as a result of P/T co-infections. Overall, the objective was to study the interaction of P/T co-infections and determine whether this interaction would result in severe disease outcomes as suggested by clinical studies.

4.3. MATERIALS AND METHODS

4.3.1. *IN VITRO* ANALYSIS

4.3.1.1 HCT-8 cells growth

The HCT-8 cell line (ATTCC CCL-244) were maintained and grown in Corning 75-cm^2 culture flask (Sigma-Aldrich, St. Louis, Missouri, USA). The complete culture media for growth contained Rose Park Memorial Institute (RPMI) 1640 medium with L-glutamine (Sigma-Aldrich, St. Louis, Missouri, USA), 10% foetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, Missouri, USA) and 10 U/mL penicillin with 10 µg/mL streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Cells were grown at 37 °C,

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5% CO_2 incubator. Once 90% confluency was reached, cells were passaged using 1 mL of 0.25% trypsin (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For seeding collagen-coated 12-well plates (Sigma-Aldrich, St. Louis, Missouri, USA) were seeded with cells containing complete culture media and placed at 37 °C, 5% CO_2 incubator. Once cells reached confluency of 70-80%, they were ready for infection.

4.3.1.2. Preparation of bacterial strains and infection on HCT-8 cells

EPEC E2348/69 (O127:H6) (Levine et al, 1985) and ETEC H1047 (O78:H11) (Evans and Evans, 1973) cultures were prepared directly from frozen glycerol stocks. The bacteria were grown in 5 mL DMEM high glucose (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in a static 37 °C incubator overnight. The next day the bacteria were centrifuged and resuspended in RPMI with no FBS and no antibiotics. Once optimum growth was reached, OD_{600} ~ 0.6, then the bacteria was ready for infection.

The first uninfected control group contained RPMI only, the second group contained 10 µL (1:100 MOI) (that is 4.8 $\times 10^8$ CFU/well) of ETEC, the third group contained 10 µL (1:100 MOI) of EPEC in each well. Lastly, the fourth group for co-infections contained10 µL (1:100 MOI) of ETEC and 10 µL (1:100 MOI) EPEC (added at the same time) with fresh RPMI with no antibiotic and no FBS, and incubated for 3 hours, at 37 °C in 5% CO_2 incubator.

After infection, 500 µL supernatant was collected and placed at -20 °C. Each well was washed 3 times with 1 X PBS (Thermo Fisher Scientific, Waltham, Massachusetts, USA). 180 µL of ATL buffer (Qiagen, Hilden, Germany) was added to each well and a sterile cell scrapper (Nalgene Nunc International, Rochester, New York, USA) was used to scrape off the attached bacteria and cells, then transferred into 1.5 mL eppendorf tubes and placed at -20 °C till further analysis. Other sets of infected cell culture plates were incubated for 2 hours at 37 °C, 5% CO_2. Cells were then washed 3 times with 1 X PBS and 400 µL of RLT buffer (Qiagen, Hilden, Germany) was added to each well and a sterile

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cell scrapper was used to scrape off the cells and adhered bacteria, then transferred into 1.5 mL eppendorf tube and placed at -20 °C till further analysis.

4.3.1.3. Measurement of IL-8 levels

The collected supernatant was used to measure IL-8 using human IL-8/CXCL8 ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) according to manufacturer instructions. The BCA was performed using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The results were measured in pg/mg of protein.

4.3.1.4. DNA extraction and real-time PCR

The lysed cells containing ATL buffer were analysed. The DNeasy blood and tissue kit (Qiagen, Hilden, Germany) was used to extract DNA according to manufacturer's instructions. The sensiFAST SYBR No-ROX kit (Bioline, London, United Kingdom) was used together with *eae* and *lt* primers (Table 4.1**)** to prepare the reaction mixture according to manufacturer instructions. Real-time PCR was performed using Bio-Rad CFX connect real-time PCR (Bio-Rad, Hercules, California, USA) under the following conditions: 95 °C for 3 min, followed by 40 cycles of 15 sec at 95 °C, 60 sec at 55 °C and lastly 20 sec at 72 °C.

Table 4.1: Primer sequence used to detect EPEC and ETEC

Pathogen	Primer	Sequence (5'-3')	References
EPEC	*eae* (F)	CCCGAATTCGGCACAAGCATAAGC	
	(R)	CCCGGATCCGTCTCGCCAGTATTCG	Zhang et al, 2002
ETEC	*lt* (F)	TTCCCACCGGATCACCAA	
	(R)	CAACCTTGTGGTGCATGATGA	Liu et al, 2013

4.3.1.5. RNA extraction and real-time PCR

Cells that were lysed with RLT buffer were further analysed. The RNAeasy kit (Qiagen, Hilden, Germany) was used to extract RNA according to manufacturer's instructions. The

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DNAse mix (1 µL DNAse buffer, 1 µL DNAse I and 8 µL H_2O) and 10 µL of RNA were incubated for 10 min at room temperature, then1 µL EDTA was added to stop the reaction. The samples were run on Bio-Rad iCycler Thermal cycler (Bio-Rad, Hercules, California, USA) at 65 °C for 10 min, and 4 °C hold. The iSCRIPT cDNA Synthesis kit (Bio-Rad, Hercules, California, USA) was used to synthesize cDNA according to manufacturer instructions.

The sensiFAST SYBR No-ROX kit (Bioline, London, United Kingdom) was used together with primers (Table 4.2 and Table 4.3) (*rrsB* used as house-keeping gene) to prepare the reaction mixture according to manufacturer instructions. Real-time PCR was performed using Bio-Rad CFX connect real-time PCR using the following conditions 95 °C for 3 min, followed by 40 cycles of 15 sec at 95 °C, 60 sec at 55 °C and lastly 20 sec at 72 °C

Table 4.2: Primers used for EPEC virulence gene expression.

Primer	Sequence 5'-3'	References
ler (F) (R)	TGGGATATACTAATGTGCCTGATGA ACCAGGTCTGCCCTTGTTGA	Leverton and Kaper, 2005
tir (F) (R)	GCAGAAGACGCTTCTCTGAATA CCCAACTTCAGCATATGGATTA	Leverton and Kaper, 2005
eae (F) (R)	GGCGATTACGCGAAAGATAC GATTAACCTATGCCGTTCCA	Leverton and Kaper, 2005
espA (F) (R)	GCTGCAATTCTCATGTTTGC GGGCAGTGGTTGACTCCTTA	Leverton and Kaper, 2005
bfpA (F) (R)	TGATTGAATCTGCAATGGTG AGCATTCTGCGACTTATTGG	Leverton and Kaper, 2005
rrsB (F) (R)	TGCAAGTCGAACGGTAACAG AGTTATCCCCCTCCATCAGG	Leverton and Kaper, 2005
per (F) (R)	GGGACATGGAAATTGTCGGAATCG TGCATTTCATTGAGGTTCGCAGT	Crane et al, 2007
escV (F) (R)	AGTGCTCGTTTTTCCCTTGA AGCGAAGAACTTTTGCCTCA	Leverton and Kaper, 2005
espB (F) (R)	GCTCTGATTGGTG GTGCTAT CCTGCCTTCT GTGCTAATTC	Crane et al, 2007

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Table 4.3: Primers used for ETEC virulence gene expression

Primer	Sequence 5'-3'	References
sta2 (F)	CACCCGGTACAAGCAGGATT	
(R)	TTCACCTTTCGCTCAGGATG	Bolick et al, 2018
eltA (F)	CAAAGCCGGTTTGTGTTCCT	
(R)	TGCTGACTCTAGACCCCCAGA	Bolick et al, 2018
cfa1 (F)	TCAGTGTGTCATGGGAGGA	
(R)	ACCGGCAGTTTTAGGTGCAG	Bolick et al, 2018

4.3.2. *IN VIVO* ANALYSIS

4.3.2.1. Animal husbandry

The protocols used in this study and the vivarium conditions used to keep the C57BL/6 mice is described in section 3.3.1

4.3.2.2. Rodent diet and antibiotics

The rodent diet and the antibiotics used are described in section 3.3.2

4.3.2.3. Preparation of bacterial strains and mice infection

Bacterial strains EPEC E2348/69 and ETEC H10407 were grown as described in section 3.3.3.

The control group was orally administered DMEM only, the second group was orally administered 100 µL of 10^{10} CFU/mL (10^{9} CFU/mouse in a total volume of 100µL of 10^{10} CFU/mL) EPEC, the third group was orally administered 100 µL of 10^{10} CFU/mL ETEC only. Lastly the forth group was orally administered 100 µL of 10^{10} CFU/mL EPEC and 100 µL of 10^{10} CFU/mL ETEC at the same time using sterile 22-guage feeding needles.

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During infection, mice were monitored daily for changes in weights and stool specimen were collected. At day 3 pi, mice were euthanized. Blood was immediately collected via cardiac puncture and placed in 1.5 mL eppendorf tube. The blood was then centrifuged at 8000 x g for 5 min at 4 °C. The serum was then transferred into a new eppendorf tube and placed immediately at -80 °C. Immediately after blood collection, mice were rapidly dissected and intestinal tissues sections (duodenum, jejunum, ileum and colon) were collected and flash frozen using liquid nitrogen and then placed at -80 °C till further analysis.

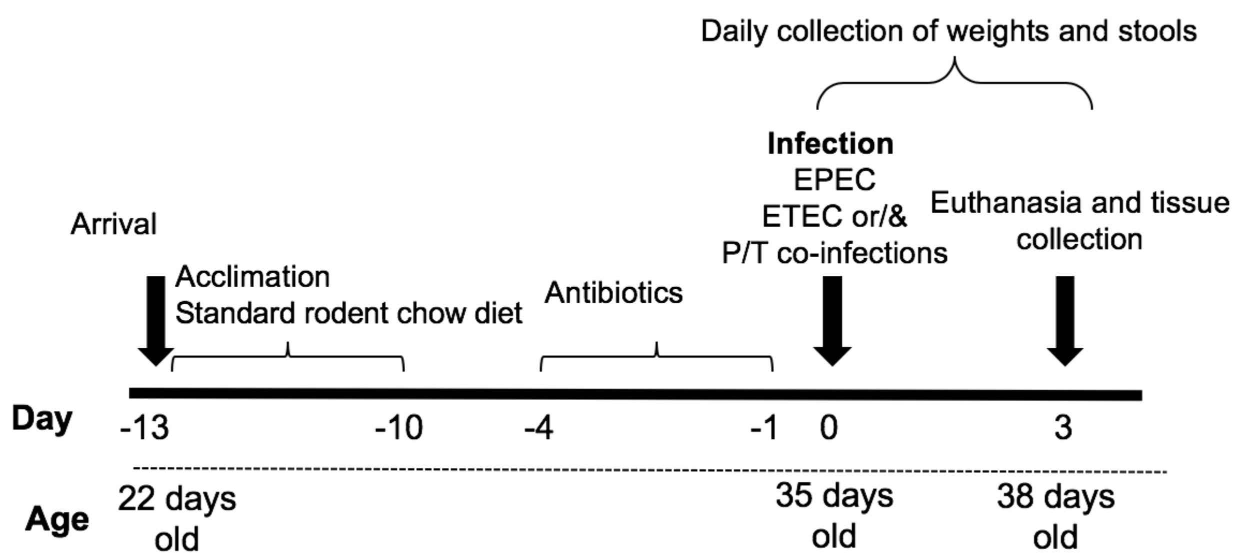

Figure 4.1: Schematic presentation of the experimental procedure of EPEC and ETEC infections in C57BL/6 mice. 22-day-old C57BL/6 mice were placed on standard rodent chow diet. Mice were administered antibiotics in drinking water 4 days prior infection, and was replaced with clean water 1-day prior infection. Mice were orally infected with either EPEC, ETEC or both pathogens. Weights were recorded and stools were collected daily. Mice were euthanised at day 3 pi.

4.3.2.4. Stool shedding and tissue burden

Stool shedding and tissue burden was determined using the procedure mentioned in section 3.3.4, using primers in Table 4.1.

4.3.2.5. Intestinal inflammatory response

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MPO and LCN-2 were evaluated using stool specimens collected at day 2 pi and cecal contents collected at day 3 pi and IL-6 was evaluated using colonic tissues collected at day 3 pi following a procedure mentioned in section 3.3.6

4.3.2.6. Systemic inflammation

SAA and CRP concentrations were measured using the protocol mentioned in section 3.3.7.

4.3.2.7. RNA extraction and real-time PCR

The RLT buffer was added to the ileal and colonic sections that were flash frozen, with the beads and the beat beater was used to homogenise the samples. The Qiagen RNeasy kit was used to extract RNA according to manufacturer instructions. The iSCRIPT cDNA synthesis kit was used to synthesize cDNA according to manufacturer instructions.

The cDNA was prepared together with the sensiFAST SYBR No-ROX kit and the primers (Table 4.2 and Table 4.3) according to manufacturer instructions. Real-time PCR was performed using Bio-Rad CFX connect real-time PCR (Bio-Rad, Hercules, California, USA) using the following conditions: 95 °C for 3 min, followed by 40 cycles of 15 sec at 95 °C, 60 sec at 55 °C and lastly 20 sec at 72 °C.

4.3.3. Statistical analysis

The experiments were conducted in triplicates. The collected data was analysed using GraphPad Prism 7 software (GraphPad, San Diego, California, USA). The data are represented as ± SEM. The results were considered significant when was $p<0.05$.

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4.4. RESULTS

4.4.1. EPEC AND ETEC *IN VITRO* ANALYSIS

4.4.1.1. Adherence and Inflammatory responses in HCT-8 cells co-infected with P/T strains

The HCT-8 cells were used to compare adherence between single infections of ETEC, EPEC and P/T co-infections. All the bacterial strains adhered to cells at $<10^8$ CFU/well (Figure 4.2A). EPEC single infections adhered to cells better than ETEC single infections ($p<0.05$). EPEC single infections were also significantly different when compared to P/T(*lt)* co-infections ($p<0.001$). Interestingly, the P/T co-infected cells adhered at a rate similar to the single infections, however, no significant difference was observed.

The HCT-8 cells were used to analyse inflammatory responses as a result of P/T co-infections. As seen in Figure 4.2B, cells infected with EPEC single infections had increased IL-8 of <1500 pg/mg protein when compared to controls and was significantly higher when compared to ETEC single infections with IL-8 measured at ≥500 pg/mg protein ($p<0.0001$). Cells infected with P/T co-infections had increased IL-8 levels of ≥1500 pg/mg protein of which was higher than that of ETEC single infections, and was significantly increased when compared to EPEC ($p<0.05$) and ETEC ($p< 0.0001$) alone, suggesting an additive effect.

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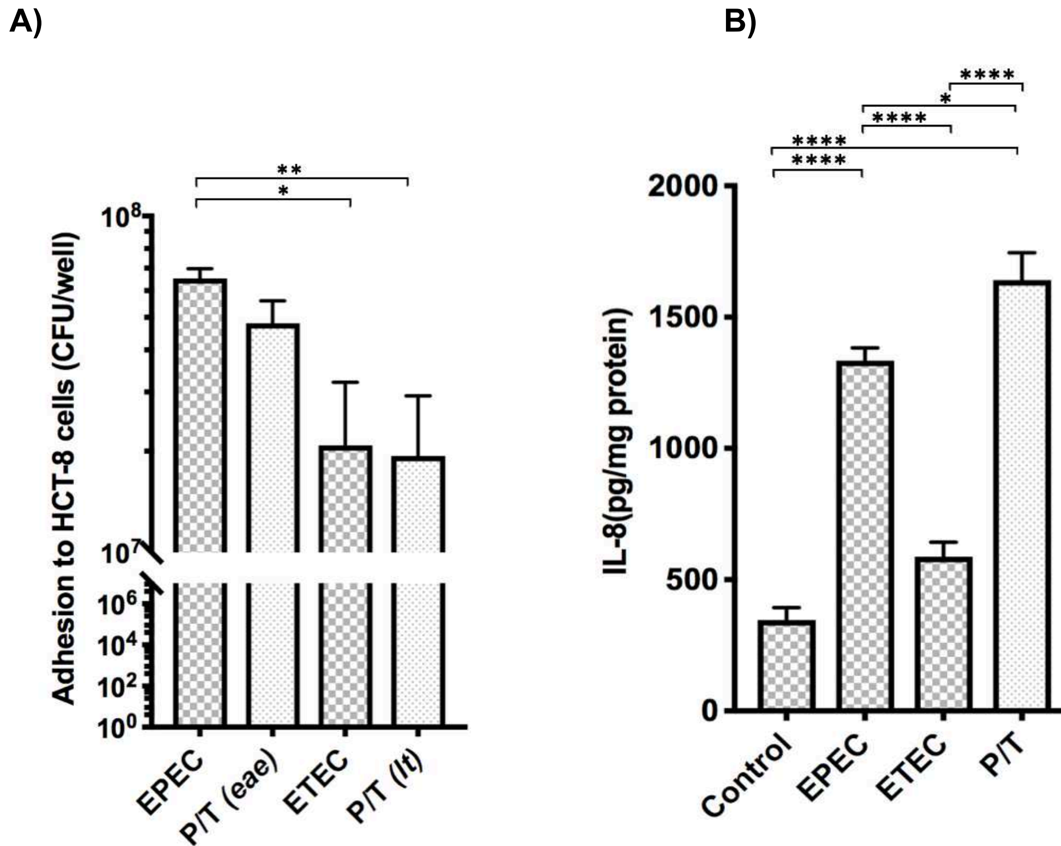

Figure 4.2: Adherence and inflammatory cytokines in P/T infected HCT-8 cells. Cells were infected with EPEC, ETEC, and P/T co-infections for 3 hours. **A**. DNA was extracted from the infected and uninfected wells and qPCR was used to determine adherence of EPEC, ETEC and P/T to the cells. **B.** Infected and uninfected cells were used to determine IL-8 levels using ELISA. Data analysed using one-way ANOVA and Tukey's *post hoc* test.*p<0.05, **p<0.001, ****p<0.0001. Key words: P/T(*eae*) = qPCR measured *eae* gene only in mice infected with EPEC and ETEC co-infections; P/T(*lt*) = qPCR measured only *lt* gene in mice infected with EPEC and ETEC co-infections.

4.4.1.2. Expression of EPEC and ETEC virulence genes in HCT-8 cells.

To explore changes in the pathogenesis mechanism of EPEC and ETEC when co-infected *in vitro*, expression of various genes involved in adherence and secretion system of these pathogens were analysed. As seen in Figure 4.3, HCT-8 cells infected with P/T co-infections had a significant increase in expression of the *bfpA* gene (involved in adherence) (p<0.05), when compared to EPEC single infections. Overall, there was no significant increase in the expression of *ler, espB, espA, eae, tir, escV* and *per* which are

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genes involved in the T3SS of EPEC pathogenesis. As seen in Figure 4.4, there was no significant difference between the genes involved in ETEC adherence (*cfa1*) and the enterotoxins produced by the T2SS (*eltA* and *sta2*).

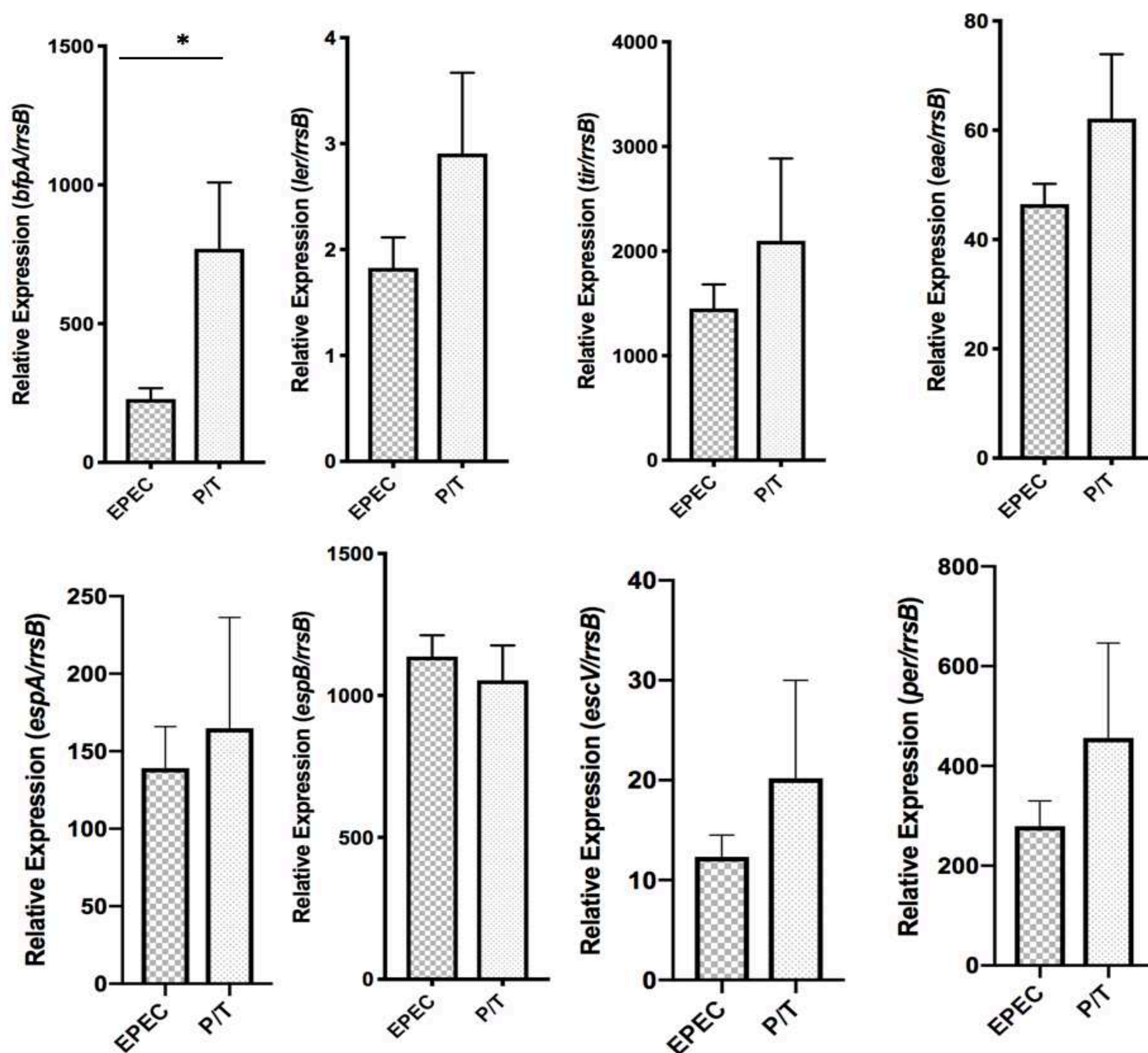

Figure 4.3: Relative expression of EPEC virulence genes in HCT-8 cells infected with P/T co-infections. HCT-8 cells infected for 2 hours, cDNA was used to determine expression of EPEC virulence genes involved in the T3SS and adherence using qPCR. Data analysed using *t-test* and Mann Whitney test, *p<0.05.

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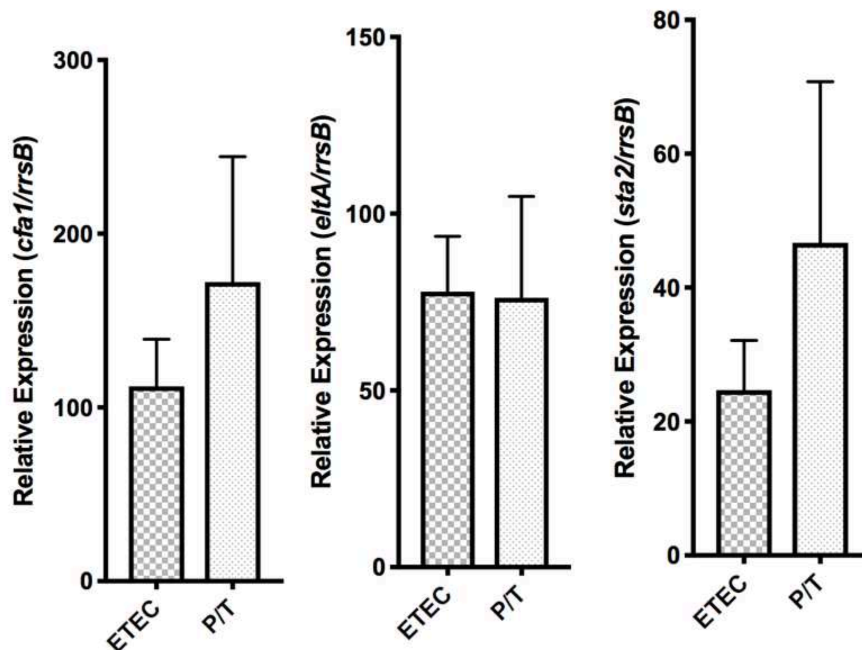

Figure 4.4: Relative expression of ETEC virulence genes in HCT-8 cells infected with P/T co-infections. HCT-8 cells infected for 2 hours, cDNA was used to determine expression of ETEC virulence genes involved in the T2SS and adherence using qPCR.

4.4.2. EPEC AND ETEC *IN VIVO* ANALYSIS

4.4.2.1. Clinical outcomes of C57BL/6 mice infected with P/T co-infections

The EPEC murine model developed in Chapter 3 was further used to determine P/T co-infections *in vivo*. Weaned mice were infected with EPEC, ETEC or/and both the strains and followed for changes in weights and other clinical outcomes. As seen in Figure 4.5A, mice infected with P/T co-infections had a significant weight change at day 1 pi when compared to ETEC single infections ($p<0.05$). At day 2 pi, all the infected mice had a significant change in weight loss when compared to control uninfected mice ($p<0.005$). At day 3 pi, mice infected with P/T co-infections also had a significant weight change compared to EPEC ($p<0.001$) and ETEC single infections ($p<0.05$) (Figure 4.5A).

All mice infected with ETEC and EPEC developed soft wet stools. The mice infected with P/T co-infections developed severe diarrhoea (watery stools), with reduced mobility. Also,

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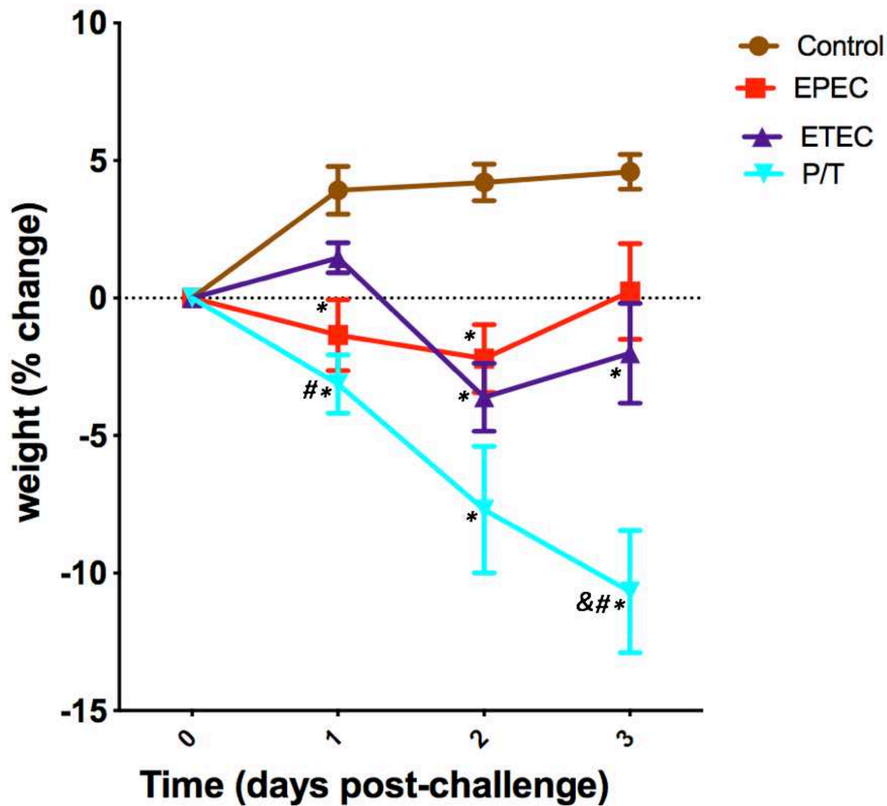
3 out of 11 P/T infected mice died during the 3-day infection period (Figure 4.5B). All the remaining mice were euthanized at day 3 pi. Due to the high weight loss recorded at day 3 pi (~10% weight change) and death, mice were euthanized in order to measure the inflammatory responses during peak of infection.

A)



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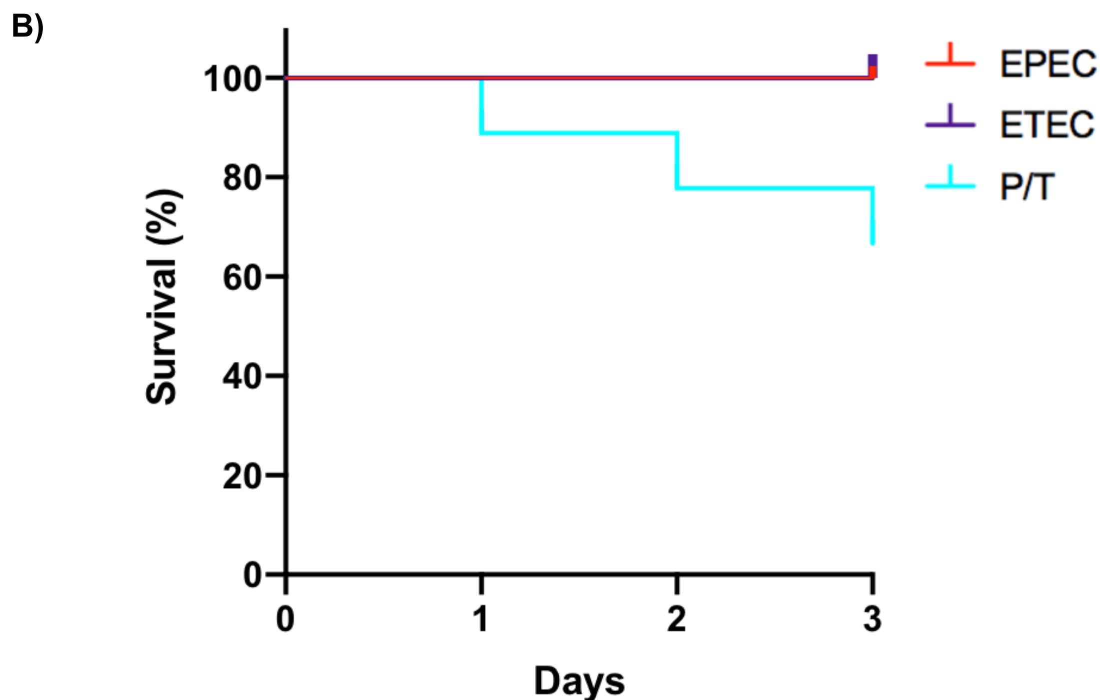

Figure 4.5: Change in weight and survival outcomes of C57BL/6 mice infected with P/T co-infections. **A)** Weaned mice infected with EPEC, ETEC and P/T (n = 11/group) co-infections were monitored daily for change in weight. Data analysed using two-way ANOVA and Tukey's *post hoc* test. *p<0.005 vs controls, # p<0.05 vs ETEC, &p<0.001 vs EPEC **B)** Survival curve following 3-day infection.

4.4.2.2. Stool shedding and tissue burden of mice infected with P/T co-infections

Stool specimens of mice infected with EPEC and ETEC single infections and P/T co-infections were collected and stool shedding was determined. As seen in Figure 4.6, ETEC (<10^{10} organisms/10 mg stool) infected mice shed more than mice infected with EPEC (<10^{8} organisms/10 mg stool) with significant difference of p<0.001 at day 1 pi. There was also a significant difference between mice infected with P/T(*eae*) co-infections and ETEC single infections (p<0.001) (Figure 4.6). Interestingly, irrespective of whether it was single or co-infections, the pathogens shed almost at the same rate.

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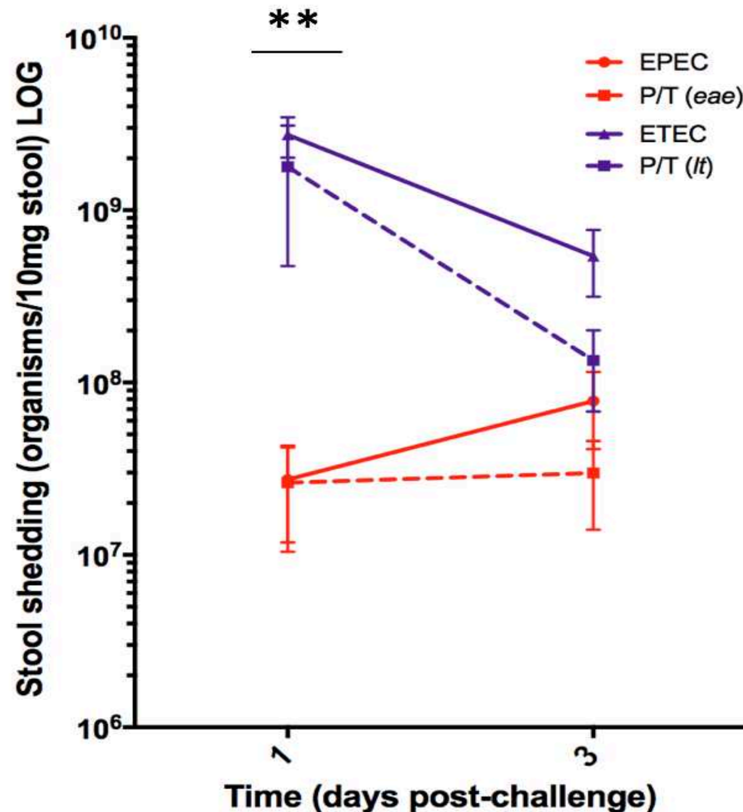

Figure 4.6: Stool shedding of mice infected with P/T co-infections. 5-week-old C57BL/6 mice were challenged orally with ETEC, EPEC, P/T co-infections and control mice were administered with DMEM (n = 11/group). DNA was extracted from collected stools and qPCR was used to determine shedding. Data analysed using two-way ANOVA and Tukey's *post hoc* test, **p<0.001. Key words: P/T(*eae*) = qPCR measured only *eae* gene in mice infected with EPEC and ETEC co-infections; P/T(*lt*) = qPCR measured only *lt* gene in mice infected with EPEC and ETEC co-infections

All uninfected and infected mice were euthanized at day 3 pi, and intestinal tissue specimen were collected and analysed for tissue burden. As seen in Figure 4.7, tissue burden was observed in all tissue sections suggesting that there was efficient colonization of the pathogenic strains in all the sections. There was a significant difference in the jejunum of mice infected with ETEC and EPEC single infections (p<0.05). Also, a significant difference was observed between in mice infected with ETEC single infections and P/T(*eae*) co-infections (p<0.05). Overall, tissue burden was higher in the ileal and colonic tissue of mice infected with single infections of ETEC and EPEC, however, no significant difference was observed. Interestingly, tissue burden of P/T co-infections was

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reduced in the ileum and colon suggesting that these pathogens were competing with each other for colonization (Figure 4.7).

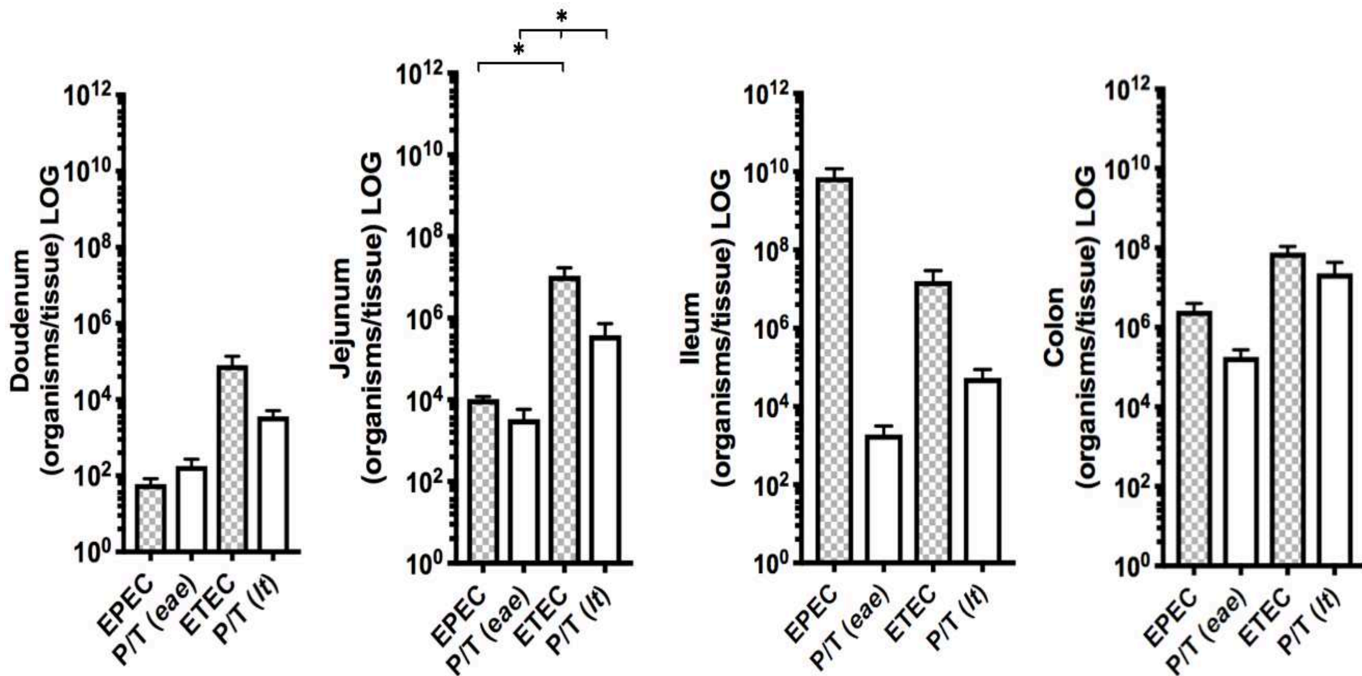

Figure 4.7: Tissue burden of P/T co-infections. Tissue sections of infected C57BL/6 mice collected at day 3 pi were homogenized, DNA was extracted and qPCR was used to determine EPEC, ETEC and P/T tissue burden. Data analysed using one-way ANOVA and Tukey's *post hoc* test, *p<0.05. Key words: P/T(*eae*) = qPCR measured only *eae* gene in mice infected with EPEC and ETEC co-infections; P/T(*lt*) = qPCR measured only *lt* gene in mice infected with EPEC and ETEC co-infections.

4.4.2.3. Inflammatory responses in mice co-infected with EPEC and ETEC strains

Stool specimens collected at day 2 pi were analysed for MPO and LCN-2 inflammatory biomarkers. As seen in Figure 4.8A, EPEC and ETEC single infections had increased MPO levels of ≥1000 pg/mg protein. Mice infected with P/T co-infections produced higher levels of MPO, ≥3000 pg/mg protein and were significantly different when compared to EPEC (p=0.0001) and ETEC (p<0.001) single infections. As seen in Figure 4.8B, mice infected with EPEC single infections had increased LCN-2 levels of ≤100 pg/mg protein,

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while mice infected with ETEC single infections produced ≤50 pg/mg protein of LCN-2. Mice infected with P/T co-infections produced higher levels of LCN-2, ≤150 pg/mg protein and were significantly different when compared to EPEC (p<0.001) and ETEC (p=0.0001) single infections.

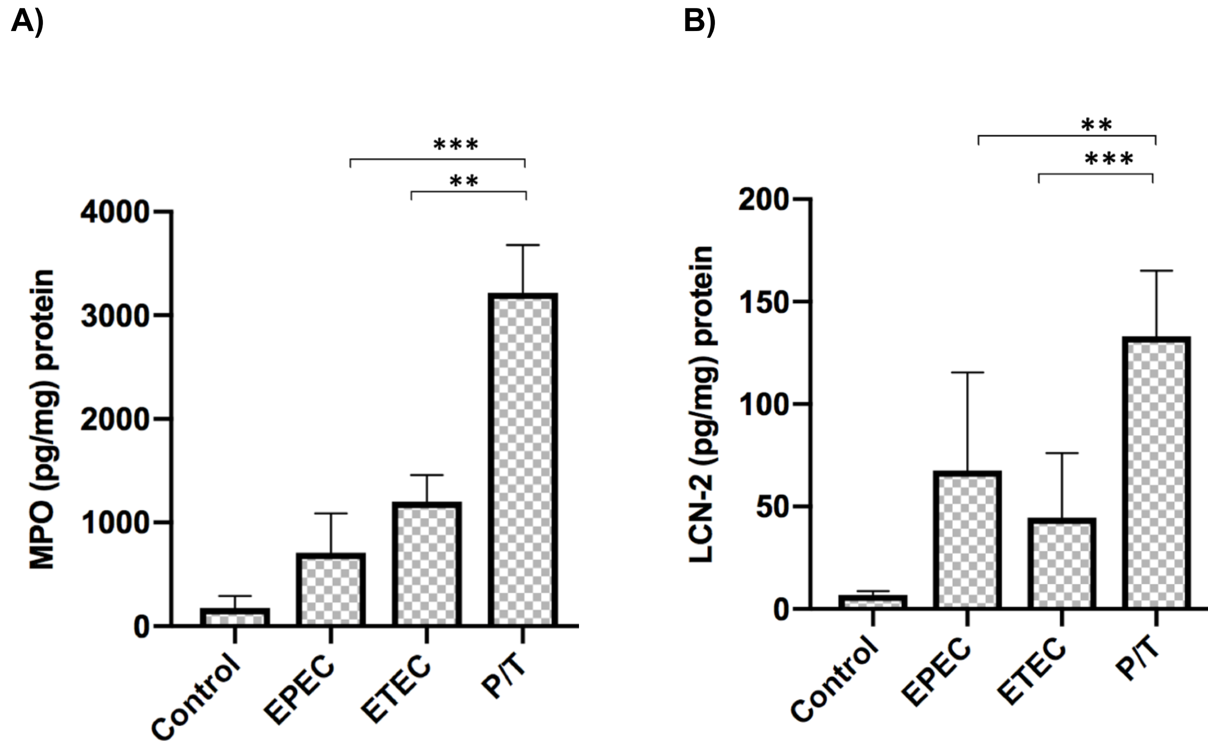

Figure 4.8: MPO and LCN-2 measured in stool specimen collected at day 2 pi. Stool protein lysates were extracted using RIPA buffer, MPO and LCN-2 were measured in control, EPEC, ETEC and P/T infections (n = 8-10/group). **A)** MPO levels **B)** LCN-2 levels. Data analysed using one-way ANOVA and Tukey's *post hoc* test, **p<0.001, ***p=0.0001.

Colonic protein lysates were used to measure pro-inflammatory cytokine, IL-6. As seen in Figure 4.9A, IL-6 levels in mice infected with P/T co-infections were produced at higher concentration of >10 pg/mg protein, and was found to be significantly different when compared to EPEC (>5 pg/mg protein) and ETEC (>5 pg/mg protein) single infections (p<0.0001). Acute-phase systematic response marker, CRP was found to be higher in mice infected with ETEC (≤3000 pg/mg protein) single infections and was significantly different when compared to mice infected with P/T co-infections (≤2000 pg/mg protein),

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p<0.05 (Figure 4.9B). Serum collected at day 3 pi was used to measure SAA. SAA was increased across all infected groups and mice infected with P/T co-infections (≤16000 pg/mL) were higher when compared to EPEC single infections (≤12000 pg/mL) with a difference of p<0.05 (Figure 4.9C).

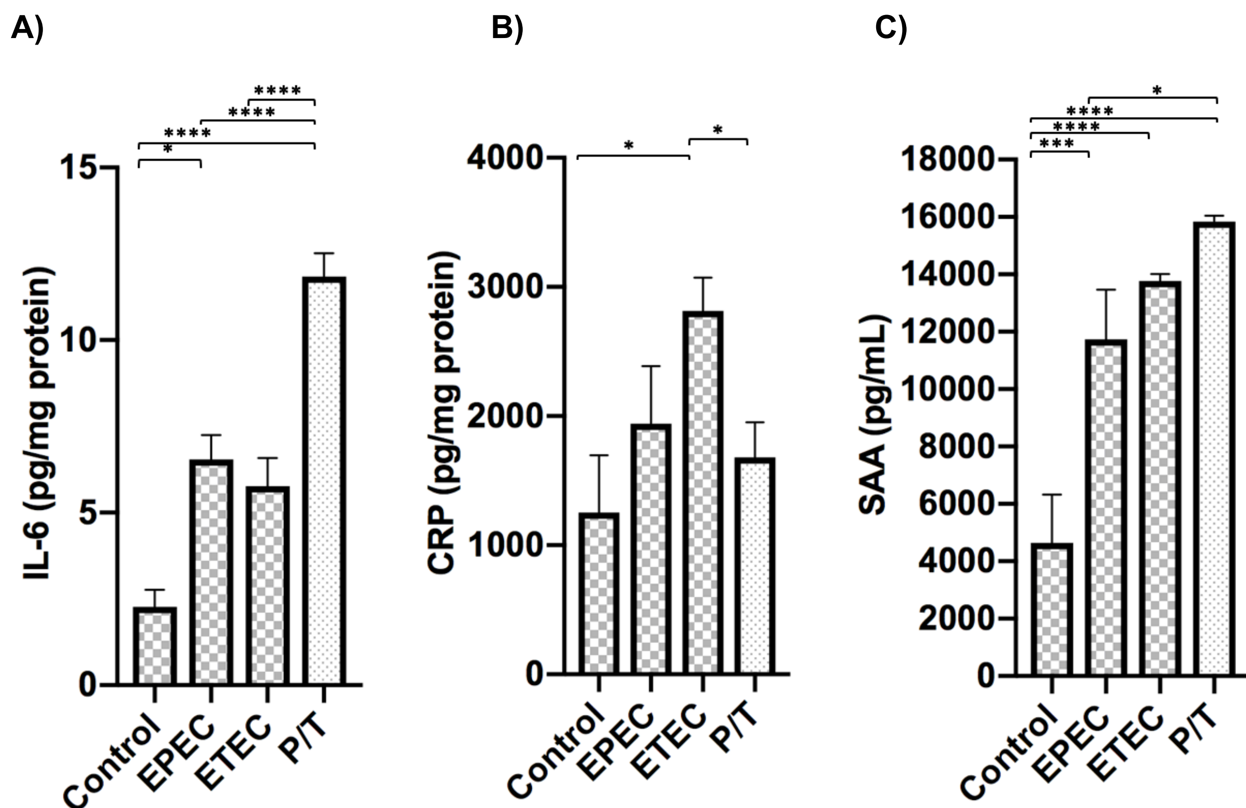

Figure 4.9: Acute phase inflammatory response of P/T co-infections. Protein lysates from colonic tissue extracted using RIPA buffer were used to measure IL-6 and CRP using ELISA, serum used to measure SAA using ELISA at day 3 pi. **A**) Increase in IL-6. **B)** Increase in CRP. **C)** Increase in SAA. Data analysed using one-way ANOVA and Tukey's *post hoc* test,*p<0.05, ***p=0.0001, ****p<0.0001.

4.4.2.4. Expression of EPEC and ETEC virulence genes *in vivo*

To explore the interaction of P/T co-infections *in vivo,* the ileal and colonic sections of mice infected with single infections of ETEC, EPEC and with both the pathogens were collected at day 3 pi and used to analyse the expression of virulence genes. As seen in

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Figure 4.10, expression of *bfpA* gene, which is involved with the initial attachment of EPEC in the host cell, although not significant was increased in the ileum of mice that were infected with P/T co-infections when compared to EPEC single infections. The *Ler* gene which is the universal regulator of EPEC and *espB* gene expressions were reduced in the ileum of mice infected with P/T co-infections. The ileal tissue sections of mice infected with ETEC single infections and P/T co-infections were also analysed for expression of ETEC virulence genes (Figure 4.11). The *cfa1* gene involved in the attachment of ETEC to host cell and *sta2* gene which releases the enterotoxin were reduced in ileal tissue of mice infected with P/T co-infections, however, no significant difference was observed.

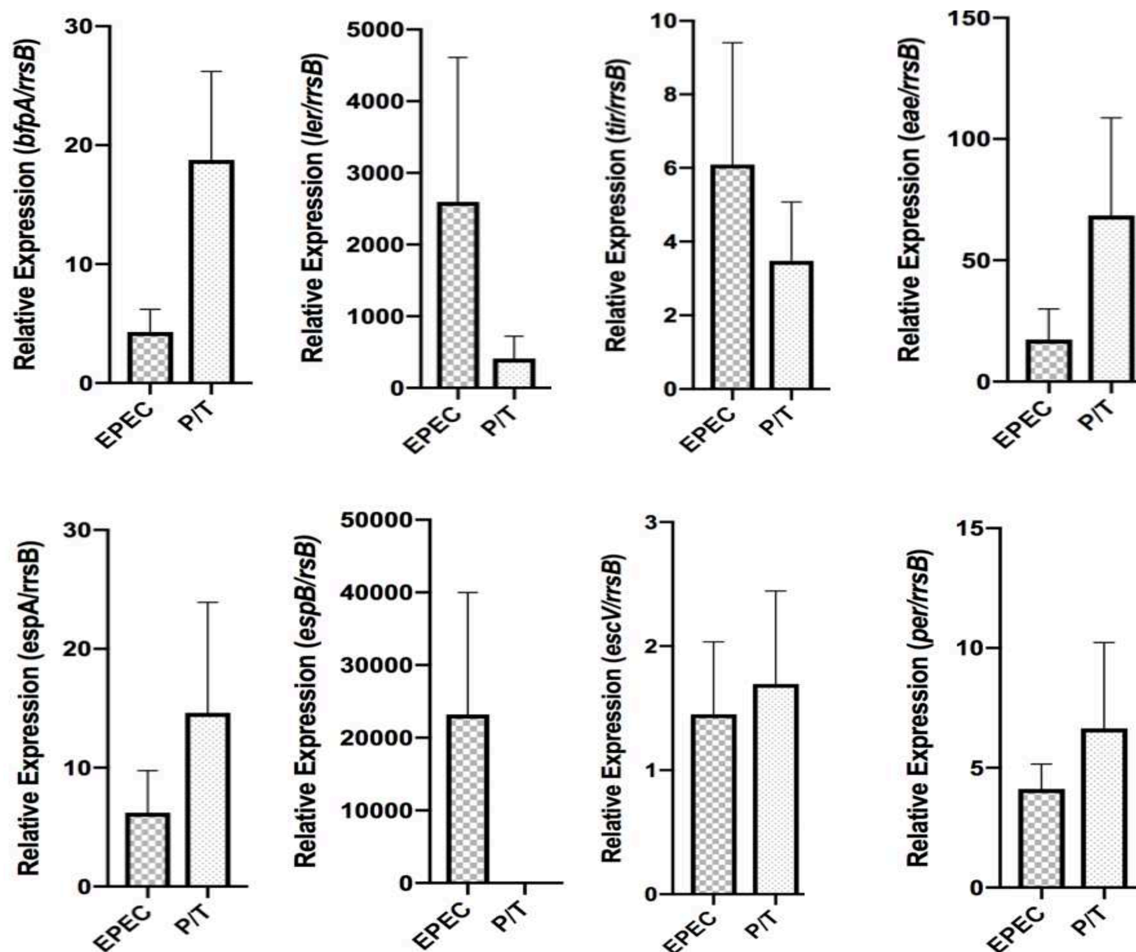

Figure 4.10: Relative expression of EPEC virulence genes in the ileum. C57BL/6 mice infected with EPEC, ETEC or P/T co-infections for 3 days, cDNA was used to determine expression of EPEC virulence genes using qPCR.

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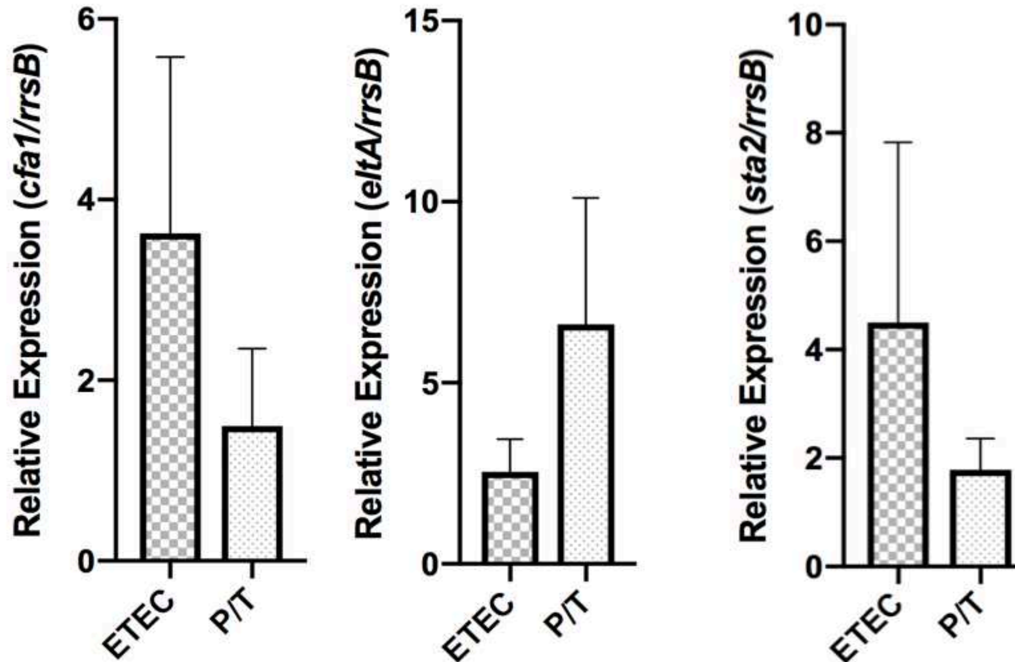

Figure 4.11: Relative expression of ETEC virulence genes in the ileal tissue. Weaned mice infected with EPEC, ETEC or P/T co-infections for 3 days, cDNA was used to determine expression of ETEC virulence genes using qPCR.

In the colon, all the genes involved in EPEC adherence and T3SS were expressed at a higher concentration in mice infected with P/T co-infections when compared to mice infected with EPEC single infections as seen in Figure 4.12, however, no significant difference was observed. Expression of all ETEC virulence genes, were reduced in mice infected with P/T co-infections when compared to ETEC single infections as seen in Figure 4.13. A significant difference was observed in the colon with *eltA* expression being reduced in the P/T co-infections when compared to ETEC single infections ($p<0.001$).

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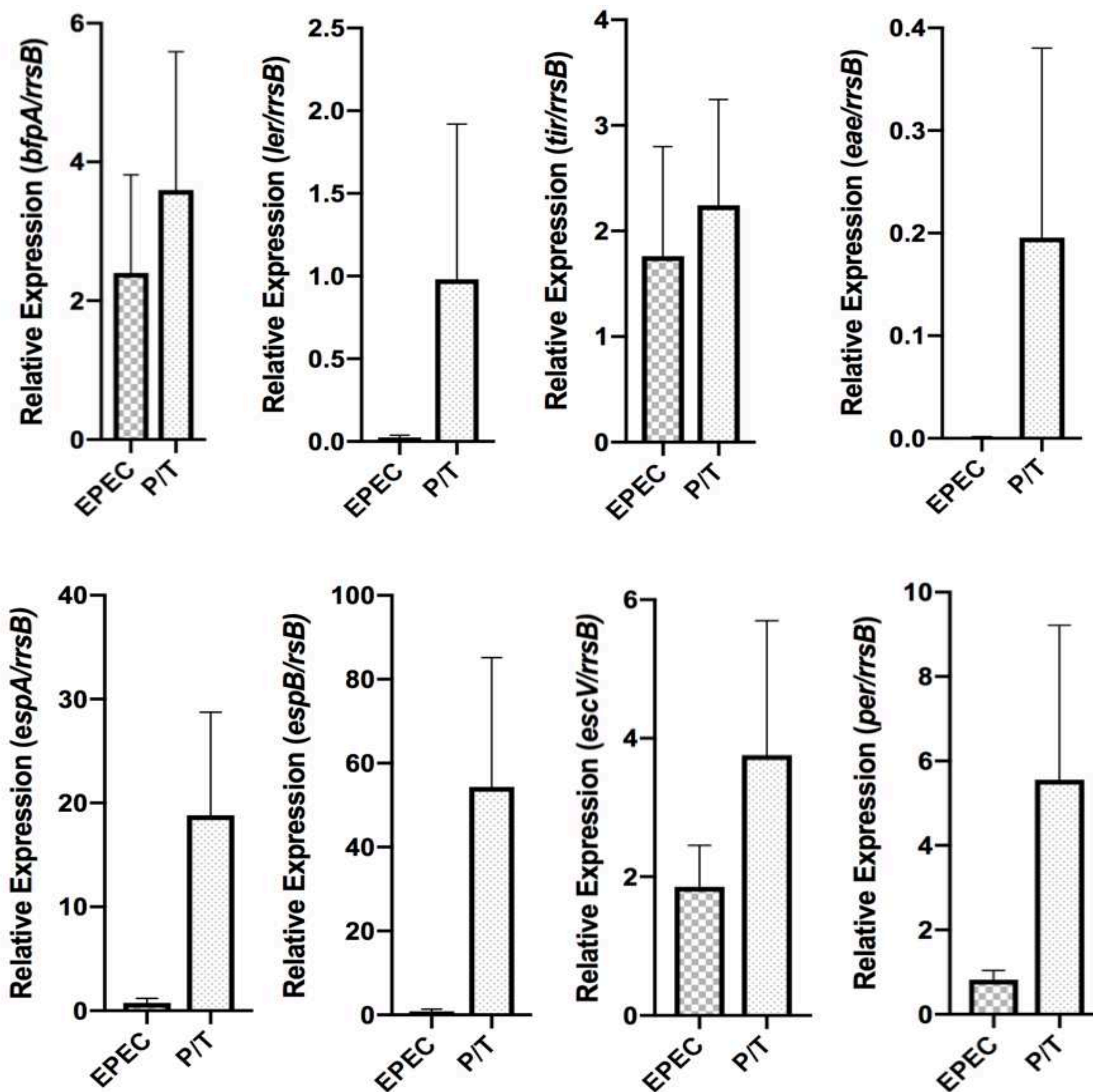

Figure 4.12: Relative expression of EPEC virulence genes measured in the colonic tissue. C57BL/6 mice infected with EPEC, ETEC or/and P/T co-infections for 3 days, cDNA was used to determine expression of EPEC virulence genes using qPCR.

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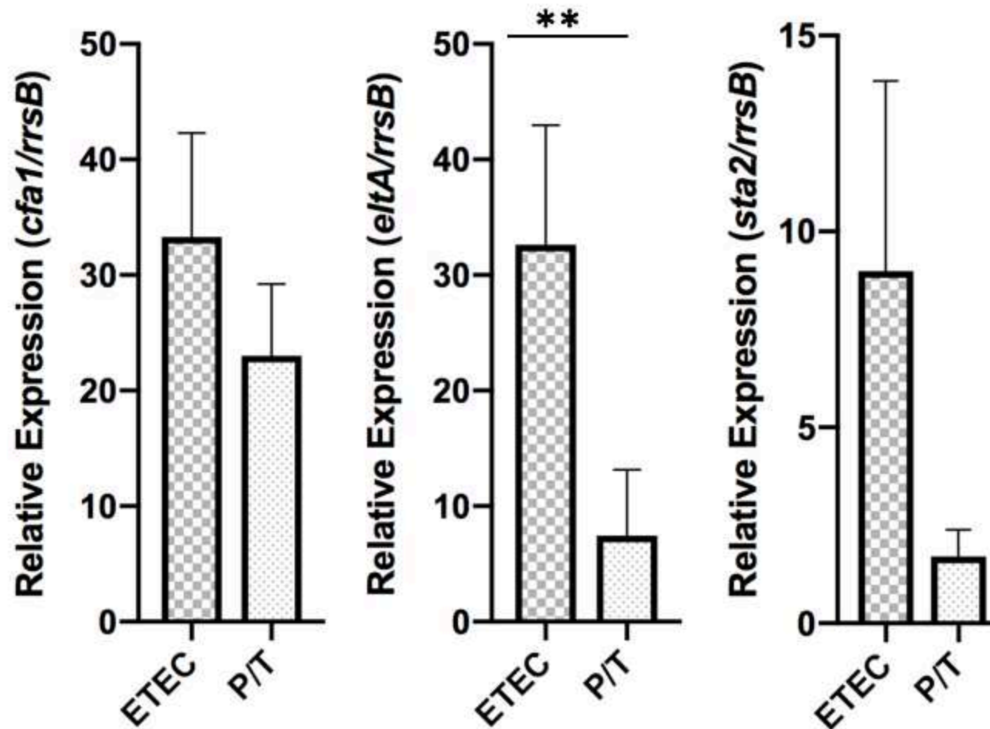

Figure 4.13: Relative expression of ETEC virulence genes measured in the colonic tissue. C57BL/6 mice infected with EPEC, ETEC or/and P/T co-infections for 3 days, c-DNA was used to determine expression of ETEC virulence genes using qPCR. *t-test* and Mann Whitney test, **p<0.0.001.

4.5. DISCUSSION

The occurrence of co-infections has been reported (Lima et al, 2018; Ledwaba et al, 2018; Mathew et al, 2019). Humans and animals are continuously exposed to different organisms and the likelihood of these organisms occurring in co-infections is relatively high (Mathew et al, 2019). The interactions of EPEC and ETEC co-infections are increasingly being recognized and have been reported in human and animal studies (Adhikari et al, 1985; Wada et al, 1996; Vilchez et al, 2009; Ledwaba et al, 2018; Mathew et al, 2019). In this study, *in vitro* and *in vivo* analyses were used to determine the

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interactions of P/T co-infections, focusing mainly on the clinical outcomes, inflammatory responses and virulence gene expression.

In vitro, ETEC attachment to colonic and epithelial cells have been observed (Darfeuille-Michaud et al, 1990; Guth et al, 1994). ETEC has been reported to not have any adherence trait (Kaper et al, 2004). On the other hand, typical EPEC has been characterized by forming LA pattern with small micro-colony formation at the site of infection (Kaper et al, 2004; Croxen et al, 2013). In this study, it can be assumed that the micro-colony formation involved during adherence, enabled EPEC to adhere better to cells when compared to ETEC single infections. Crane and colleagues (2006) were the first to report on the interaction of P/T co-infections *in vitro,* using human T-84 cells, and this has provided a great tool in understanding the interaction between these pathogens. Crane et al (2006) found that T-84 cells that were infected with EPEC and treated with ETEC filtrates produced a higher ATP response in cells that were co-infected than those that had only single infections. It was suggested that the LT enterotoxin enhanced EPEC to produce more ATP response (Crane et al, 2006). Based on these previous findings, interestingly the same observation was also seen in this study, in which the IL-8 response in cells infected with P/T co-infections was higher than the single infections, suggesting that ETEC might have had an additive effect, resulting in these pathogens acting synergistically together. Galván-Moroyoqui and colleagues (2008) studied the interaction of *E. histolytica* and EPEC co-infections *in vitro,* and found that *E. histolytica* strains that have phagocyted enteropathogenic bacteria adhered more to cells than the single infections, and this led to increased IL-8 inflammatory cytokine being released as a result of severe cell damage.

EPEC and ETEC interactions were further analysed *in vivo*. As reported in Chapter 3, an EPEC infectious murine model has been developed and was able to produce clinical outcomes, inflammation and metabolome perturbations. This model was further used to understand the interaction that might be involved during P/T co-infections. Changes in the gut microbiota have been reported to be the cause of increased clinical outcomes in children infected with EPEC and other pathogen co-infections (Mathew et al, 2019). In

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this study, all the infected groups developed diarrhoea and had increased weight loss. Disruption of the gut microbiota in mice infected with P/T co-infections resulted in mice developing severe diarrhoea that was accompanied with increased weight loss, reduced mice activity and some of the mice died during the infection period. Interaction of P/T co-infections has also been reported in post-weaning pigs that died as a result of these infections (Wada et al, 1996). These piglets developed severe diarrhoea that was accompanied by dehydration and sunken eyes, the intestines had severe lesions with increased mucosal haemorrhages and the brush border was not visible (Wada et al, 1996).

Allen et al (2006), reported on ETEC infection in adult mouse model that was treated with streptomycin and found that the ETEC H10407 strain colonized the small intestine with no increase in inflammatory responses. Recently, Bolick and colleagues (2018) reported on ETEC H10407 infection in weaned mice treated with antibiotic cocktail (similar to this study) and found that infection resulted in increased MPO and LCN-2. In this study, ETEC single infections also resulted in increased MPO and LCN-2 as previously reported. Interestingly, the inflammatory biomarkers (MPO and LCN-2) were very high in the P/T co-infections suggesting that the infected mice developed severe inflammation. This was further observed with increased pro-inflammatory IL-6 cytokine, that acts as a host defence mechanism stimulating the acute-phase response (Tanaka, 2014). The P/T co-infections have been reported to act synergistically and the infection is said to be greater when compared to the single infections and this poses a serious health risk (Crane et al, 2006). Bartelt et al (2017), reported on the interaction of *Giardia lamblia* and EAEC co-infections in protein deficient mice pretreated with antibiotic cocktail and found that this interaction resulted in increased inflammatory cytokines such as IL-1 which was reported to be higher than EAEC and *G. lamblia* single infections.

The CRP and SAA, markers of acute inflammatory response were increased in all the infected groups, however, in the P/T co-infected mice, SAA was produced at greater concentrations. It was surprising to note that CRP was produced at a lower concentration in the mice that were infected with the P/T co-infection at day 3 pi even though the

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infection was observed to be severe. This could have been due to a number of reasons, firstly, CRP was measured only in the colon and not in the ileum (as seen in objective 1), secondly the time for harvesting the tissues might have been after peak of infection.

It has been suggested that interaction with more than one pathogen in a host can result in increased virulence expression (Crane et al, 2006; Galván-Moroyoqui, et al, 2008; Mosser et al, 2015; Bose et al, 2016) Galván-Moroyoqui and colleagues (2008) studied the interaction of *E. histolytica* and EPEC co-infections that resulted in increased expression of *E. histolytica* virulence genes that are involved in phagocytosis during host cell infection. In this study, expression of virulence genes was observed *in vitro* and *in vivo*. The EPEC *bfpA* gene is essential for initial attachment to intestinal cells and assists in microcolony formation (Giron et al, 1991). In this study, expression of the *bfpA* gene was produced at a higher concentration in mice infected with P/T co-infections than EPEC single infections. This data suggests that the presence of ETEC enhanced the expression of *bfpA*, enabling EPEC to adhere more to cells during P/T co-infections.

In this study, genes that are involved in the secretion system of EPEC and ETEC were measured. EPEC uses the T3SS, which involves injection of needle-like structure in to the host cell and this enables a series of genes to be translocated into the cells (Frankel et al, 1998). The *ler* gene which acts as a major transcriptional regulator (Friedberg et al, 1999) was reduced in the ileum, and not in the colon, suggesting EPEC infection was enhanced only in the colon. The *espA* (assist in filament formation of the needle complex), *espB* (assists with formation of pores in the host cell) genes are involved in translocation (Guerrant et al, 2011; Scaletsky, 2019). Gorelik and colleagues (2019) studied the expression of EPEC virulence genes during EPEC E2348/69 and *Vibrio cholerae* co-infections. It was found that EPEC and *V. cholerae* co-cultures had increased expression of *espB* and *espA* genes. It was also observed that *escJ* and *tir* genes were expressed at higher concentration when compared to EPEC pure cultures (Gorelik et al, 2019). These results suggested that EPEC is more virulent when co-infected with *V. cholerae* than when it is alone.

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ETEC requires the T2SS for pathogenesis (Tauschek et al, 2002). The ETEC H10407 strain produces both the LT and ST enterotoxins (Kaper et al, 2004). In this study, a significant difference was observed in the expression of ETEC *eltA* gene between ETEC and P/T co-infections *in vivo*. It was interesting to also note that all the ETEC virulence genes were reduced in the colon and the same trend was also observed in the ileum except for increase in *eltA* gene. The *eltA* gene encodes LT toxin and has been widely detected in clinical studies (Kotloff et al, 2013; Norton et al, 2015). The LT enterotoxin has been reported be a major cause of childhood diarrhoea (Kotloff et al, 2013). The ST enterotoxin on the other hand, has also been reported to cause diarrhoea at a lesser extent compared to the LT enterotoxin (Kotloff et al, 2013; Platts-Mills-2015). These results suggest that during single infections of ETEC in mice treated with antibiotic cocktail, the LT enterotoxin is produced more in the colon and this might lead to an increase in CFTR (due to changes in the chlorine channel), and thus causing diarrhoea in mice infected with P/T co-infections.

Pathogens are evolving and this increases their ability to interact with one another, thus sharing the same niche and this increases the chances of competition (Crane et al, 2006; Gorelik et al, 2019). An outbreak of EPEC and Shiga-toxin producing *E. coli* mixed infections have been reported in Finland, with patients developing severe disease outcome (Kinnula et al, 2018). Andersson et al (2018) recently reported on the association of diarrhoeal pathogens interacting with each other in East-Africa, and found that children presenting with clinical symptoms of diarrhoea were more likely to be infected with P/T co-infections than the EPEC and ETEC single strains (Andersson et al, 2018). *E. coli* pathotypes are closely related, and this might enable them to acquire certain genes from each other (Bielaszewska et al, 2007, Bando et al, 2009; Ruiz et al, 2014; Dutta et al, 2015; Hazen et al, 2017). Dutta and colleagues (2015) reported on a P/T hybrid strain discovered from a child with severe diarrhoea, the strain had the ability to produce both the *elt* and *eae* genes and was said to have evolved from a parent EPEC strain. ETEC and EPEC co-infections have been reported to interact synergistically by intensifying the interaction between both the pathogens (Crane et al, 2006). This positive association

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between co-infecting pathogens renders them to be more virulent (Andersson et al, 2018) and these combinations as seen in this study, induced more severity in disease outcome.

In conclusion, the data in this study showed severe weight loss and diarrhoea as a result of P/T co-infections in weaned mice. The P/T co-infections in mice resulted in detrimental inflammation in the intestine and was accompanied by increased acute-phase systemic inflammatory biomarkers and changes in virulence gene expression. The results presented here have provided further understanding in the interaction of P/T co-infections resulting in synergistic effects. This model can further be used to explore the pathogen-pathogen interaction between other *E. coli* pathotypes in a host in order to understand the mechanism that might be involved during co-infections.

4.6. REFERENCES

Adhikari, M., Coovadia, Y. and Hewitt, J., 1985. Enteropathogenic *Escherichia coli* (EPEC) and enterotoxigenic (ETEC) related diarrhoeal disease in a neonatal unit. *Annals of Tropical Paediatrics*, **5**(1), 19-22.

Albert, M.J., Faruque, A.S.G., Faruque, S.M., Sack, R.B. and Mahalanabis, D., 1999. Case-control study of enteropathogens associated with childhood diarrhea in Dhaka, Bangladesh. *Journal of Clinical Microbiology*, **37**(11), 3458-3464.

Allen, K.P., Randolph, M.M. and Fleckenstein, J.M., 2006. Importance of heat-labile enterotoxin in colonization of the adult mouse small intestine by human enterotoxigenic *Escherichia coli* strains. *Infection and Immunity*, **74**(2), 869-875.

Andersson, M., Kabayiza, J.C., Elfving, K., Nilsson, S., Msellem, M.I., Mårtensson, A., Björkman, A., Bergström, T. and Lindh, M., 2018. Coinfection with Enteric Pathogens in

© University of Venda

East African Children with Acute Gastroenteritis—Associations and Interpretations. *American Journal of Tropical Medicine and Hygiene*, **98**(6), 1566-1570.

Bando, S.Y., Andrade, F.B., Guth, B.E., Elias, W.P., Moreira-Filho, C.A. and Pestana de Castro, A.F., 2009. Atypical enteropathogenic *Escherichia coli* genomic background allows the acquisition of non-EPEC virulence factors. *FEMS Microbiology Letters*, **299**(1), 22-30.

Bartelt, L.A., Bolick, D.T., Mayneris-Perxachs, J., Kolling, G.L., Medlock, G.L., Zaenker, E.I., Donowitz, J., Thomas-Beckett, R.V., Rogala, A., Carroll, I.M. and Singer, S.M., 2017. Cross-modulation of pathogen-specific pathways enhances malnutrition during enteric co-infection with *Giardia lamblia* and enteroaggregative *Escherichia coli*. *PLoS Pathogens*, **13**(7).

Bernal-Reynaga, R., Thompson-Bonilla, R., Lopez-Saucedo, C., Pech-Armenta, M., Estrada-Parra, S. and Estrada-Garcia, T., 2013. C57-CD40 ligand deficient mice: a potential model for enterotoxigenic *Escherichia coli* (H10407) colonization. *Veterinary and Immunology Immunopathology*, **152**(1-2), 50-56.

Bielaszewska, M., Prager, R., Köck, R., Mellmann, A., Zhang, W., Tschäpe, H., Tarr, P.I. and Karch, H., 2007. Shiga toxin gene loss and transfer *in vitro* and *in vivo* during enterohemorrhagic *Escherichia coli* O26 infection in humans. *Applied Environmental Microbiology*, **73**(10), 3144-3150.

Boisen, N., Melton-Celsa, A.R., Hansen, A.M., Zangari, T., Smith, M., Russo, L.M., Scheutz, F., O'Brien, A.D. and Nataro, J.P., 2019. The role of the AggR regulon in the virulence of the Shiga Toxin-producing enteroaggregative *Escherichia coli* Epidemic O104: H4 Strain in Mice. *Frontiers in Microbiology*, **10**, 1824.

Bolick, D.T., Medeiros, P.H.Q.S., Ledwaba, S.E., Lima, A.A., Nataro, J.P., Barry, E.M. and Guerrant, R.L., 2018. Critical role of zinc in a new murine model of enterotoxigenic *Escherichia coli* diarrhea. *Infection and Immunity*, **86**(7), e00183-18.

© University of Venda

Bolick, D.T., Roche, J.K., Hontecillas, R., Bassaganya-Riera, J., Nataro, J.P. and Guerrant, R.L., 2013. Enteroaggregative *Escherichia coli* strain in a novel weaned mouse model: exacerbation by malnutrition, biofilm as a virulence factor and treatment by nitazoxanide. *Journal of Medical Microbiology*, **62**(Pt 6), 896.

Bonkoungou, I.J.O., Lienemann, T., Martikainen, O., Dembelé, R., Sanou, I., Traoré, A.S., Siitonen, A., Barro, N. and Haukka, K., 2012. Diarrhoeagenic *Escherichia coli* detected by 16-plex PCR in children with and without diarrhoea in Burkina Faso. *Clinical Microbiology and Infection*, **18**(9), 901-906.

Bose, J., Kloesener, M.H. and Schulte, R.D., 2016. Multiple-genotype infections and their complex effect on virulence. *Zoology*, **119**(4), 39-349.

Crane, J.K., Choudhari, S.S., Naeher, T.M. and Duffey, M.E., 2006. Mutual enhancement of virulence by enterotoxigenic and enteropathogenic *Escherichia coli*. *Infection and Immunity*, **74**(3), 1505-1515.

Crane, J.K., Naeher, T.M., Shulgina, I., Zhu, C. and Boedeker, E.C., 2007. Effect of zinc in enteropathogenic *Escherichia coli* infection. *Infection and Immunity*, **75**(12), 5974-5984.

Croxen, M.A., Law, R.J., Scholz, R., Keeney, K.M., Wlodarska, M. and Finlay, B.B., 2013. Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clincal Microbiology Reviews*, **26**(4), 822-880.

Darfeuille-Michaud, A., Aubel, D., Chauviere, G., Rich, C., Bourges, M., Servin, A. and Joly, B., 1990. Adhesion of enterotoxigenic *Escherichia coli* to the human colon carcinoma cell line Caco-2 in culture. *Infection and Immunity*, **58**(4), 893-902.

Dupont, A., Sommer, F., Zhang, K., Repnik, U., Basic, M., Bleich, A., Kühnel, M., Bäckhed, F., Litvak, Y., Fulde, M. and Rosenshine, I., 2016. Age-dependent susceptibility to enteropathogenic *Escherichia coli* (EPEC) infection in mice. *PLoS Pathogens*, **12**(5).

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Dutta, K., Pal, B., Mandal, R., Mullick, S. and Mandal, P., 2018. Circulating rotavirus types and drug-resistant diarrheagenic *Escherichia coli* causing enteric infection in under-five children in rural west Bengal, India. *Journal of Bioengineering and Biomedical Sciences*, **8**(255), 2.

Dutta, S., Pazhani, G.P., Nataro, J.P. and Ramamurthy, T., 2015. Heterogenic virulence in a diarrheagenic *Escherichia coli*: evidence for an EPEC expressing heat-labile toxin of ETEC. *International Journal Medical Microbiol*ogy, **305**(1), 47-54.

Estrada-Garcia, T., Lopez-Saucedo, C., Thompson-Bonilla, R., Abonce, M., Lopez-Hernandez, D., Santos, J.I., Rosado, J.L., DuPont, H.L. and Long, K.Z., 2009. Association of diarrheagenic *Escherichia coli* pathotypes with infection and diarrhea among Mexican children and association of atypical enteropathogenic *E. coli* with acute diarrhea. *Journal of Clinical Microbiology*, **47**(1), 93-98.

Evans, D.J. and Evans, D.G., 1973. Three characteristics associated with enterotoxigenic *Escherichia coli* isolated from man. *Infection and Immunity*, **8**(3), 322-328.

Frankel, G. Phillips, A.D., Rosenshine, I., Dougan, G., Kaper, J.B. and Knutton, S., 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Molecular Microbiology* **30**, 911-921.

Friedberg, D., Umanski, T., Fang, Y. and Rosenshine, I., 1999. Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic *Escherichia coli. Molecular Microbiology* **34**, 941-952.

Galván-Moroyoqui, J.M., Del Carmen Domínguez-Robles, M., Franco, E. and Meza, I., 2008. The interplay between Entamoeba and enteropathogenic bacteria modulates epithelial cell damage. *PLoS Neglected Tropical Diseases*, **2**(7).

Giron, J. A., Ho, A. S. & Schoolnik, G. K., 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. *Science,* **254**, 710-713.

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Gorelik, O., Levy, N., Shaulov, L., Yegodayev, K., Meijler, M.M. and Sal-Man, N., 2019. *Vibrio cholerae* autoinducer-1 enhances the virulence of enteropathogenic *Escherichia coli*. *Scientific Reports*, **9**(1), 1-13.

Guerrant, R.L., Walker, D.H. and Weller, P.F., 2011. In *Trop Infect Dis: Principles, Pathogens and Practice E-Book*. Elsevier Health Sciences.

Guth, B.E.C., Aguiar, E.G., Griffin, P.M., da Silva Ramos, S.R.T. and Gomes, T.A.T., 1994. Prevalence of colonization factor antigens (CFAs) and adherence to HeLA cells in enterotoxigenic *Escherichia coli* isolated from feces of children in São Paulo. *Microbiology and Immunology*, **38**(9), 695-701.

Hazen, T.H., Michalski, J., Luo, Q., Shetty, A.C., Daugherty, S.C., Fleckenstein, J.M. and Rasko, D.A., 2017. Comparative genomics and transcriptomics of *Escherichia coli* isolates carrying virulence factors of both enteropathogenic and enterotoxigenic *E. coli*. *Scientific Reports*, **7**(1), 1-17.

Iijima, Y., Oundo, J.O., Hibino, T., Saidi, S.M., Hinenoya, A., Osawa, K., Shirakawa, T., Osawa, R. and Yamasaki, S., 2017. High prevalence of diarrheagenic *Escherichia coli* among children with diarrhea in Kenya. *Japan Journal of Infectious Diseases*, **70**, 80-83.

Kaper, J.B., Nataro, J.P. and Mobley, H.L., 2004. Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*, **2**(2), 123.

Kinnula, S., Hemminki, K., Kotilainen, H., Ruotsalainen, E., Tarkka, E., Salmenlinna, S., Hallanvuo, S., Leinonen, E., Jukka, O. and Rimhanen-Finne, R., 2018. Outbreak of multiple strains of non-O157 Shiga toxin-producing and enteropathogenic *Escherichia coli* associated with rocket salad, Finland, autumn 2016. *Eurosurveillance*, **23**(35).

Kotloff, K.L., Nataro, J.P., Blackwelder, W.C., Nasrin, D., Farag, T.H., Panchalingam, S., Wu, Y., Sow, S.O., Sur, D., Breiman, R.F. and Faruque, A.S., 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global

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Enteric Multicenter Study, GEMS): a prospective, case-control study. *The Lancet*, **382**(9888), 209-222.

Law, R.J., Gur-Arie, L., Rosenshine, I. and Finlay, B.B., 2013. *In vitro* and *in vivo* model systems for studying enteropathogenic *Escherichia coli* infections. *Cold Spring Harbor Perspectives in Medicine*, **3**(3), a009977.

Ledwaba, S.E., Kabue, J.P., Barnard, T.G., Traore, A.N. and Potgieter, N., 2018. Enteric pathogen co-infections in the paediatric population from rural communities in the Vhembe District, South Africa. *South African Journal of Child Health*, **12**(4), 170-174.

Leverton, L.Q. and Kaper, J.B., 2005. Temporal expression of enteropathogenic *Escherichia coli* virulence genes in an *in vitro* model of infection. *Infection and Immunity*, **73**(2), 1034-1043.

Levine, M.M., Nataro, J.P., Karch, H., Baldini, M.M., Kaper, J.B., Black, R.E., Clements, M.L. and O'Brien, A.D., 1985. The diarrheal response of humans to some classic serotypes of enteteropathogenic *Escherichia coli* is dependent on a plasmid encoding the enteroadhesiveness factor. *Journal of Infectious Diseases* **152**, 550-559.

Lima, A.A., Soares, A.M., José Filho, Q.S., Havt, A., Lima, I.F., Lima, N.L., Abreu, C.B., Junior, F.S., Mota, R., Pan, W.K.Y. and Troeger, C., 2018. Enteroaggregative *Escherichia coli* subclinical infection and coinfections and impaired child growth in the MAL-ED Cohort Study. *Journal of Pediatric Gastroenterology and Nutrition*, **66**(2), 325-333.

Liu, J., Gratz, J., Amour, C., Kibiki, G., Becker, S., Janaki, L., Verweij, J.J., Taniuchi, M., Sobuz, S.U., Haque, R. and Haverstick, D.M., 2013. A laboratory-developed TaqMan Array Card for simultaneous detection of 19 enteropathogens. *Journal of Clinical Microbiology*, **51**(2), 472-480.

Manko, A., Motta, J.P., Cotton, J.A., Feener, T., Oyeyemi, A., Vallance, B.A., Wallace, J.L. and Buret, A.G., 2017. Giardia co-infection promotes the secretion of antimicrobial

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peptides beta-defensin 2 and trefoil factor 3 and attenuates attaching and effacing bacteria-induced intestinal disease. *PLoS One*, **12**(6).

Mathew, S., Smatti, M.K., Al Ansari, K., Nasrallah, G.K., Al Thani, A.A. and Yassine, H.M., 2019. Mixed viral-bacterial infections and their effects on gut microbiota and clinical Illnesses in children. *Scientific Reports*, **9**(1), 1-12.

Mosser, T., Talagrand-Reboul, E., Colston, S.M., Graf, J., Figueras, M.J., Jumas-Bilak, E. and Lamy, B., 2015. Exposure to pairs of Aeromonas strains enhances virulence in the *Caenorhabditis elegans* infection model. *Frontiers in Microbiology*, **6**, 1218.

Nataro, J.P., Kaper, J.B., Robins-Browne, R.O.Y., Prado, V., Vial, P. and Levine, M.M., 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. *Pediatric Infectious Disease Journal*, **6**(9), 829-831.

Norton, E.B., Branco, L.M. and Clements, J.D., 2015. Evaluating the A-subunit of the heat-labile toxin (LT) as an immunogen and a protective antigen against enterotoxigenic *Escherichia coli* (ETEC). *PLoS One*, **10**(8).

Platts-Mills, J.A., Babji, S., Bodhidatta, L., Gratz, J., Haque, R., Havt, A., McCormick, B.J., McGrath, M., Olortegui, M.P., Samie, A. and Shakoor, S., 2015. Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). *The Lancet Global health*, **3**(9), e564-e575.

Ritchie, J.M., 2015. Animal models of enterohemorrhagic Escherichia coli infection. In *Enterohemorrhagic Escherichia coli and other Shiga Toxin-Producing E. coli*. *American Society of Microbiology Press*. 175-195

Ruiz, R.C., Melo, K., Rossato, S.S., Barbosa, C.M., Corrêa, L.M., Elias, W.P. and Piazza, R.M., 2014. Atypical enteropathogenic *Escherichia coli* secretes plasmid encoded toxin. *BioMed Research International*, **2014**.

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Savkovic, S.D., Villanueva, J., Turner, J.R., Matkowskyj, K.A. and Hecht, G., 2005. Mouse model of enteropathogenic *Escherichia coli* infection. *Infection and Immunity*, **73**(2), 1161-1170.

Scaletsky, I.C.A., 2019. Enteropathogenic *Escherichia coli*. In *The Universe of Escherichia coli*. IntechOpen.

Tanaka, T., Narazaki, M. and Kishimoto, T., 2014. IL-6 in inflammation, immunity, and disease. *Cold Spring Harbor Perspectives in Biology,* **6**(10), a016295.

Tauschek, M., Gorrell, R.J., Strugnell, R.A. and Robins-Browne, R.M., 2002. Identification of a protein secretory pathway for the secretion of heat-labile enterotoxin by an enterotoxigenic strain of *Escherichia coli*. *Proceedings of the National Academy of Sciences*, **99**(10), 7066-7071.

Toledo, C.C., Arvidsson, I. and Karpman, D., 2011. Cross-reactive protection against enterohemorrhagic *Escherichia coli* infection by enteropathogenic *E. coli* in a mouse model. *Infection and Immunity*, **79**(6), 2224-2233.

Vargas, M., Gascon, J., Gallardo, F., De Anta, M.J. and Vila, J., 1998. Prevalence of diarrheagenic *Escherichia coli* strains detected by PCR in patients with travelers' diarrhea. *Clinical Microbiology and Infection*, **4**(12), 682-688.

Vilchez, S., Reyes, D., Paniagua, M., Bucardo, F., Möllby, R. and Weintraub, A., 2009. Prevalence of diarrhoeagenic *Escherichia coli* in children from Leon, Nicaragua. *Journal of Medical Microbiology*, **58**(5), 630-637.

Wada, Y., Nakaoka, Y., Kondo, H., Nakazawa, M. and Kubo, M., 1996. Dual infection with attaching and effacing *Escherichia coli* and enterotoxigenic *Escherichia coli* in post-weaning pigs. *Journal of Comparative Pathology*, **114**(1), 93-99.

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Zhang, W.L., Köhler, B., Oswald, E., Beutin, L., Karch, H., Morabito, S., Caprioli, A., Suerbaum, S. and Schmidt, H., 2002. Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. *Journal of Clinical Microbiology*, **40**(12), 4486-4492.

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CHAPTER 5

OBJECTIVE 3: **DETERMINE THE INTERACTION BETWEEN EAEC AND EPEC CO-INFECTIONS**

The objective was to determine the interaction between EAEC and EPEC co-infections *in vitro,* using HeLA cells and HCT-8 cells to determine adherence traits, inflammatory responses and expression of virulence genes. To also determine interaction between EAEC and EPEC co-infections *in vivo* using C57BL/6 mice by assessing clinical outcomes and inflammatory responses

5.1. ABSTRACT

Typical enteropathogenic *E. coli* causes infantile diarrhoea and Enteroaggregative *E. coli* causes persistent diarrhoea. Co-infections with these pathogens have been reported in children with and without diarrhoea. HCT-8 cells were infected with EAEC, EPEC and both the pathogens at the same time in order to determine adherence, inflammation and expression of virulence genes. HeLa cells were used to determine adherence traits using immunofluorescence microscopy. Weaned C57BL/6 mice were infected with EAEC, EPEC and both the pathogens at the same time in order to determine clinical outcomes, and inflammatory responses. *In vitro*, adherence of EAEC/EPEC (A/P) co-infections was the same, and inflammatory IL-8 was higher in HCT-8 cells infected with EAEC single infections. Virulence genes involved in adherence and type 3 secretion system of EPEC were significantly reduced in HCT-8 cells infected with A/P co-infections. EPEC localized adherence traits in infected HeLa cells was altered when infected with EAEC. *In vivo,* A/P co-infected mice had diminished weight loss. The LCN-2 and MPO levels in the co-infected mice did not have any additive effect. In conclusion, interaction between A/P co-infections leads to enteropathy.

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5.2. INTRODUCTION

In developing countries, the incidences of diarrhoeal causing pathogens are common (Platts-Mills et al, 2015). Microorganisms such as typical EPEC have been reported to causes infantile diarrhoea in children under the age of 2 years (Kotloff et al, 2013; Platts-Mills et al, 2015). EAEC infections have been reported to cause persistent diarrhoea (Okhuysen and DuPont, 2010; Hebbelstrup Jensen et al, 2017) and enteropathy (Rogawski et al, 2017) especially in children under the age of 5 years. Infections caused by EAEC results in chronic inflammation affecting the intestinal brush border and long-term effects have been associated with malnutrition (Opintan et al, 2010; Roche et al, 2010; Platts-Mills et al, 2015).

The occurrence of co-infections in epidemiological studies has been reported for many years. The microbe-microbe interactions have been suggested to result in increased or diminished disease outcomes in an infected host. Co-infecting pathogens compete with each other for nutrients and this might lead to increased virulence between the pathogens or one pathogen suppressing the virulence of another pathogen, and this might have a negative effect on the infected host. Interaction between A/P co-infections have been reported by clinical studies in symptomatic and asymptomatic cases (Itoh et al, 1997; Yatsuyanagi et al, 2002; Iijima et al, 2017; Broujerdi et al, 2018; Dutta et al, 2018; Ledwaba et al, 2018; Lima et al, 2018). It has been reported that the occurrence of co-infections might lead to growth failure and suppressed immune system (Yatsuyanagi et al, 2002; Griffiths et al, 2011; Stockmann et al, 2017; Lima et al, 2018).

The host-pathogen interactions (involving a single pathogen) has been widely reported using models in order to study disease outcome, immune response (Bartelt et al, 2017), pathogenesis mechanism (Savkovic et al, 2005; Schiebel et al, 2017), changes in metabolites (Sugiharto et al, 2014) and microbiota (Dupont et al, 2016). Single infections involving EAEC and EPEC have been studied using *in vitro* (Law et al, 2013; Elias and Navarro-Garcia, 2016) and *in vivo* (Law et al, 2013; Zhang et al, 2010; Dupont et al, 2016) analyses. However, interaction involving A/P co-infections using models is underreported.

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Co-infections involving pathogens such as *G. lamblia* and EAEC have been reported to result in reduced weight gain and increased inflammatory responses in C57BL/6 mice (Bartelt et al, 2017). *Giadia* species have been reported to have protective effect when co-infected with either *C. rodentium* or EPEC *in vitro*, by inhibiting growth of these enteropathogens (Manko et al, 2017). The EPEC murine developed in Objective 1 has been used to study clinical outcomes and inflammatory responses produced during A/P co-infections. Overall, the objective was to study the interaction of A/P co-infections using *in vitro* and *in vivo* analyses to determine disease outcome between the single and co-infections.

5.3. MATERIALS AND METHODS

5.3.1. *IN VITRO* ANALYSIS

5.3.1.1. HCT-8 cells growth

The HCT-8 cells growth was done following the procedure described in section 4.3.1.1.

5.3.1.2. Preparation of bacterial strains and infection on HCT-8 cells

EPEC E2348/69 (O127:H6) (Levine et al, 1985) and EAEC 042 (O44:H18) (Nataro et al, 1987) cultures were prepared following a procedure mentioned in section 4.3.1.2.

The control group contained RPMI only, the second group was infected with 10 µL (1:100 MOI) (4.8 x10^8 CFU/well) of EAEC, the third group infected with 10 µL (1:100 MOI) of EPEC in each well containing cells with confluency of 70-80%. Lastly, the fourth group for co-infections cells were infected with 10 µL (1:100 MOI) of EAEC and 10 µL (1:100 MOI) of EPEC (added at the same time). The experiment was performed in triplicates and all the wells contained fresh RPMI with no antibiotic and no serum. All the plates were incubated for 3 hours at 37 °C, 5% CO_2. Figure 5.1 shows the schematic presentation summarising the experimental procedure on HCT-8 cells.

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After infection, the samples were preserved following the procedure mentioned in section 4.3.1.2.

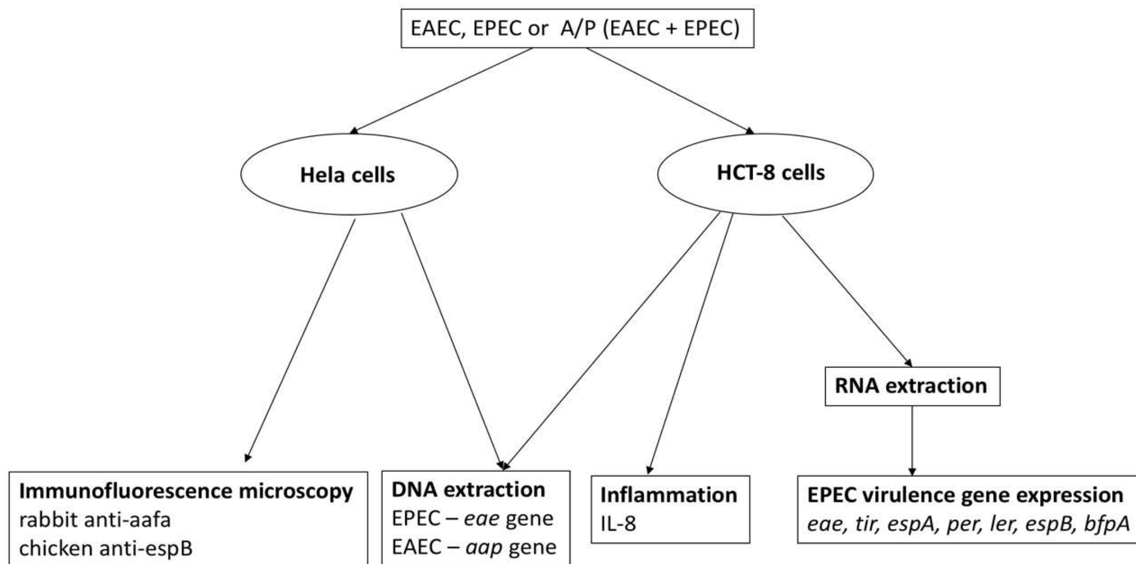

Figure 5.1. Schematic experimental presentation of EPEC, EAEC and A/P co-infections on HCT-8 and HeLA cells. HeLa cells and HCT-8 cells infected with either EAEC, EPEC or both pathogens for 3 hours. After HeLa cells infection, DNA was extracted from the infected cells and qPCR was used to analyse adhered bacteria and immunofluorescence microscopy was used to analyse adherence traits. After HCT-8 infection, supernatant was collected and IL-8 was measured. DNA was also extracted from infected cells and qPCR was used to analyse adhered bacteria. RNA was also extracted from the infected cells and determine expression of EPEC virulence genes. Keywords: A/P= EAEC and EPEC IL-8= interleukin-8, *eae*= intimin, *tir*= translocated intimin receptor, *espA*= EPEC secreted protein A, *bfpA*= bundle forming pili A, aafa= aggregative adherence fimbria A.

5.3.1.3. Measurement of IL-8 levels

Inflammatory cytokine, IL-8 was determined following a procedure mentioned in section 4.3.1.3.

5.3.1.4. HeLA cells growth

HeLA (ATCC CCL-2) cell line were maintained and grown in Corning 75-cm^2 culture flask (Sigma-Aldrich, St. Louis, Missouri, USA). The complete culture media for growth

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contained DMEM/F12 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, Missouri, USA), L-glutamine (Sigma-Aldrich, St. Louis, Missouri, USA),10 U/mL penicillin and 10 mg/mL streptomycin (ThermoFisher Scientific, Waltham, Massachusetts, USA). Cells were grown at 37 °C, 5% CO_2 incubator. Once 90% confluency was reached in the flask, cells were passaged using 1 mL of 0.25% trypsin (ThermoFisher Scientific, Waltham, Massachusetts, USA). For seeding, collagen-coated 24-well plates (Sigma-Aldrich, St. Louis, Missouri, USA) with autoclaved cover slips (Sigma-Aldrich, St. Louis, Missouri, USA) carefully placed in each well were used. Each well was seeded with cells containing complete culture media and placed at 37 °C, 5% CO_2 incubator. Once cells have reached confluency of 70-80%, they were ready for infection.

5.3.1.5. Preparation of bacterial strains and infection on HeLA cells

The EAEC 042 and EPEC E2348/69 strains were separately streaked on LB agar plates (ThermoFisher Scientific, Waltham, Massachusetts, USA) and incubated overnight at 37 °C. Single colonies were picked and inoculated in 3 mL DMEM low glucose (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and grown overnight at 37 °C in a shaking incubator. The next day, each well containing cells was washed with 1 X PBS and 1 mL DMEM low glucose containing 0.5% mannose was added. Figure 5.1 shows the schematic summarising the experimental procedure on HeLA cells.

For infection, the control group contained only DMEM, the second group was infected with 10 µL (1:100 MOI) (4.8 x10^8 CFU/well) EAEC, the third group was infected with 10 µL (1:100 MOI) EPEC. The fourth group was infected with 10 µL (1:100 MOI) EAEC and 10 µL (1:100 MOI) EPEC at the same time. All the plates were incubated at 37 °C for 3 hours, 5% CO_2.

Cells were then washed 3 times with 1 X PBS and 500 µL of 2% formalin/PBS was added to fix the samples. In another set of wells, after cells were washed 3 times with 1 X PBS and 180 µL of ATL buffer was added to each well and a sterile cell scrapper was used scrape off the adhered cells and bacteria and placed at -20 °C till further analysis.

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5.3.1.6. DNA extraction and real-time PCR

DNA extraction and real-time PCR were determined as described in section 4.3.1.4, using primers in Table 5.1.

Table 5.1. Primer sequence used to detect EPEC and EAEC

Pathogen	Primer	Sequence (5'-3'	Reference
EPEC	*eae* (F)	CCCGAATTCGGCACAAGCATAAGC	
	(R)	CCCGGATCCGTCTCGCCAGTATTCG	Zhang et al, 2002
EAEC	*aap* (F)	CTTGGGTATCAGCCTGAATG	
	(R)	AACCCATTCGGTTAGAGCAC	Cerna et al, 2003

5.3.1.7. RNA extraction and real-time PCR

RNA extraction and EPEC virulence gene expression were determined following a procedure described in section 4.3.1.5.

5.3.1.8. Adherence assay using immunofluorescence microscopy

Cells that were fixed with formalin were analysed further. The cover slips were washed 3 times with 1 X PBS. 300 µL of 0.1% Triton X-100 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) (diluted with 1 X PBS) was added and incubated for 5 min at room temperature. Cells were then washed and 300 µL RNAse A (Thermo Fisher Scientific, Waltham, Massachusetts, USA) diluted to 1:500 in 1 X PBS-10% horse serum (PBS-HS) was added and incubated for 30 min at 37 °C. Cover slips were then incubated with the primary antibody [for EPEC, chicken anti-EspB and for EAEC, rabbit anti-aafa (provided by JA Giron)] for 1 hr. Cells were then washed and incubated with the secondary antibody 1:5000 Alexa fluor 448 donkey anti-rabbit IgG and Alexa fluor 555 goat anti-chicken IgY (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 1 hour in the dark. Cover slips were washed and 300 µL of 1:2000 dapi (Invitrogen, Carlsbad, California, USA) was added and incubated for 5 min at room temperature. Cells were then washed and 2 µL of Fluoroshield with p-phenylenediamine (Sigma-Aldrich, St Louis, Missouri, USA) was added to the coverslip and then mounted on the slide using cytoseal (Thermo Fisher

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Scientific, Waltham, Massachusetts, USA). The slides were viewed using Zeiss Fluorescent microscope (Zeiss, Oberkochen, Germany).

5.3.2. *In vivo* analysis

5.3.2.1. Animal husbandry

The protocols used in this study and the vivarium conditions used to keep the C57BL/6 mice is described in section 3.3.1

5.3.2.2. Rodent diet and antibiotics

The rodent diet and antibiotics used are mentioned in section 3.3.2.

5.3.2.3. Preparation of bacterial strains and mice infection

EPEC E2348/69 and EAEC 042 cultures were prepared following the procedure mentioned in section 3.3.3.

Four groups of mice were used: control uninfected mice were orally administered DMEM only, the second group was administered 100µl of 10^{10} CFU/mL (each mouse received 10^{9} CFU in a total volume of 10^{10} CFU/mL) of EAEC and third group was administered 100µl of 10^{10} CFU/mL EPEC. The last group was orally administered 100µl of 10^{10} CFU/mL EAEC and 100µl of 10^{10} CFU/mL EPEC (at the same time) using a sterile 22-guage feeding needles. Figure 5.2 shows the schematic procedure of the overall infection.

Changes in weights and disease outcome were monitored and stools were collected daily. On day 10 pi, mice were euthanized according to the protocol. The cecum contents collected at day 3 pi were flash frozen using liquid nitrogen and then placed at -80 °C.

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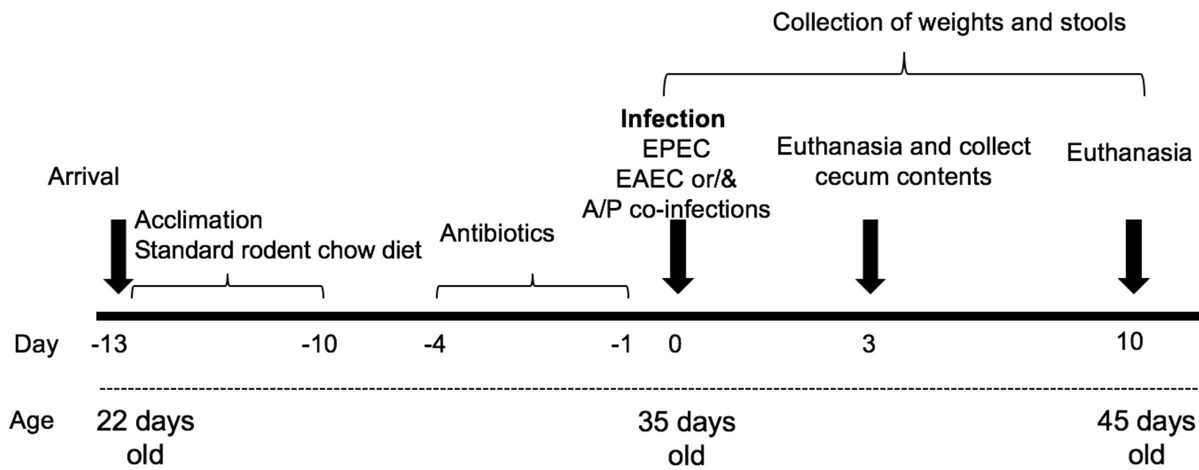

Figure 5.2: Schematic experimental design of EPEC, EAEC and A/P co-infections in C57BL/6 mice. 22-day-old mice were placed on standard rodent chow diet upon arrival. Mice were administered drinking water containing antibiotic cocktail 4 days prior infection, and clean water was replaced 1-day prior infection. Mice were orally infected with either EPEC, EAEC or both the pathogens at the same time, and followed daily for changes in weights and stool collection. Mice were euthanised at days 3 and 10 pi.

5.3.2.4. Stool shedding

Stool shedding was determined using a procedure described in section 3.3.4, using primers in Table 5.1.

5.3.2.5. Intestinal inflammatory response

The stool specimens collected at day 2 pi and were used to determine MPO and LCN-2 using the procedure described in section 3.3.6.

5.3.3. Statistical analysis

All experiments were conducted in triplicates. The collected data was analysed using GraphPad Prism 7 software (Graph Pad, San Diego, California, USA). The data are represented as $\pm$ SEM. The results were considered significant when $p<0.05$.

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5.4. RESULTS

5.4.1. EAEC and EPEC *IN VITRO* ANALYSIS

5.4.1.1. Adherence and inflammatory responses in HCT-8 cells infected with A/P co-infections

HCT-8 cells were used to compare difference in adherence between single infections of EAEC, EPEC and A/P co-infections. As seen in Figure 5.3A, all the bacterial pathogens adhered to cells. There was a significant decrease in adherence of EPEC in infected cells when compared to A/P (*eae*) ($p<0.001$), suggesting that EPEC adherence was reduced when co-infected with EAEC strain. There was also a significant difference in adherence of cells infected with EPEC when compared to EAEC single infections ($p=0.0001$).

HCT-8 cells were used to analyse inflammatory cytokine, IL-8 as a result of A/P co-infections. As seen in Figure 5.3B, all the infected cells had a significant increase IL-8 levels when compared to control uninfected cells ($p<0.0001$). Cells infected with single infections of EAEC had a significant increase in IL-8 levels when compared to EPEC single infections ($p=0.0001$) and A/P co-infections ($p<0.0001$). Interestingly, A/P co-infections and EPEC infections had similar levels of IL-8, $\leq$300 pg/mg protein.

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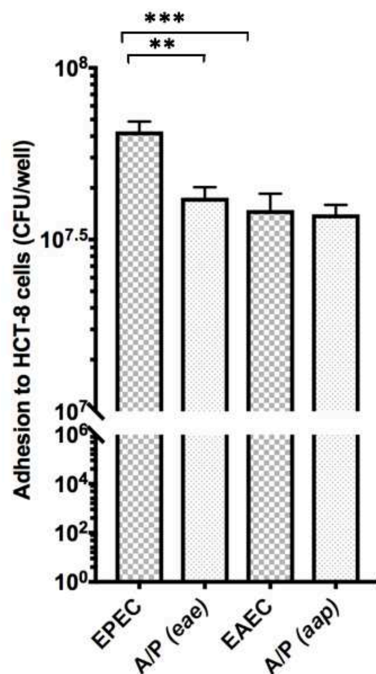
A) B)



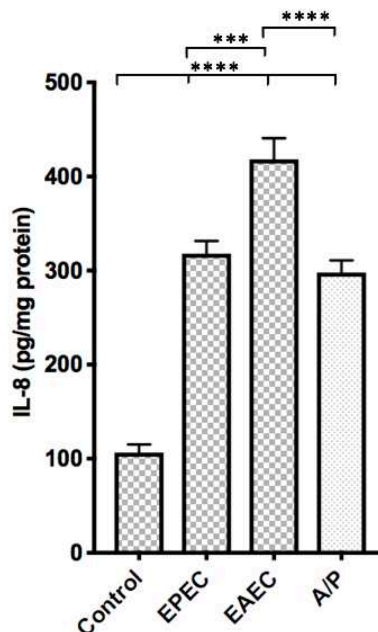


Figure 5.3: Adherence assay and inflammatory responses in HCT-8 cells infected with A/P co-infections. Cells were infected with EAEC, EPEC and A/P co-infections for 3 hours. **A)** DNA was extracted from infected and uninfected cells and qPCR was used to determine EAEC, EPEC and A/P co-infections on adhered cells. **B)** IL-8 levels measured in HCT-8 cells infected with EAEC, EPEC and A/P co-infections using ELISA. Data analysed using one-way ANOVA and Tukey's *post hoc* test, **p<0.001, ***p=0.0001, ****p<0.00001. Keywords A/P (*aap*) = qPCR measured only *aap* gene in mice infected with EAEC and EPEC co-infections; A/P (*eae*) = qPCR measured only *eae* gene in mice infected with EAEC and EPEC co-infections.

5.4.1.2. Adherence traits of A/P co-infections on HeLA cells

HeLA cells were used to compare difference in adherence between single infections of EAEC, EPEC and A/P co-infections. As seen in Figure 5.4, EAEC single infections adhered better to cells when compared to EPEC single infections. A/P co-infections adhered to HeLa cells at the same rate similar to the single infections, and no significant difference was observed.

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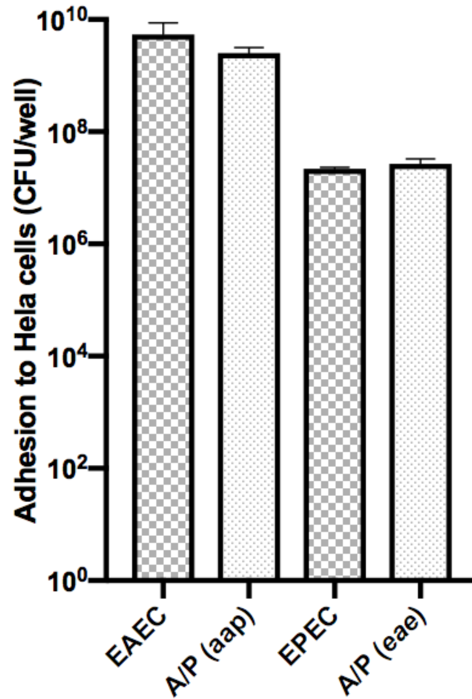

Figure 5.4: Adherence assay in HeLA cells infected with A/P co-infections. HeLA cells were infected with EAEC, EPEC single infections and A/P co-infections for 3 hours. DNA was extracted from all the infected groups and qPCR was used to determine adherence of EAEC, EPEC and A/P co-infections to cells. Keywords A/P (*aap*) = qPCR measured only *aap* gene in mice infected with EAEC and EPEC co-infections; A/P (*eae*) = qPCR measured only *eae* gene in mice infected with EAEC and EPEC co-infections.

In order to assess difference in adherence traits between cells infected with single and co-infections, immunofluorescence microscopy was performed on HeLA cells that were infected with either EAEC, EPEC and A/P co-infections. Adherence of EAEC was observed as stacked-brick-like pattern (Figure 5.5B). while EPEC adherence was observed as LA pattern (Figure 5.5C) on infected cells. However, cells infected with A/P co-infections had a change in adherence pattern. EAEC was observed to form stacked-brick pattern around EPEC (Figure 5.5D), that is, altering the LA adherence pattern that is normally seen when EPEC is infected alone.

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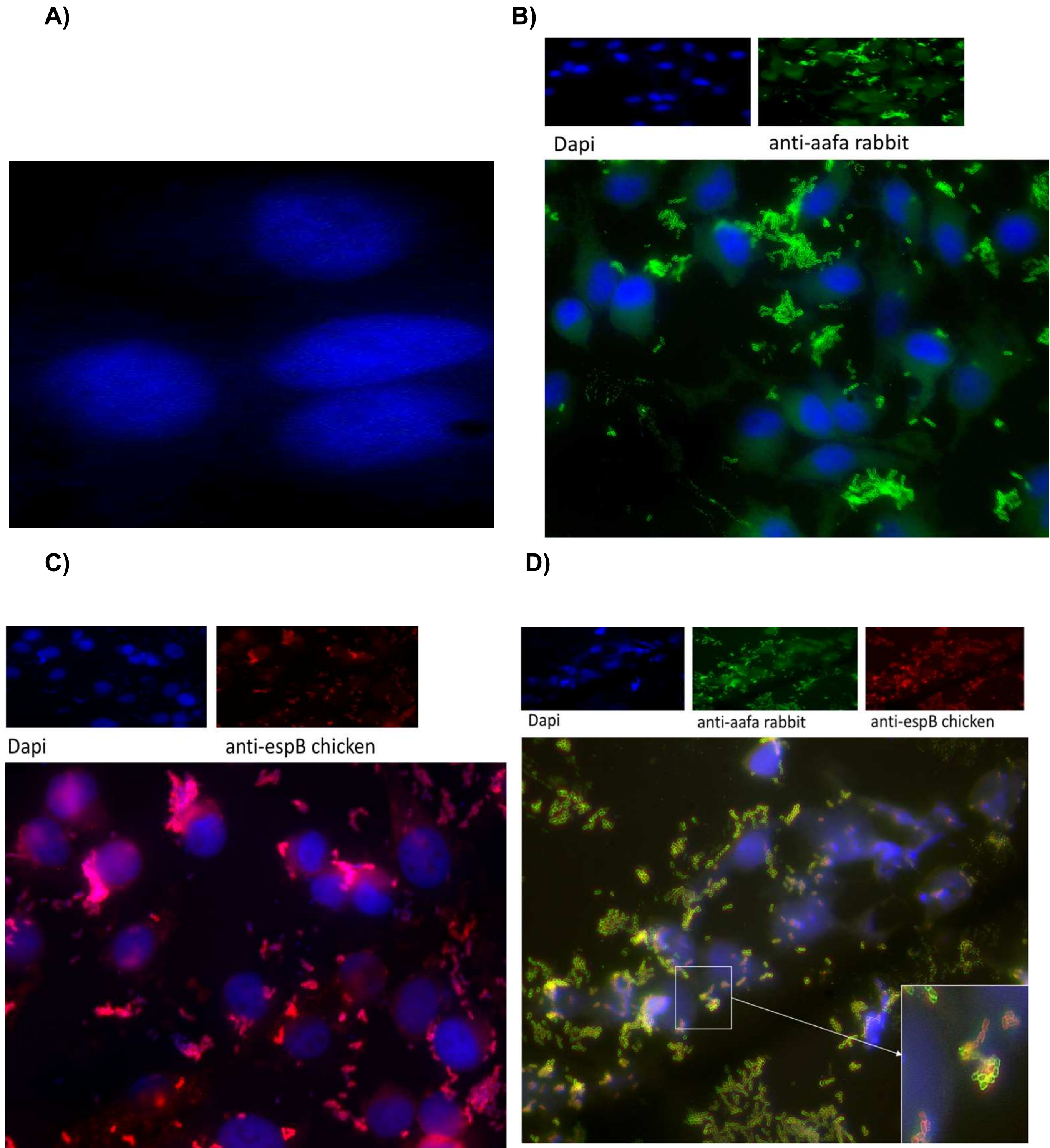

Figure 5.5: Immunofluorescence microscopy on HeLA cells. HeLA cells infected for 3 hours with EAEC, EPEC and A/P co-infections and Immunofluorescence microscopy was used to determine adherence traits. **A)** Uninfected HeLA cells (blue) (**B)** EAEC adherence showing stacked brick-like (green) on HeLA cells (blue). **C)** EPEC adherence showing localized adherence (red) on

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HeLA cells (blue). **D)** A/P co-infections adherence EAEC (green) forming stacked brick-like pattern around EPEC (red) on HeLA cells (blue).

5.4.1.3. Expression of EPEC virulence genes on HCT-8 cells

To evaluate changes in the pathogenesis mechanism of EPEC infection when co-infected with EAEC in HeLA cells, expression of genes involved in adhesion and secretion system of EPEC were analysed. As seen in Figure 5.6, the expression of the *bfpA* gene (involved in initial attachment to cells) was significantly decreased in cells infected with A/P co-infections when compared to EPEC single infections ($p<0.001$). The *eae* and *tir* genes (involved in intimate adherence) were also significantly decreased in cells infected with A/P co-infections when compared to EPEC single infections ($p<0.001$). The *ler* (major transcriptional regulator), *espA* and *espB* (form part of the T3SS injector) were also significantly reduced in cells infected with A/P co-infections when compared to EPEC single infections ($p<0.05$ and $p<0.001$). Overall, all the genes involved in EPEC pathogenesis (adherence and T3SS) were greatly affected when co-infected with EAEC.

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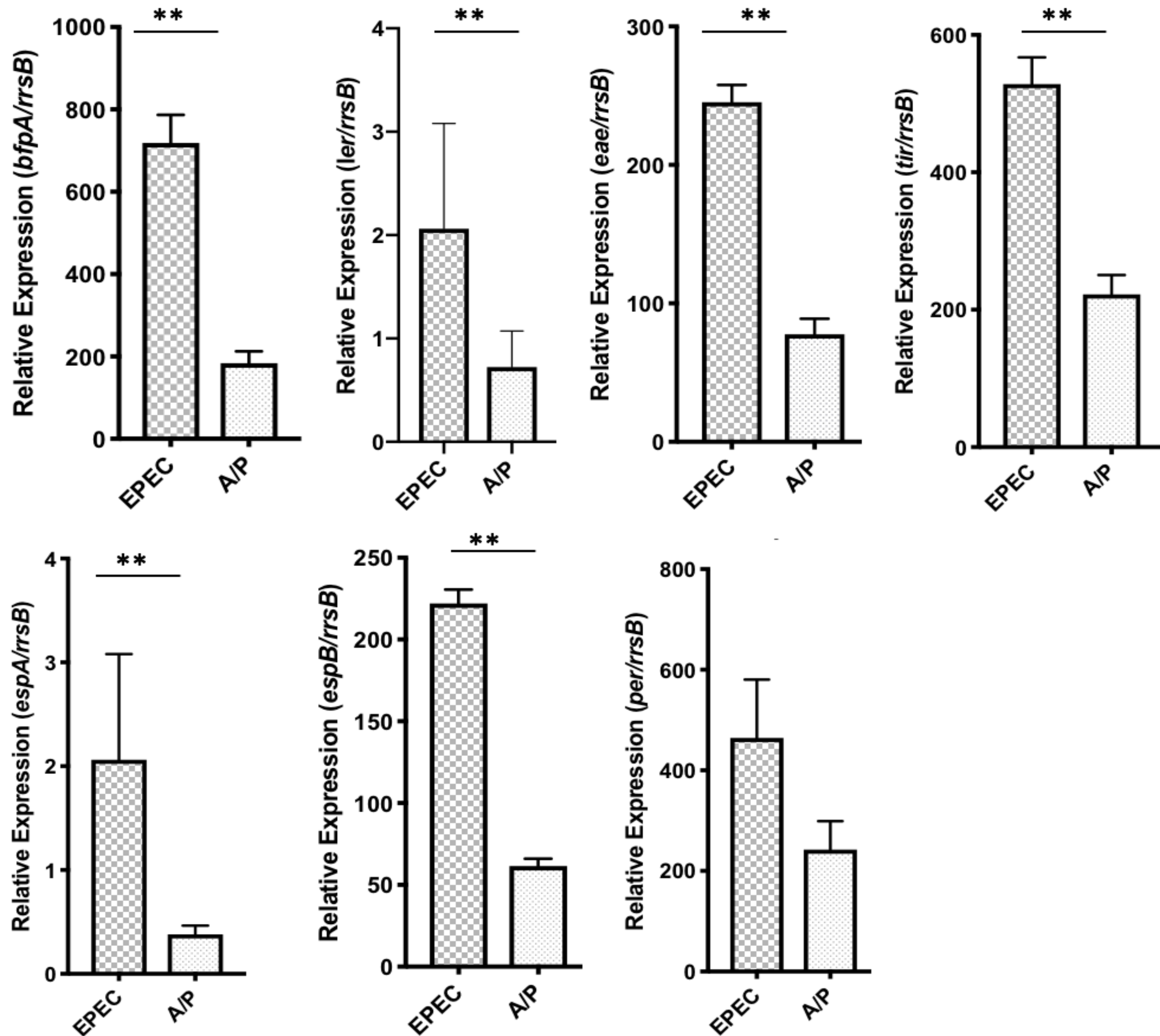

Figure 5.6: EPEC virulence gene expression on HCT-8 cells infected with A/P co-infections. HCT-8 cells infected with EAEC, EPEC and A/P were analysed for EPEC virulence gene expression following 2-hour infection. Data analysed using *t-test* and Mann Whitney test, *p<0.05, **p<0.001.

5.4.2. EAEC and EPEC co-infections *in vivo* analysis

5.4.2.1. Clinical outcomes of mice infected with A/P co-infections

The EPEC murine model developed in Chapter 3 was used to determine the interaction of A/P co-infections *in vivo*. Weaned mice infected with EAEC, EPEC and A/P co-

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infections were followed for changes in weight and clinical outcomes. Mice infected with EAEC and EPEC single infections developed wet stools. Mice infected with A/P co-infections developed wet stools only at day 1 pi and no other symptoms were observed throughout the 10-day infection period. As seen in Figure 5.7, at day 1 pi, a significant difference in weight changes was observed between control mice and A/P co-infected mice (p<0.05). At day 2 pi, there was a significant difference in weight changes between control mice and EPEC infected mice (p<0.001). The EAEC infected mice recovered quickly from the infection and the growth was similar to the control group (from day 4 pi to day 10 pi). By day 8 pi all the infected groups had recovered their body weights (Figure 5.7).

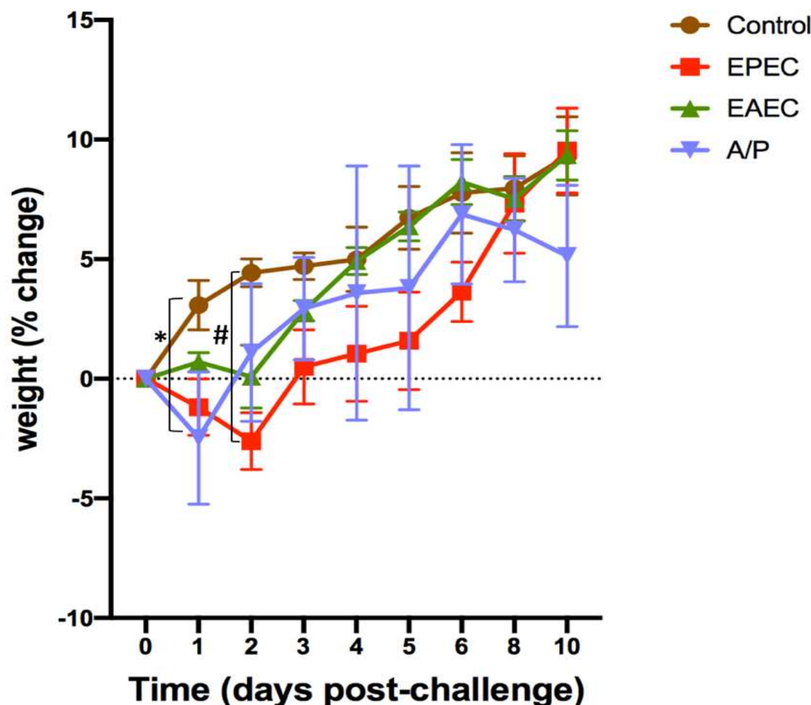

Figure 5.7: Change in weight of C57BL/6 mice infected with A/P co-infections. Weaned mice infected with EAEC, EPEC and A/P (n = 8/group) were monitored for change in weight. Data analysed using two-way ANOVA and Tukey's *post hoc* test, pi *p<0.05, # p<0.001.

5.4.2.2. Stool shedding of A/P co-infected mice

Stool specimens of mice infected with EAEC, EPEC single infections and P/T co-infections were analysed for stool shedding during the 10-day infection period. As seen

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in Figure 5.8, even though there was no significant difference, EAEC shedding at day 1 pi was higher (≥10^8 organisms/10mg stool) when compared to EPEC infected mice (≥10^6 organisms/10mg stool). At day 1 pi, there was a significant difference of p<0.05 between mice infected with EAEC single infections and A/P (*eae*) co-infected mice. EAEC infected mice also had a significant difference of p<0.05 when compared to A/P (*aap*) co-infections. At day 5 pi, mice infected with EPEC had a significant difference of p<0.05 when compared to mice infected with A/P (*aap*). Also, at day 5 pi, in the co-infected groups, A/P (*eae*) shedding was lower (≥10^8 organisms/10mg stool) than A/P (*aap*) (≤10^6 organisms/10mg stool) with significant difference of p<0.05. At day 10 pi, EPEC shedding was no longer detected in the single and A/P (*eae*) co-infections. (Figure 5.8).

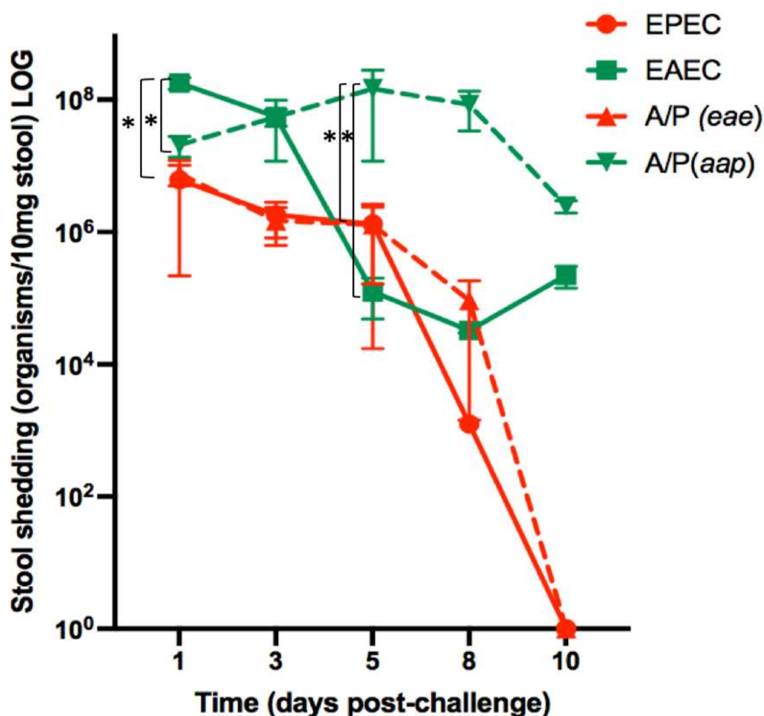

Figure 5.8: Stool shedding of mice infected with A/P co-infections. Weaned mice were challenged orally with EAEC, EPEC, A/P co-infections and controls with DMEM (n = 8/group). DNA was extracted from stool specimen and qPCR was used to determine EAEC, EPEC and A/P co-infections. Data analysed using two-way ANOVA and Tukey's *post hoc* test, * p<0.05. Keywords A/P (*aap*) = qPCR measured only *aap* gene in mice infected with EAEC and EPEC co-infections; A/P (*eae*) = qPCR measured only *eae* gene in mice infected with EAEC and EPEC co-infections.

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5.4.2.3. Biomarkers of inflammatory responses in mice infected with A/P co-infections

To assess increase in inflammation as a result of A/P co-infections in mice, MPO and LCN-2 were used as faecal biomarkers of inflammatory response. As seen in Figure 5.9A, all the infected groups had an increase in faecal MPO, however, no significant difference was observed. LCN-2 levels in mice infected with single infections of EPEC (≤80 pg/mg protein) were significant higher when compared to mice infected with EAEC (<20 pg/mg protein) alone, $p<0.05$. At day 3 pi, half of all the infected and uninfected mice groups were euthanized and cecal contents were collected and analysed for MPO and LCN-2 using ELISA. As seen in Figure 5.9B, mice infected with only EAEC had a significant increase in MPO levels when compared to control uninfected mice with >10000 pg/mg protein ($p<0.05$). EPEC infected mice had low levels of LCN-2 (≤5000 pg/mg protein) when compared to A/P infected mice (≤10000 pg/mg protein), however, no significant difference was observed. Mice infected with EAEC also had a significant increase in LCN-2 levels when compared to control uninfected group ($p<0.05$) with <800 pg/mg protein. Mice infected with A/P co-infections produced about ≤600 pg/mg protein when compared to EPEC single infections levels of ≤200 pg/mg protein of LCN-2, however, no significant difference was observed.

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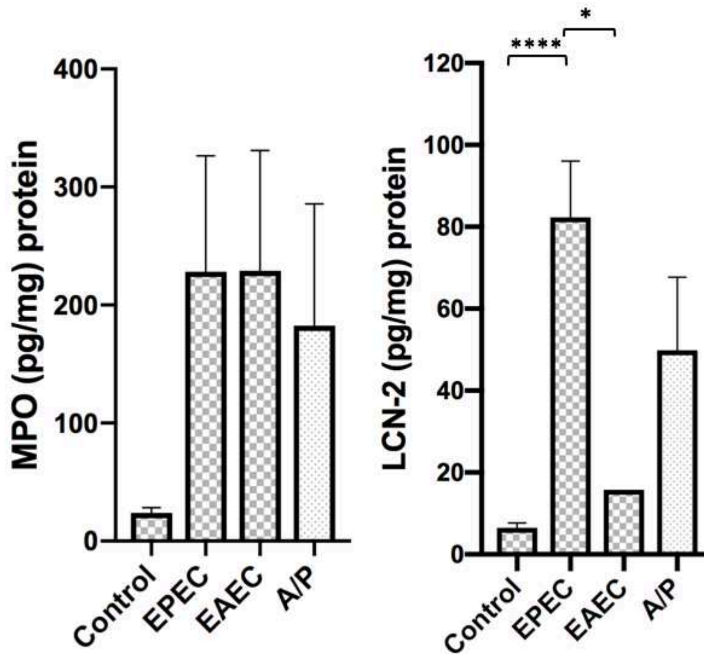
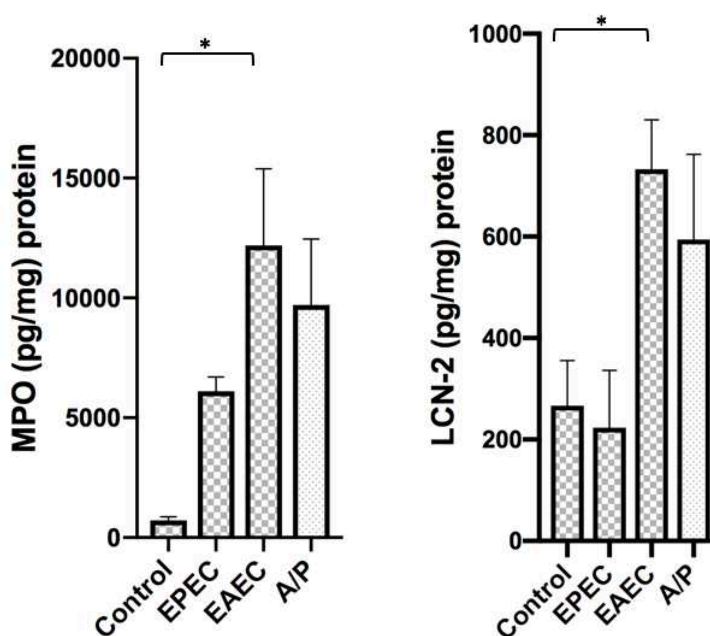
A)



B)



<u>Figure 5.9:</u> Inflammatory biomarkers MPO and LCN-2 in mice infected with A/P co-infections. Stool specimen and cecal contents were collected, lysates were extracted using RIPA buffer and MPO and LCN-2 were measured. **A)** MPO and LCN-2 levels in stool specimen of mice infected with EAEC, EPEC and A/P collected at day 2 pi. **B).** MPO and LCN-2 in cecal contents of mice

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collected at day 3 pi. Data analysed using one-way ANOVA and Tukey *post hoc* test, *p<0.05; ****p<0.0001

5.5. DISCUSSION

Typical EPEC and EAEC infections cause diarrhoea mainly in children under the age of 2 years (Kotloff et al, 2013; Platts-Mills et al, 2015). The interaction between these pathogens are increasingly being reported in clinical studies (Itoh et al, 1997; Yatsuyanagi et al, 2002; Iijima et al, 2017; Broujerdi et al, 2018; Dutta et al, 2018; Ledwaba et al, 2018; Lima et al, 2018). Infection models of EPEC and EAEC have been developed (Savkovic et al, 2005; Bolick et al, 2013; Dupont et al, 2016). However, limited data on how both of these pathogens interact in relation to disease outcome is underreported. In this study, the interaction of A/P co-infections were studied using *in vitro* and *in vivo* analyses in order to determine adherence traits, inflammatory responses and expression of EPEC virulence genes.

In vitro EPEC is characterized by forming LA pattern at the site of infection (Scaletsky et al, 1984; Kaper et al, 2004, Dow et al, 2006; Ochoa and Contreras, 2011), while EAEC is characterized by biofilm formation and stacked-brick-like pattern (AA) at the site of infection (Kaper et al, 2004; Dow et al, 2006; Gomes et al, 2016). It was interesting to note that when EAEC was co-infected with EPEC there was a change in the adherence traits of both the pathogens. In this study, EPEC localized adherence pattern was altered and EAEC was observed surrounding EPEC on HeLa cells. The data suggested that during A/P co-infections, EAEC competes with EPEC for adherence and this results in EAEC altering the adherence pattern of EPEC by aggregating aggressively around EPEC. Manko and colleagues (2017) reported on *G. duodenalis* and EPEC co-infections in Caco-2 cells. Infection between these pathogens was inhibited, with *G. duodenalis* preventing growth of EPEC (Manko et al, 2017). As observed in this study, even though there was a change in the adherence pattern of the A/P strains, it was also interesting to note that the single infections of EAEC and EPEC adhered to cells at the same rate similar to the A/P co-infections. Millette and colleagues (2019) studied interaction of *P.*

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aeruginosa and *Staphylococcus aureus* co-infections *in vitro*. This study also found that interaction between these pathogens resulted in changes in colony formation; *S. aureus* colonies were observed colonizing around *P. aeruginosa* (Millette et al, 2019). Infection between EPEC and *Saccharomyces boulardii* has been reported to also have antagonistic effects *in vitro* (Czerucka et al, 2000). EPEC infection in T84 cells resulted in disruption of tight junction and increased cell apoptosis. However, co-infection of EPEC with *S. boulardii* was found to reduce disruption of tight junction and delayed cell apoptosis, by affecting the signal transduction pathways of EPEC (Czerucka et al, 2000). Interestingly, adhesion between the two strains was not affected, similar to this study.

EAEC and EPEC single infections have been reported to result in increased IL-8 levels *in vitro* studies (Huang et al, 2004; Ruchaud-Sparagano et al, 2007; Sanchez-Villamil et al, 2016; Park et al, 2017; Haarmann et al, 2018). In this study, single infections of EAEC and EPEC in cells resulted in increased levels of IL-8. However, the interaction between both these pathogens did not result in additive effects, that is higher levels of IL-8 were not observed in cells infected with A/P co-infections. Thus, instead of the cells producing increased inflammatory responses due to the presence of two pathogens; the pathogens were instead competing with each other, as observed with altered adherence, resulting in reduced IL-8 levels. In another study, chickens infected with *Eimeria maxima* and *C. perfringens* co-infections resulted in reduced IL-8 levels due to presence of supplemented diet (Oh et al, 2019). Reece and colleagues also reported on antagonistic effects between *Aspergillus fumigatus* and *P. aeruginosa* co-infections *in vitro*. This interaction was reported to result in reduced biofilm formation and IL-8 levels were not enhanced by the presence of both pathogens (Reece et al, 2018).

In evolution of virulence, it has been predicted that increase in number of pathogens within a host leads to increased virulence (Ebert, 2000). The analysis of virulence gene expression in infected cells highlighted an interesting outcome of A/P co-infections when compared to EPEC alone. The EPEC virulence genes analysed in this study were all down regulated in cells infected with A/P co-infections. The *bfpA* gene, involved in initial attachment and formation of LA pattern (Giron et al, 1991) was greatly reduced,

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suggesting that the LA pattern formation was affected. This data is also supported by the immunofluorescence microscopy of A/P co-infections, with the image showing EAEC surrounding EPEC with altered LA pattern. Intimin is encoded by the *eae* gene and requires tir in order for EPEC to intimately adhere to cells (Frankel et al, 1998). Both these genes were greatly reduced in cells infected with A/P co-infections when compared to EPEC alone. The *ler* gene, a major transcriptional regulator of the T3SS and crucial for EPEC pathogenesis (Frankel et al, 1998; Friedberg et al, 1999), and is activated by *per* (Scaletsky, 2019). In this study, it was also noted that both these genes were also down regulated in cells infected with A/P co-infections when compared to EPEC alone. The *espA* and *espB* genes were also down-regulated, these produce secretory proteins that assists in filament formation and translocation of other proteins into the cells via the T3SS. These proteins cause changes in the cytoskeletal structure of the cells leading to actin accumulation at the site of infection (Dean and Kenny, 2009). Other studies have also reported on co-infecting pathogens resulting in reduced virulence expression (Garbutt et al, 2011; Reece et al, 2018).

The interaction of A/P co-infections were further analysed *in vivo* using C57BL/6 mice. The EPEC infectious mouse model developed in Chapter 3 was used to analyse the interaction of A/P co-infections. In this study A/P co-infections resulted in diminished weight changes and increased inflammatory responses. Mice infected with EAEC and EPEC single infections developed wet stools between day 1 and 3 pi, while mice infected with A/P co-infections developed soft stools only at day 1 pi. In this study, mice infected with A/P strains recovered quickly at day 2 pi while mice infected with single infections of EPEC recovered the weights at day 4 pi. Animal models have shown that diet plays an important role during disease outcome (Medeiros et al, 2013; Bolick et al, 2013; Mayneris-Perxachs et al, 2016). In this study, EAEC infection in mice fed standard rodent chow diet (nourished diet) did not have any effect on weight change. EAEC infection has been previously studied in mice fed malnourished diet and found that there was a significant weight loss than in mice fed nourished diet (Roche et al, 2010; Bolick et al, 2013). Medeiros and colleagues (2013) also reported on the importance of zinc deficiency during EAEC infection and weight loss was reported to occur up to day 7 pi when compared to

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mice administered zinc supplementation (Medeiros et al, 2013). Therefore, further studies are required to study A/P co-infections in mice with malnourished diet to determine the disease outcome.

In this study, EPEC shedding in A/P co-infections was also observed until day 8 pi. On the other hand, EAEC shedding in the A/P co-infections was observed up to day 10 pi. This suggested that during A/P co-infections, the presence of EPEC enabled EAEC to adhere better to the intestinal cells, enabling it to shed longer at a higher rate. Increase in faecal intestinal biomarker such as MPO and LCN-2 in children have been associated with growth impairment (Guerrant et al, 2016; Prata et al, 2016). The MAL-ED Cohort study recently studied EAEC co-infections in children under 6 months of age and found that EAEC infections with more than 2 pathogens had increased MPO concentrations in the stool specimens than in EAEC infection alone (Lima et al, 2018). In this study, MPO concentrations in the stools and cecal contents were also increased across all infected groups. Increase in LCN-2 levels was found to be higher in A/P co-infections when compared to EAEC alone. Pathogens such as EAEC are associated with enteropathy leading to growth impairment in children (Bartelt et al, 2013). EAEC and EPEC have been detected in non- diarrhoeal stool samples of children and have been found to cause mucosal disruption with increased MPO faecal biomarker (Kosek et al, 2017). Exposure to these pathogens leads to increased inflammation, growth failure and abnormal gut permeability (Kosek et al, 2017).

The microbe-microbe interaction with the host is complex (Mideo, 2009) because there are many factors within the host and the microbes that determine disease outcome. Recently, the interaction of EAEC and EPEC co-infections with other viral pathogens have been studied in clinical settings, and was found that alteration in gut microbiota resulted in increased disease outcome (Mathew et al, 2019). Antagonistic effects with other pathogen co-infections have also been reported (Shen et al, 2019). Zhu and colleagues (2017) reported on *S. typhimurium* and *Schistosoma japonicum* co-infection model in mice and found that symptoms of schistosomiasis were greatly reduced during co-

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infections, and that the presence of *S. typhimurium* greatly reduced the metabolic disturbances (Zhu et al, 2017).

In conclusion, based on the findings, it can be suggested that not all co-infections result in detrimental disease outcome. EPEC pathogenesis was suppressed when co-infected with EAEC, causing EAEC to be dominant.

5.6. REFERENCES

Bartelt, L.A., Bolick, D.T., Mayneris-Perxachs, J., Kolling, G.L., Medlock, G.L., Zaenker, E.I., Donowitz, J., Thomas-Beckett, R.V., Rogala, A., Carroll, I.M. and Singer, S.M., 2017. Cross-modulation of pathogen-specific pathways enhances malnutrition during enteric co-infection with *Giardia lamblia* and enteroaggregative *Escherichia coli*. *PLoS Pathogens*, **13**(7).

Bartelt, L.A., Lima, A.A., Kosek, M., Yori, P.P., Lee, G. and Guerrant, R.L., 2013. “Barriers" to Child Development and Human Potential: The Case for Including the “Neglected Enteric Protozoa"(NEP) and Other Enteropathy-Associated Pathogens in the NTDs. *PLoS Neglected Tropical Diseases*, **7**(4).

Bhunia, A.K., 2018. *Foodborne microbial pathogens: mechanisms and pathogenesis*. Springer.

Bolick, D.T., Roche, J.K., Hontecillas, R., Bassaganya-Riera, J., Nataro, J.P. and Guerrant, R.L., 2013. Enteroaggregative *Escherichia coli* strain in a novel weaned mouse model: exacerbation by malnutrition, biofilm as a virulence factor and treatment by nitazoxanide. *Journal of Medical Microbiology*, **62**(Pt 6), 896.

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Broujerdi, S.M., Ardakani, M.R. and Rezatofighi, S.E., 2018. Characterization of diarrheagenic *Escherichia coli* strains associated with diarrhea in children, Khouzestan, Iran. *The Journal of Infection in Developing Countries*, **12**(08), 649-656.

Cerna, J.F., Nataro, J.P. and Estrada-Garcia, T., 2003. Multiplex PCR for detection of three plasmid-borne genes of enteroaggregative *Escherichia coli* strains. *Journal of Clinical Microbiology*, **41**(5), 2138-2140.

Czerucka, D., Dahan, S., Mograbi, B., Rossi, B. and Rampal, P., 2000. Saccharomyces boulardii preserves the barrier function and modulates the signal transduction pathway induced in enteropathogenic *Escherichia coli*-infected T84 cells. *Infection and Immunity*, **68**(10), 5998-6004.

Dean, P. and Kenny, B., 2009. The effector repertoire of enteropathogenic *E. coli*: ganging up on the host cell. *Current Opinion in Microbiology*, **12**(1), 101-109.

Dow, M.A., Toth, I., Malik, A., Herpay, M., Nogrady, N., Ghenghesh, K.S. and Nagy, B., 2006. Phenotypic and genetic characterization of enteropathogenic *Escherichia coli* (EPEC) and enteroaggregative *E. coli* (EAEC) from diarrhoeal and non-diarrhoeal children in Libya. *Comparative Immunology, Microbiology and Infectious Diseases*, **29**(2-3), 100-113.

Dupont, A., Sommer, F., Zhang, K., Repnik, U., Basic, M., Bleich, A., Kühnel, M., Bäckhed, F., Litvak, Y., Fulde, M. and Rosenshine, I., 2016. Age-dependent susceptibility to enteropathogenic *Escherichia coli* (EPEC) infection in mice. *PLoS Pathogens* **12**(5).

Dutta, K., Pal, B., Mandal, R., Mullick, S. and Mandal, P., 2018. Circulating Rotavirus Types and Drug-Resistant Diarrheagenic *Escherichia coli* Causing Enteric Infection in Under-Five Children in Rural West Bengal, India. *Journal of Bioengineering and Biomedical Sciences*, **8**(255), 2.

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Ebert, D., 2000. Experimental evidence for rapid parasite adaptation and its consequences for the evolution of virulence. *Evolutionary biology of host-parasite relationships: theory meets reality*, **32**, 163-184.

Elias, W.P. and Navarro-Garcia, F., 2016. Enteroaggregative *Escherichia coli* (EAEC). In *Escherichia coli in the Americas,* (27-57). Springer, Cham.

Frankel, G., Phillips, A.D., Rosenshine, I., Dougan, G., Kaper, J.B. and Knutton, S., 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Molecular Microbiology*, **30**(5), 911-921.

Friedberg, D., Umanski, T., Fang, Y. and Rosenshine, I., 1999. Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic *Escherichia coli. Molecular Microbiology* **34**, 941-952.

Garbutt, J., Bonsall, M.B., Wright, D.J. and Raymond, B., 2011. Antagonistic competition moderates virulence in *Bacillus thuringiensis*. *Ecology Letters*, **14**(8), 765-772.

Giron, J. A., Ho, A. S. and Schoolnik, G. K., 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. *Science,* **254**, 710-713.

Gomes, T.A., Elias, W.P., Scaletsky, I.C., Guth, B.E., Rodrigues, J.F., Piazza, R.M., Ferreira, L.C. and Martinez, M.B., 2016. Diarrheagenic *Escherichia coli*. *Brazilian Journal of Microbiology*, **47**, 3-30.

Griffiths, E.C., Pedersen, A.B., Fenton, A. and Petchey, O.L., 2011. The nature and consequences of coinfection in humans. *Journal of Infection*, **63**(3), 200-206.

Guerrant, R.L., Leite, A.M., Pinkerton, R., Medeiros, P.H., Cavalcante, P.A., DeBoer, M., Kosek, M., Duggan, C., Gewirtz, A., Kagan, J.C. and Gauthier, A.E., 2016. Biomarkers of environmental enteropathy, inflammation, stunting, and impaired growth in children in northeast Brazil. *PLoS One*, **11**(9).

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Haarmann, N., Berger, M., Kouzel, I.U., Mellmann, A. and Berger, P., 2018. Comparative virulence characterization of the Shiga toxin phage-cured *Escherichia coli* O104: H4 and enteroaggregative *Escherichia coli*. *International Journal of Medical Microbiology*, **308**(7), 912-920.

Hebbelstrup Jensen, B., Poulsen, A., Hebbelstrup Rye Rasmussen, S., Struve, C., Engberg, J.H., Friis-Møller, A., Boisen, N., Jønsson, R., Petersen, R.F., Petersen, A.M. and Krogfelt, K.A., 2017. Genetic virulence profile of enteroaggregative *Escherichia coli* strains isolated from Danish children with either acute or persistent diarrhea. *Frontiers in Cellular and Infection Microbiology*, **7**, 230.

Huang, D.B., DuPont, H.L., Jiang, Z.D., Carlin, L. and Okhuysen, P.C., 2004. Interleukin-8 response in an intestinal HCT-8 cell line infected with enteroaggregative and enterotoxigenic *Escherichia coli*. *Clinical and Diagnostic Laboratory Immunology*, **11**(3), 548-551.

Iijima, Y., Oundo, J.O., Hibino, T., Saidi, S.M., Hinenoya, A., Osawa, K., Shirakawa, T., Osawa, R. and Yamasaki, S., 2017. High prevalence of diarrheagenic *Escherichia coli* among children with diarrhea in Kenya. *Japan Journal of Infectious Diseases*, **70**, 80-83.

Itoh, Y., Nagano, I., Kunishima, M. and Ezaki, T., 1997. Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable: H10 associated with a massive outbreak of gastrointestinal illness. *Journal of Clinical Microbiology*, **35**(10), 2546-2550.

Kaper, J.B., Nataro, J.P. and Mobley, H.L., 2004. Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*, **2**(2), 123.

Kosek, M.N., Ahmed, T., Bhutta, Z., Caulfield, L., Guerrant, R., Houpt, E., Kang, G., Kosek, M., Lee, G., Lima, A. and McCormick, B.J., 2017. Causal pathways from enteropathogens to environmental enteropathy: findings from the MAL-ED birth cohort study. *EBioMedicine*, **18**,109-117.

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Kotloff, K.L., Nataro, J.P., Blackwelder, W.C., Nasrin, D., Farag, T.H., Panchalingam, S., Wu, Y., Sow, S.O., Sur, D., Breiman, R.F. and Faruque, A.S., 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *The Lancet*, **382**(9888), 209-222.

Law, R.J., Gur-Arie, L., Rosenshine, I. and Finlay, B.B., 2013. *In vitro* and *in vivo* model systems for studying enteropathogenic *Escherichia coli* infections. *Cold Spring Harbor Perspectives in Medicine*, **3**(3), a009977.

Ledwaba, S.E., Kabue, J.P., Barnard, T.G., Traore, A.N. and Potgieter, N., 2018. Enteric pathogen co-infections in the paediatric population from rural communities in the Vhembe District, South Africa. *South African Journal of Child Health*, **12**(4), 170-174.

Levine, M. M., Nataro, J.P., Karch, H., Baldini, M.M., Kaper, J.B., Black, R.E., Clements, M.L. and O'Brien, A.D., 1985. The diarrheal response of humans to some classic serotypes of enteteropathogenic *Escherichia coli* is dependent on a plasmid encoding the enteroadhesiveness factor. *Journal of Infectious Diseases,* **152**(3), 550-559.

Lima, A.A., Soares, A.M., José Filho, Q.S., Havt, A., Lima, I.F., Lima, N.L., Abreu, C.B., Junior, F.S., Mota, R., Pan, W.K.Y. and Troeger, C., 2018. Enteroaggregative *Escherichia coli* subclinical infection and coinfections and impaired child growth in the MAL-ED Cohort Study. *Journal of Pediatric Gastroenterology and Nutrition*, **66**(2), 325-333.

Manko, A., Motta, J.P., Cotton, J.A., Feener, T., Oyeyemi, A., Vallance, B.A., Wallace, J.L. and Buret, A.G., 2017. Giardia co-infection promotes the secretion of antimicrobial peptides beta-defensin 2 and trefoil factor 3 and attenuates attaching and effacing bacteria-induced intestinal disease. *PLoS One*, **12**(6).

Mathew, S., Smatti, M.K., Al Ansari, K., Nasrallah, G.K., Al Thani, A.A. and Yassine, H.M., 2019. Mixed viral-bacterial infections and their effects on gut microbiota and clinical Illnesses in children. *Scientific Reports*, **9**(1), 1-12.

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Mayneris-Perxachs, J., Bolick, D.T., Leng, J., Medlock, G.L., Kolling, G.L., Papin, J.A., Swann, J.R. and Guerrant, R.L., 2016. Protein-and zinc-deficient diets modulate the murine microbiome and metabolic phenotype. *American Journal of Clinical Nutrition*, **104**(5), 1253-1262.

Medeiros, P., Bolick, D.T., Roche, J.K., Noronha, F., Pinheiro, C., Kolling, G.L., Lima, A. and Guerrant, R.L., 2013. The micronutrient zinc inhibits EAEC strain 042 adherence, biofilm formation, virulence gene expression, and epithelial cytokine responses benefiting the infected host. *Virulence,* **4**(7), 624-633.

Mideo, N., 2009. Parasite adaptations to within-host competition. *Trends in Parasitology*, **25**(6), 261-268.

Millette, G., Langlois, J.P., Brouillette, E., Frost, E.H., Cantin, A.M. and Malouin, F., 2019. Despite antagonism *in vitro*, *Pseudomonas aeruginosa* enhances *Staphylococcus aureus* colonization in a murine lung infection model. *Frontiers in Microbiology*, **10**.

Nataro, J.P., Kaper, J.B., Robins-Browne, R.O.Y., Prado, V., Vial, P. and Levine, M.M., 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. *Pediatric Infectious Disease Journal*, **6**(9), 829-831.

Ochoa, T.J. and Contreras, C.A., 2011. Enteropathogenic *E. coli* (EPEC) infection in children. *Current Opinion in Infectious Diseases*, **24**(5), 478.

Oh, S., Lillehoj, H.S., Lee, Y., Bravo, D. and Lillehoj, E., 2019. Dietary antibiotic growth promoters down-regulate intestinal inflammatory cytokine expression in chickens challenged with LPS or co-infected with *Eimeria maxima* and *Clostridium perfringens*. *Frontiers in Veterinary Science*, **6**, 420.

Okhuysen, P.C. and DuPont, H.L., 2010. Enteroaggregative *Escherichia coli* (EAEC): a cause of acute and persistent diarrhea of worldwide importance. *Journal of Infectious Diseases,* **202**(4), 503-505.

© University of Venda

Opintan, J.A., Newman, M.J., Ayeh-Kumi, P.F., Affrim, R., Gepi-Attee, R., Sevilleja, J.E., Roche, J.K., Nataro, J.P., Warren, C.A. and Guerrant, R.L., 2010. Pediatric diarrhea in southern Ghana: etiology and association with intestinal inflammation and malnutrition. *American Journal of Tropical Medicine and Hygiene*, **83**(4), 936-943.

Park, S.H., Yu, M., Kim, J. and Moon, Y., 2017. C/EBP homologous protein promotes NSAID-activated gene 1-linked pro-inflammatory signals and enterocyte invasion by enteropathogenic *Escherichia coli*. *Microbes and Infection*, **19**(2), 110-121.

Platts-Mills, J.A., Babji, S., Bodhidatta, L., Gratz, J., Haque, R., Havt, A., McCormick, B.J., McGrath, M., Olortegui, M.P., Samie, A. and Shakoor, S., 2015. Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). *The Lancet Global Health*, **3**(9), e564-e575.

Prata, M.D.M.G., Havt, A., Bolick, D.T., Pinkerton, R., Lima, A.A.M. and Guerrant, R.L., 2016. Comparisons between myeloperoxidase, lactoferrin, calprotectin and lipocalin-2, as fecal biomarkers of intestinal inflammation in malnourished children. *Journal of Translational Science*, **2**(2), 134.

Reece, E., Doyle, S., Greally, P., Renwick, J. and McClean, S., 2018. Aspergillus fumigatus inhibits Pseudomonas aeruginosa in co-culture: implications of a mutually antagonistic relationship on virulence and inflammation in the CF airway. *Frontiers in Microbiology*, **9**, 1205.

Roche, J.K., Cabel, A., Sevilleja, J., Nataro, J. and Guerrant, R.L., 2010. Enteroaggregative *Escherichia coli* (EAEC) impairs growth while malnutrition worsens EAEC infection: a novel murine model of the infection malnutrition cycle. *Journal of Infectious Diseases*, **202**(4), 506-514.

Rogawski, E.T., Guerrant, R.L., Havt, A., Lima, I.F., Medeiros, P.H., Seidman, J.C., McCormick, B.J., Babji, S., Hariraju, D., Bodhidatta, L. and Shrestha, J., 2017.

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Epidemiology of enteroaggregative *Escherichia coli* infections and associated outcomes in the MAL-ED birth cohort. *PLoS Neglected Tropical Diseases*, **11**(7), e0005798.

Ruchaud-Sparagano, M.H., Maresca, M. and Kenny, B., 2007. Enteropathogenic *Escherichia coli* (EPEC) inactivate innate immune responses prior to compromising epithelial barrier function. *Cellular Microbiology*, **9**(8), 1909-1921.

Sanchez-Villamil, J., Tapia-Pastrana, G. and Navarro-Garcia, F., 2016. Pathogenic lifestyles of *E. coli* pathotypes in a standardized epithelial cell model influence inflammatory signaling pathways and cytokines secretion. *Frontiers in Cellular and Infection Microbiology*, **6**, 120.

Savkovic, S.D., Villanueva, J., Turner, J.R., Matkowskyj, K.A. and Hecht, G., 2005. Mouse model of enteropathogenic *Escherichia coli* infection. *Infection and Immunity*, **73**(2), 1161-1170.

Scaletsky, I.C., Silva, M.L. and Trabulsi, L.R., 1984. Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLA cells. *Infection and Immunity*, **45**(2), pp.534-536.

Scaletsky, I.C.A., 2019. Enteropathogenic *Escherichia coli*. In *The Universe of Escherichia coli*. IntechOpen.

Schiebel, J., Böhm, A., Nitschke, J., Burdukiewicz, M., Weinreich, J., Ali, A., Roggenbuck, D., Rödiger, S. and Schierack, P., 2017. Genotypic and phenotypic characteristics in association with biofilm formation in different pathotypes of human clinical *Escherichia coli* isolates. *Applied Environmental Microbiology*, AEM-01660.

Shen, S.S., Qu, X.Y., Zhang, W.Z., Li, J. and Lv, Z.Y., 2019. Infection against infection: parasite antagonism against parasites, viruses and bacteria. *Infectious Disease of Poverty*, **8**(1), 49.

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Stockmann, C., Pavia, A.T., Graham, B., Vaughn, M., Crisp, R., Poritz, M.A., Thatcher, S., Korgenski, E.K., Barney, T., Daly, J. and Rogatcheva, M., 2017. Detection of 23 gastrointestinal pathogens among children who present with diarrhea. *Journal of Pediatric Infectious Diseases Society*, **6**(3), pp.231-238.

Sugiharto, S., Hedemann, M.S. and Lauridsen, C., 2014. Plasma metabolomic profiles and immune responses of piglets after weaning and challenge with *E. coli*. *Journal of Animal Science and Biotechnology*, **5**(1), 17.

Trabulsi, L.R., Keller, R. and Gomes, T.A.T., 2002. Typical and atypical enteropathogenic *Escherichia coli*. *Emerging Infectious Diseases*, **8**(5), 508.

Yatsuyanagi, J., Saito, S., Sato, H., Miyajima, Y., Amano, K.I. and Enomoto, K., 2002. Characterization of enteropathogenic and enteroaggregative *Escherichia coli* isolated from diarrheal outbreaks. *Journal of Clinical Microbiology*, **40**(1), 294-297.

Zhang, Q., Li, Q., Wang, C., Liu, X., Li, N. and Li, J. 2010. Enteropathogenic *Escherichia coli* changes distribution of occludin and ZO-1 in tight junction membrane microdomains in vivo. *Microbial Pathogens,* **48**, 28-34.

Zhang, W.L., Köhler, B., Oswald, E., Beutin, L., Karch, H., Morabito, S., Caprioli, A., Suerbaum, S. and Schmidt, H., 2002. Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. *Journal of Clinical Microbiology*, **40**(12), 4486-4492.

Zhu, X., Chen, L., Wu, J., Tang, H. and Wang, Y., 2017. *Salmonella typhimurium* infection reduces *Schistosoma japonicum* worm burden in mice. *Scientific Reports*, **7**(1), 1-10.

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CHAPTER 6

SUMMATIVE COMMENTS AND RECOMMENDATIONS

6.1. SUMMATIVE COMMENTS

Diarrhoeal infections cause deaths in young children (Troeger et al, 2018). DEC pathotypes such as EAEC, EPEC and ETEC have been reported to cause moderate to severe diarrhoea in children under 2 years of age (Kotloff et al, 2013). Occurrence of co-infections has been reported (Crane et al, 2006; Bartelt et al, 2017; Ledwaba et al, 2018; Lima et al, 2018). The interaction of co-infecting pathogens has opened a whole new perspective, and studies have been done in order to understand the microbe-microbe interactions within the host (Crane et al, 2006; Estrada et al, 2012; Bartelt et al, 2017). *In vitro* and *in vivo* models are of great importance as they enable analysis of pathogens in closed controlled environmental settings. The use of animal models that mimic clinical outcomes of human infections act as a gateway in understanding the interaction of pathogens in a living host.

Typical EPEC causes severe diarrhoea leading to deaths in children under 12 months of age (Kotloff et al, 2013). This pathogen is characterized by distinct LA pattern forming A/E lesions at the site of infection (Croxen et al, 2013). EPEC has been studied extensively *in vitro*, with studies highlighting the pathogenesis mechanism and inflammatory responses produced during infection (Law et al, 2013). EPEC has also been studied *in vivo* using different animal models, however, these models have failed to report on a small animal model of EPEC infection resulting in clinical outcomes. The first objective of this study was to develop an EPEC murine model using weaned C57BL/6 mice that were treated with antibiotic cocktail to enable colonization, clinical outcomes,

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inflammatory responses (similar to humans) and metabolic disruptions. The following were observed

- An EPEC infectious murine model has been developed. Weaned C57BL/6 mice pretreated with antibiotic cocktail were orally infected with typical EPEC. Infection resulted in clinical outcomes of changes in faecal appearance and transient weight loss.
- Infection resulted in increased inflammatory biomarkers (MPO, LCN-2) similar to children. Acute systematic inflammatory responses were also observed with an increase in IL-6, SAA and CRP markers.
- Metabolic perturbations were observed with the TCA cycle being greatly affected
- The EPEC murine model will be the first study to report on wet stools in mice infected with WT EPEC E2348/69 strain and bloody stools in mice infected with UMD901 E2348/69 strain.
- This model also highlighted the importance of the *bfp* (involved in adherence) and *escN* (assisting in activation of the T3SS system) virulence genes involved in EPEC pathogenesis
    - A functional T3SS system is essential for EPEC pathogenesis. Mice infected with Δ*escN* CVD425 strain resulted in diminished weight changes and no clinical and inflammatory responses were observed during infection.
    - Mice infected with the mutant UMD901 strain resulted in severe disease outcome. Colonization was observed across all tissue sections with inflammatory and systemic markers higher in mice infected with the *bfp* mutant than the WT strain. This data highlighted the importance of atypical EPEC strains that are increasingly being detected in clinical settings.

Co-infections such as P/T and A/P pathogens have been reported in humans and animals (Wada et al, 1996; Ledwaba et al, 2018; Lima et al, 2018). ETEC is characterized by production of LT and ST enterotoxins that result in diarrhoea in travellers and children (Croxen et al, 2013). EAEC is characterized by distinct biofilm formation and secretion of

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enterotoxins (Croxen et al, 2013). The interaction of co-infections has been suggested to result in increased disease outcome. The second and third objectives were to study the interactions between P/T and A/P co-infections using *in vitro* and *in vivo* analyses. The following were observed:

- Interactions between P/T co-infections *in vitro* resulted in synergistic effects, the IL-8 response was higher than the single infections. The *bfpA* gene involved in adherence was greatly increased during co-infections suggesting that ETEC enhanced EPEC pathogenesis resulting in increased adherence.
- P/T co-infections resulted in increased severe disease outcome *in vivo*. Infection in the co-infected mice resulted in severe weight loss, diarrhoea with reduced movement and some of the mice died as a result of infection. Inflammatory biomarkers (MPO and LCN-2), IL-6 and acute systemic marker (SAA) were higher in the P/T infected mice, suggesting that infection resulted in detrimental disease outcome as suggested by clinical studies.
- The interaction of A/P co-infections *in vitro* resulted in antagonistic effects. EAEC altered EPEC localized adherence during A/P co-infections. This was further observed with EPEC virulence genes involved in pathogenesis being downregulated during co-infections
- A/P co-infections *in vivo* resulted in diminished weight loss and diarrhoea. EAEC colonization was enhanced by the presence of EPEC. However, interactions between these pathogens did not result in additive effects on intestinal inflammation (that is MPO and LCN-2 levels were not higher than the single infections).

Overall, the research questions were answered and the objectives of the study were achieved. That is, an EPEC murine model was developed. The interaction between A/P and P/T co-infections were studied. The results in this study have provided an understanding in the interaction of co-infecting bacterial pathogens in a host.

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6.2. LIMITATIONS

This study has provided insight in understanding the mechanisms involved during EPEC pathogenesis and interaction of co-infecting pathogens, however, there are limitations in the study. The metabolic analysis was only conducted on the WT EPEC strain and not on the mutant strains. In A/P co-infection analyses, the EAEC virulence gene expression was not assessed and the conclusions were made only on analysis of EPEC virulence gene expression.

6.3. RECOMMENDATIONS AND FUTURE STUDIES

- A new EPEC murine model has been developed showing the importance of the bundle forming pili involved in adherence and T3SS mechanism. Further studies are therefore required to study other genes that are involved during EPEC pathogenesis in order to facilitate in development of vaccines.
- The interaction between P/T and A/P co-infections in C57BL/6 mice have provided an understanding in disease outcome using prototype strains. Further studies are therefore, required to understand the mechanism that might be involved during co-infections in the host using clinical strains.
- The co-infection models used in this study can further be used to explore therapeutic interventions, such as studying the genes involved in increased virulence and use the model to reduce the disease outcome.
- *In vitro* and *in vivo* models are strictly controlled when compared to clinical settings. Strains used in this study were administered at the same time-point during co-infections. Further studies are therefore, required to administer the pathogens at different time points to determine if there will be a difference in disease outcome.
- The EPEC pathogenesis mechanism was greatly affected during EAEC and EPEC co-infections *in vitro.* The EAEC virulence mechanism involved during these co-infections needs further assessment.

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6.4. REFERENCES

Bartelt, L.A., Bolick, D.T., Mayneris-Perxachs, J., Kolling, G.L., Medlock, G.L., Zaenker, E.I., Donowitz, J., Thomas-Beckett, R.V., Rogala, A., Carroll, I.M. and Singer, S.M., 2017. Cross-modulation of pathogen-specific pathways enhances malnutrition during enteric co-infection with *Giardia lamblia* and enteroaggregative *Escherichia coli*. *PLoS Pathogens*, **13**(7).

Crane, J.K., Choudhari, S.S., Naeher, T.M. and Duffey, M.E., 2006. Mutual enhancement of virulence by enterotoxigenic and enteropathogenic *Escherichia coli*. *Infection and Immunity*, **74**(3), 1505-1515.

Croxen, M.A., Law, R.J., Scholz, R., Keeney, K.M., Wlodarska, M. and Finlay, B.B., 2013. Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clinical Microbiology Reviews*, **26**(4), 822-880.

Estrada, A.E.R., Jonkers, W., Kistler, H.C. and May, G., 2012. Interactions between Fusarium verticillioides, Ustilago maydis, and Zea mays: an endophyte, a pathogen, and their shared plant host. *Fungal Genetic and Biology*, **49**(7), 578-587.

Kotloff, K.L., Nataro, J.P., Blackwelder, W.C., Nasrin, D., Farag, T.H., Panchalingam, S., Wu, Y., Sow, S.O., Sur, D., Breiman, R.F. and Faruque, A.S., 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *The Lancet*, **382**(9888), 209-222.

Law, R.J., Gur-Arie, L., Rosenshine, I. and Finlay, B.B., 2013. *In vitro* and *in vivo* model systems for studying enteropathogenic *Escherichia coli* infections. *Cold Spring Harbor Perspectives in Medicine*, **3**(3), a009977.

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Ledwaba, S.E., Kabue, J.P., Barnard, T.G., Traore, A.N. and Potgieter, N., 2018. Enteric pathogen co-infections in the paediatric population from rural communities in the Vhembe District, South Africa. *South African Journal of Child Health*, **12**(4), 170-174.

Lima, A.A., Soares, A.M., José Filho, Q.S., Havt, A., Lima, I.F., Lima, N.L., Abreu, C.B., Junior, F.S., Mota, R., Pan, W.K.Y. and Troeger, C., 2018. Enteroaggregative *Escherichia coli* subclinical infection and coinfections and impaired child growth in the MAL-ED Cohort Study. *Journal of Pediatric Gastroenterology and Nutrition*, **66**(2), 325-333.

Troeger, C., Blacker, B.F., Khalil, I.A., Rao, P.C., Cao, S., Zimsen, S.R., Albertson, S.B., Stanaway, J.D., Deshpande, A., Abebe, Z. and Alvis-Guzman, N., 2018. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of diarrhoea in 195 countries: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet Infectious Diseases*, **18**(11), 1211-1228.

Wada, Y., Nakaoka, Y., Kondo, H., Nakazawa, M. and Kubo, M., 1996. Dual infection with attaching and effacing *Escherichia coli* and enterotoxigenic *Escherichia coli* in post-weaning pigs. *Journal of Comparative Pathology*, **114**(1), 93-99.

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APPENDIX

APPENDIX A: SUPPLEMENTARY DATA FOR CHAPTER 3

A1: The overall change in weight of mice infected with WT EPEC, *bfp*- and △*escN* mutants followed until day 8 pi.

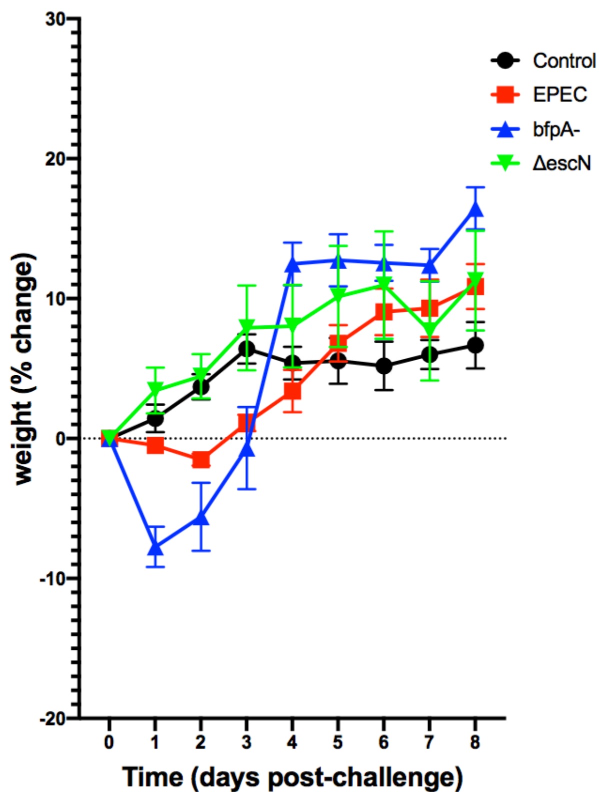

Figure 1: Weight change of mice infected with WT EPEC, *bfpA*- and Δ*escN* (n = 12/group) at day 0 pi, mice were euthanized at day 8 pi.

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A2: Haemoglobin levels of mice infected with WT EPEC, *bfp*- and ∆*escN* at day 4 pi.

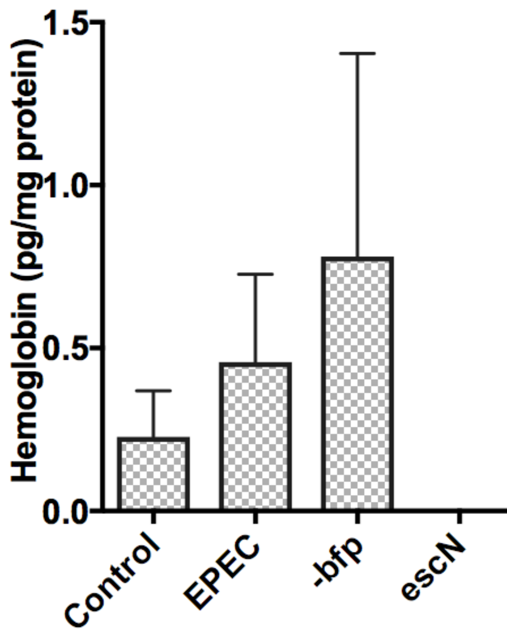

Figure 2: Haemoglobin levels in stools. Stool protein lysates of uninfected and infected mice were used to determine increase in haemoglobin using mouse hemoglobin ELISA (R&D systems) according to manufacturer instructions (n = 8). Increased haemoglobin levels were observed in stools of mice infected with *bfpA*-.

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A3: Additional TEM showing ileal tissue of mice infected with EPEC

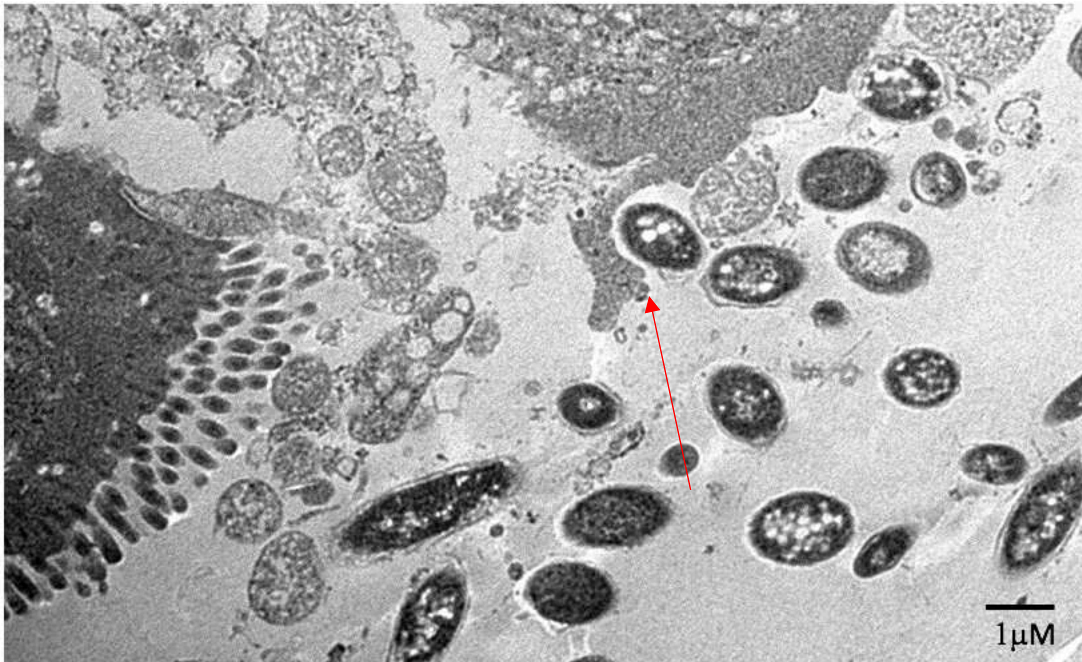

Figure 3: Transmission electron microscope (TEM) images showing ileum infected with WT EPEC at day 3 pi with disrupted microvilli (red arrow).

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APPENDIX B: LISTS OF ABSTRACTS AND CONFERENCES ATTENDED

B.1. Conference abstract of the poster presentation at

Carey-Marshall-Thorner scholar's and research day
University of Virginia USA, May 11th 2016

Title: **Distribution of virulence-related genes and pathogenesis of Diarrheagenic *E. coli* strains in Limpopo Province, South Africa**

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Background: Globally diarrhoea in children less than 5 years of age is still a major problem in public health. In developing countries including South Africa diarrhoea caused by Diarrheagenic *E. coli* (DEC) occurs due to poor sanitation and hygiene behaviour. Increase in exposure to DEC can lead to occurrence of different strains that result in co-infections.

Objectives: To determine virulence patterns of genes that might be involved in infections and determine pathogenesis using murine models

Hypothesis: Diarrheagenic *E. coli* (DEC) co-infections result from interchange of virulence genes

Research questions: What are the genes involved in virulence of DEC strains during host infection? What are the pathogenesis mechanisms involved in DEC co-infections?

Methods: Diarrheal stool specimens from children attending Primary Health Facilities (clinics) were collected in rural communities of Limpopo province, South Africa. Bacterial DNA was extracted and a published m-PCR protocol was used to determine the prevalence of DEC strains (EAEC, ETEC, EIEC, EHEC, a-EPEC, t-EPEC).

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Results: Overall Diarrheagenic *E. coli* strains were detected in 48.5% of the specimens. Bacterial co-infections were prevalent and seen in 32.9% of the participants.

Way forward: To determine specific virulence patterns in single and in co-infections and explore their pathogenesis using murine models.

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B.2. Conference abstract of the poster presentation at

Infectious Disease and Biodefense Research Day
University of Virginia USA, April 18th 2016

Title: **Epidemiology and outcomes of multiple infections with Enteropathogenic, Enterotoxigenic and Enteroaggregative *E. coli***

Solanka E. Ledwaba[1,2,3], Araceli E. Santiago[2], David Bolick[3], Richard Guerrant[3], Afsatou N. Traoré[1] Natasha Potgieter[1] and James P. Nataro[2]

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Background: Globally diarrhoea in children less than 5 years of age is still a major problem in public health. Diarrheagenic *E. coli* infections occur primarily in settings with poor sanitation and hygiene and limited access to safe drinking water. In some parts of South Africa there are several communities that lack access to improved water and sanitation services. In a previous study, 237 diarrheal specimens of children under the age of 5 years were collected and the prevalence of Diarrheagenic *E. coli* (DEC) strains was determined. ETEC (31%), EAEC (26%), Atypical EPEC (16%) and Typical EPEC (16) strains were found to be the most prevalent strains. Several DEC combinations were also seen. Reports from epidemiological and *in vitro* studies have stated that the certain DEC combinations such as ETEC, EAEC and EPEC co-infections are likely to cause more severe diarrheal diseases in infected patients.

Objective: Characterization of single and multiple infections of pathogenic *E. coli in vitro* and *in vivo.*

Methods: HeLA cells will be exposed to single infections of Atypical EPEC, Typical EPEC, EAEC, and ETEC or combinations of enteropathogenic bacteria. Bacterial adherence and cytotoxicity will be determined in infected cells. These pathogens will further be tested on T84 cells for confirmation of adherence assay, cytokine release and

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trans-epithelial resistance. Co-infections that show increased interaction *in vitro*, will further be analysed *in vivo* using murine models.

Expected outcomes: Determination of the interaction of EPEC, ETEC and EPEC single and co-infections *in vitro* and also the pathogenesis mechanism *in vivo* in order to assess the severity of these co-infections.

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B.3. Conference abstract of the poster presentation at

Infectious diseases and biodefence research day
University of Virginia, USA, March 13th 2017

Title: **Modeling EPEC and ETEC co-infections in a mouse model**

Ledwaba SE[1,2,3], Santiago AE[2], Bolick D[3], Nataro JP[2], Potgieter N[1], Traore AN and Guerrant RL[3]

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Background: Diarrheal diseases are a major cause of morbidity and mortality in children worldwide. Epidemiological studies in both developed and developing countries have shown that children infected with Diarrheagenic *E. coli* suffer from different series of clinical outcomes. Enteropathogenic *E. coli* (EPEC), and Enterotoxigenic *E. coli* (ETEC) are recognized as major causes of acute diarrhea and dual infections with these pathogens have been reported in different parts of the world. The aim of the study is to determine the role of diet and disease severity during single infection of EAEC, EPEC and ETEC and also during EAEC/EPEC and EPEC/ETEC co-infections *in vitro* and *in vivo*.

Method: Single infections of EPEC, EAEC, and ETEC together with the co-infections will be infected on HeLA cells to determine adherence pattern and inflammatory cytokines. These pathogens will further be analyzed to determine expression virulence genes involved during infection. C57BL/6 mice on specific diets will be used to determine infection outcome during single and co-infections *in vivo*.

Expected outcomes: The results of this study will lead to increased knowledge on the role of diet and interaction of EAEC, EPEC and ETEC single and co-infections. Development of EAEC/EPEC co-infection model. Lastly observe the clinical outcomes as would be seen in infected children.

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B.4. Conference abstract of the poster presentation at

Carey-Marshall-Thorner scholar's and research day
University of Virginia, May 10th 2017

Title: **Interactions of Diarrheagenic *E. coli* infections in a murine model**

Ledwaba SE[1,2,3], Bolick D[3], Santiago AE[2], Nataro JP[2], Potgieter N[1] and Guerrant RL[3]

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Background: Diarrheal diseases are a major cause of morbidity and mortality in children worldwide. Enteropathogenic *E. coli (*EPEC), Enteroaggregative *E. coli* (EAEC) and Enterotoxigenic *E. coli* (ETEC) are recognized as major causes of acute diarrhea, and dual infections with these pathogens have been fairly reported in different parts of the world. The goal of the study is to determine the role of diet and disease severity during single infections of EAEC, EPEC and ETEC and also during co-infections with these pathogens.

Method: Weaned C57BL/6 mice on specific diets were infected with single infections (EAEC, EPEC, ETEC) and co-infections (EAEC/EPEC and EPEC/ETEC). During infection, mice were monitored for change in weight and stools were collected daily. Mice were euthanized on the third day of infection, intestinal tissue and cecum contents were collected. Stool shedding and tissue burden were analyzed using qPCR. Proteins lysates extracted from cecum contents and stools were used to determine inflammatory biomarkers myeloperoxidase (MPO) and lipocalin-2 (LCN- 2).

Results: Disease outcome of EPEC/ETEC co-infection was worse compared to the single infections. During EAEC/EPEC co-infection, EAEC inhibited EPEC effect on weight loss and diminished EPEC shedding. Inflammatory biomarkers MPO and LCN-2 levels in

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EPEC/ETEC coinfections were significantly higher compared to EPEC or ETEC alone, while MPO levels were diminished during EAEC/EPEC co-infection.

Conclusions: EPEC/ETEC co-infection resulted in synergistic effect with mice experiencing severe outcome with increased weight loss, diarrhea, and increased inflammatory MPO and LCN-2. However, EAEC/EPEC co-infection resulted in antagonistic effect with no weight loss, and reduced inflammatory LCN-2 and MPO. Future directions are to determine expression of EPEC, ETEC and EAEC virulence genes.

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B.5. Conference abstract of the poster presentation at

29th Annual University of Virginia children's hospital symposium and research trainee competition
University of Virginia, USA, May 25th 2017

Title: **Interactions of Enteropathogenic *E. coli* and Enteroaggregative *E. coli* infections *in vitro* and *in vivo***

Solanka E. Ledwaba[1,2,3], David T. Bolick[3], Pedro HQS. Medeiros[3] Araceli E. Santiago[2], Natasha Potgieter[1] and Richard L. Guerrant[3], James P. Nataro[2]

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Background: Enteroaggregative *E. coli* (EAEC), and Enteropathogenic *E. coli* (EPEC) are recognized as major causes of acute diarrhea in children living in poor social conditions in developing countries. Epidemiological studies have reported on the dual infections with these pathogens suggesting interaction of EPEC and EAEC might result in altered disease outcomes.

Objective: To determine interaction of EAEC, EPEC and EAEC/EPEC co-infections *in vitro* and *in vivo*.

Design/Methods: Single infections of EPEC E2348/69 and EAEC 042 together with co-infections (EAEC/EPEC) were infected on HeLA cells to determine inflammatory cytokines. cDNA from infected HeLA cells was used to determine expression of EPEC virulence genes. Interaction of EAEC/EPEC co-infections were further analyzed in *vivo* using weaned C57BL/6 mice on standard chow diet.

Results: HeLA cells infected with EPEC resulted in increased IL6 and IL8 cytokines; cells infected with EAEC/EPEC had reduces IL6 and IL8. Expression of EPEC was greatly reduced during EAEC/EPEC co-infections. *In vivo*, there was diminished weight loss and

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diarrhea in EAEC/EPEC infected mice. EAEC stool shedding was higher than EPEC shedding in co-infected mice with reduced inflammatory Myeloperoxidase (MPO).

Conclusions: EAEC/EPEC co-infection resulted in antagonistic effect with reduced IL6 and IL8 cytokines. *In vivo*, EAEC inhibited EPEC effect on weight loss and diminished EPEC shedding. Future directions are to determine expression of EPEC and EAEC virulence genes during co-infections.

Summary:

Objective: To determine interaction of EAEC, EPEC and EAEC/EPEC co-infections *in vitro* and *in vivo*.

Conclusions: EAEC/EPEC co-infections result in diminished weight loss and diarrhea, suggesting EAEC inhibits effect of EPEC during infection

Implications for children: EAEC is protective against EPEC infection

This work was funded by NIH grants AI- 033096 and U19AI090873 to JPN. Global Infectious Diseases and Research Training (GIDRT) Program at UVA Award by NIH D43 TW006578.

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B.6. Conference abstract of the poster presentation at

29th Annual University of Virginia children's hospital symposium and research trainee competition
University of Virginia, USA, May 25th 2017

Title: **Modeling of Enteropathogenic *E. coli* and Enterotoxigenic *E. coli* co-infection in a mouse model**

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Background: Diarrheal diseases are a major cause of morbidity and mortality in children worldwide. Enteropathogenic *E. coli* (EPEC), and Enterotoxigenic *E. coli* (ETEC) are recognized as major causes of acute diarrhea and dual infections with these pathogens have been reported in different parts of the world.

Objective: To determine disease severity during single infections of EPEC and ETEC and also during EPEC/ETEC co-infections.

Design/Methods: Weaned C57BL/6 mice on standard chow diet were infected with single infections of EPEC E-2348/69, ETEC H10407 and co-infections (EPEC/ ETEC). During infection, mice were monitored daily for weights and stools were collected. These mice were euthanized on the third day of infection. Bacterial DNA was extracted from stools tissue sections; and shedding was determined using qPCR. Protein lysates from day 2 stool samples were used to determine intestinal inflammatory biomarkers Myeloperoxidase (MPO) and Lipoalin-2 (LCN-2).

Results: All mice developed diarrhea on the first day post-infection. There was significant weight loss on day 2 and 3 post infection in mice infected with EPEC, ETEC and

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EPEC/ETEC co-infections. Stool shedding of ETEC was significantly higher in single and co-infections. ETEC colonized better in all sections of the intestine with increased tissue burden in the ileum and colon.

Inflammatory biomarkers MPO and LCN-2 levels in EPEC/ETEC co-infections were significantly higher compared to EPEC or ETEC alone.

Conclusions: EPEC together with ETEC have a synergistic effect with mice experiencing more severe disease outcomes than the single EPEC and ETEC infections. These findings suggest that EPEC and ETEC co-infections are worse than either single respective infection alone and warrant further study in the field.

Summary:

- **Objective:** To determine disease severity during single infections of EPEC and ETEC and also during EPEC/ETEC co-infections.
- **Conclusions:** Disease outcome of co-infections (EPEC/ETEC) was worse compared to the single EPEC and ETEC infections in weaned C57BL/6 mice.
- **Implications for children:** Interaction of EPEC/ETEC co-infections results in severe diarrhea and weight loss.

This work was funded by NIH grants AI- 033096 and U19AI090873 to JPN. Global Infectious Diseases and Research Training (GIDRT) Program at UVA Award by NIH D43 TW006.

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B.7. Conference abstract of the poster presentation at

American Society for Tropical Medicine and Hygiene
Baltimore, Maryland, USA, November 5th – 9th 2017

Title: **Synergistic and Antagonistic effects of Diarrheagenic *E. coli* co-infections in a murine model**

<u>Solanka E. Ledwaba</u>[1], David T. Bolick[2], Pedro H. Medeiros[2], Afsatou N. Traore[1], Natasha Potgieter[1], James P. Nataro[2], Richard L. Guerrant[2]

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Diarrheal diseases are major causes of morbidity in children worldwide. Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC) and Enterotoxigenic *E. coli* (ETEC) are major causes of acute diarrhea; dual infections pathogens have been reported in different parts of the world. The goal of the study is to determine the role of diet and disease severity during single infections of EAEC, EPEC and ETEC and also during co-infections. Four-week old weaned C57BL/6 mice on standard chow diet were placed on antibiotic cocktail water for 3 days. These mice were administered orally (10^{10} CFU/mouse) single infections (EAEC 042, EPEC E2348/69, ETEC H10407) and co-infections (EAEC/EPEC and EPEC/ETEC) (10^{10} CFU/mouse per pathogen). During infection, mice were monitored for change in weight and stools were collected daily. At peak of infection; serum, intestinal tissue and cecum contents were collected. Bacterial DNA was extracted from stools and tissue, and qPCR was used to determine shedding and tissue burden. Proteins lysates extracted from cecum contents and stools were used to determine inflammatory biomarkers myeloperoxidase (MPO) and lipocalin-2 (LCN-2). All mice developed diarrhea on the first day post-infection except EAEC/EPEC co-infections. There was significant weight loss on day 2 and 3 post-infection in mice infected with EPEC or ETEC alone or with EPEC/ETEC co-infections, but not for those infected with EAEC/EPEC. MPO and LCN-2 levels in EPEC/ETEC co-infections were significantly higher compared to EPEC or ETEC alone, while MPO levels

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were diminished during EAEC/EPEC co-infection. Disease outcome of EPEC/ETEC co-infection was worse compared to the single infections. In addition, EPEC/ETEC co-infection resulted in worse outcomes with mice experiencing severe disease outcome with increased weight loss, diarrhea and increased MPO and LCN-2. EAEC/EPEC co-infection resulted in antagonistic effects with no weight loss and reduced inflammatory LCN-2 and MPO. Future directions include assessing expression of virulence genes during co-infections to better understand these potentially important differences in outcomes.

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B.8. Research abstract of the poster presentation at

Research Open Day

University of Venda, Thohoyandou, SA, October 4th 2019

Title: **Typical and atypical Enteropathogenic *E. coli* (EPEC) infection cause enteropathy and diarrhea in weaned C57Bl/6 mice**

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Introduction: Typical and atypical Enteropathogenic *E. coli* (EPEC) are recognized as one of the leading bacterial causes of infantile diarrhea worldwide.

Methods: Weaned C57BL/6 mice pretreated with antibiotics were challenged orally with wild type EPEC, *bfp* and *escN* mutants (lacking bundle forming pili or type 3 secretion respectively) in order to determine colonization, inflammatory responses and clinical outcomes during infection.

Results: Antibiotic disruption of intestinal microbiota led to efficient colonization of EPECwt and EPEC*bfp*-, resulting in weight loss and diarrhea in mice. Increases in inflammatory biomarkers were observed in the intestinal tissue. EPEC*escN*- colonized in the mice, however no weight changes or clinical outcomes were observed. Metabolome changes were also observed in EPEC infected mice with changes in the TCA cycle with increased creatine and TMA.

Conclusion: A small animal model treated with antibiotics has been developed to mimic clinical outcomes as observed in children with EPEC infection. This model can therefore be used to further understand mechanisms that are involved during pathogenesis of EPEC infections and thus assist in development of potential therapeutic interventions.

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