

**UNDERSTANDING PATTERNS OF *ESCHERICHIA COLI* O157:H7
SHEDDING AND COLONISATION IN CATTLE AND THEIR ROLE IN
TRANSMISSION**

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Thesis submitted for the Degree of

Doctor of Philosophy

in

Environmental Science

Environment

University of York

August 2013

Abstract

Escherichia coli O157:H7 is a human pathogen capable of causing severe disease due to the release of the phage-encoded exotoxin Shiga toxin (Stx). The primary reservoir of *E. coli* O157:H7 is cattle from which the organism is shed asymptotically and colonises specifically at the terminal rectum (TR). Prevalence rates in cattle vary and shedding is transient making determination of transmission routes difficult. This thesis aims to gain further understanding of shedding patterns and transmission of *E. coli* O157:H7 in cattle to inform future control strategies.

Rates of *E. coli* O157:H7 replication in gut contents and exceptional replication rates in TR mucus revealed that passive shedding could explain both low and high faecal counts observed in epidemiological studies and that replication in TR mucus coincides with high rates of attachment to bovine terminal rectum epithelial (BTRE) cells. The rumen was identified as a critical point of control of bacterial numbers which could be exploited in future control strategies which should also consider the potential for passive shedding and environmental replication to maintain *E. coli* O157:H7 populations on farms in the absence of a colonised animal.

Transmission studies showed that while useful for studying colonisation of cattle, Stx⁻ strains of *E. coli* O157:H7 are unable to transmit effectively compared to Stx⁺ strains and are not appropriate for use in experimental transmission studies. Differences observed between shedding and transmission of phage type (PT) 21/28 and PT 32 strains could explain why PT 21/28 is more common in cattle and humans.

Studies of replication and colonisation of the strains from the transmission studies revealed that PT 21/28 is better able to replicate, attach and increase in number on BTRE cells compared to PT 32 and Stx⁻ W3. These advantages to survival and colonisation indicate how PT 21/28 strains could out-compete other strains to persist in cattle populations.

Contents

Abstract	2
List of Tables.....	8
List of Figures	9
Acknowledgements	10
Author's Declaration.....	11
Chapter 1 Introduction	12
1.1 An Introduction to <i>Escherichia coli</i> O157:H7	13
1.1.1 <i>Escherichia coli</i> (<i>E. coli</i>) and its basic properties.....	13
1.1.2 Intestinal Pathotypes of <i>E. coli</i>	13
1.1.3 Human disease and pathogenesis	14
1.1.4 Sources of human infection.....	17
1.2 Cattle as reservoirs of <i>E. coli</i> O157:H7.....	19
1.2.1 Reservoirs of disease.....	19
1.2.2 The bovine gut as a reservoir of <i>E. coli</i> O157:H7.....	20
1.2.3 Prevalence of <i>E. coli</i> O157:H7 in cattle.....	23
1.2.4 Shedding patterns of <i>E. coli</i> O157:H7 in cattle.....	24
1.3 Transmission of <i>E. coli</i> O157:H7 in cattle	26
1.3.1 Routes of disease transmission in animals.....	26
1.3.2 Faecal-oral transmission of <i>E. coli</i> O157:H7 in cattle	28
1.3.3 Experimental studies of transmission of <i>E. coli</i> O157:H7.....	30
1.3.4 The importance of correctly defining colonisation and transmission of <i>E. coli</i> O157:H7 in cattle	32
1.4 The Thesis	34
1.4.1 Aims	34
1.4.2 Thesis structure	34

Chapter 2 Impact of <i>E. coli</i> O157:H7 replication and colonisation in the bovine gut on shedding patterns in cattle	36
2.1 Preface	37
2.2 Introduction	37
2.2.1 Aim.....	39
2.3 Materials and Methods	40
2.3.1 Culture and storage of bacteria	40
2.3.2 Derivation of spontaneous antibiotic-resistant strains	40
2.3.3 Collection and storage of samples.....	40
2.3.4 Isolation of primary bovine terminal rectum epithelial (BTRE) cells .	41
2.3.5 Primary BTRE growth	42
2.3.6 <i>E. coli</i> O157:H7 replication in bovine gut contents, faeces and terminal rectum mucus.....	42
2.3.7 <i>E. coli</i> O157:H7 replication rates in fresh rumen fluid.....	43
2.3.8 Estimation of potential challenge dose at terminal rectum and rates of passive shedding	44
2.3.9 Attachment rates on primary BTRE cells from mucus	44
2.3.10 Progression of colonisation on primary BTRE cells.....	45
2.3.11 Statistical analysis	46
2.4 Results	47
2.4.1 <i>E. coli</i> O157:H7 survival and replication in the bovine gut.....	47
2.4.2 Investigation of <i>E. coli</i> O157:H7 growth in fresh rumen contents	48
2.4.3 Estimation of potential attachment dose at terminal rectum and rates of passive shedding	50
2.4.4 Rates of <i>E. coli</i> O157:H7 attachment to BTRE cells.....	51
2.4.5 Progression of <i>E. coli</i> O157:H7 colonisation on BTRE cells	51
2.5 Discussion	53

Chapter 4 Rates of replication and colonisation in the bovine gut of <i>E. coli</i> O157:H7 strains with and without Stx.....	77
4.1 Preface	78
4.2 Introduction	78
4.2.1 Aims	80
4.3 Methods	81
4.3.1 Culture and storage of bacteria	81
4.3.2 Collection and storage of samples.....	81
4.3.3 Survival and replication in the rumen of <i>E. coli</i> O157:H7 strains with or without Stx bacteriophage	82
4.3.4 Replication rates of <i>E. coli</i> O157:H7 strains with and without Stx bacteriophage in terminal rectum mucus	82
4.3.5 Attachment rates of Stx ⁻ and Stx ⁺ <i>E. coli</i> O157:H7 strains from mucus	83
4.3.6 Progression of colonisation of Stx ⁻ and Stx ⁺ <i>E. coli</i> O157:H7 strains following attachment to primary BTRE cells	84
4.3.7 Statistical analysis	84
4.4 Results	86
4.4.1 Survival and replication of Stx ⁺ and Stx ⁻ <i>E. coli</i> O157:H7 strains in fresh rumen contents	86
4.4.2 Replication rates of <i>E. coli</i> O157:H7 strains in terminal rectum mucus..	87
4.4.3 Attachment rates of <i>E. coli</i> O157:H7 strains on BTRE cells from mucus	90
4.4.4 Progression of colonisation of <i>E. coli</i> O157:H7 strains on BTRE cells	91
4.5 Discussion	93

Chapter 5 General Discussion.....	96
5.1 Summary of thesis aims	97
5.2 Role of passive and active shedding in understanding <i>E. coli</i> O157:H7 epidemiology	97
5.3 Implications of this work for future research and possibilities for control .	99
5.4 Conclusions	102
Abbreviations	104
References	106

List of Tables

Chapter 1

Table 1.1 Experimental <i>E. coli</i> O157:H7 transmission studies	31
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Chapter 2

Table 2.1 <i>E. coli</i> O157:H7 strains used in Chapter 2.....	40
Table 2.2 Generation rate and doubling time of <i>E. coli</i> O157:H7 in different gut locations	47
Table 2.3. <i>E. coli</i> O157:H7 generation rate and doubling time in rumen contents....	49
Table 2.4 Calculation of potential counts of <i>E. coli</i> O157:H7 in different gut compartments based on replication rates.	50
Table 2.5. Potential <i>E. coli</i> O157:H7 faecal output/challenge at TR due to passive shedding.	51

Chapter 3

Table 3.1 <i>E. coli</i> O157:H7 strains used in Chapter 3.....	60
Table 3.2 Primers used in this study	61

Chapter 4

Table 4.1 <i>E. coli</i> O157:H7 strains used in Chapter 4.....	81
Table 4.2. Generation rate and doubling time of phage types 21/28 and 32 and W3 strains in mucus.....	88
Table 4.3. Generation rate and doubling time of W1, W3 Stx ⁻ and W3 Stx ⁺ strains in mucus.	90
Table 4.4 Generation rate and doubling time of <i>E. coli</i> O157:H7 strains once attached.	92

List of Figures

Chapter 2

Figure 2-1. <i>E. coli</i> O157:H7 replication in gut contents and mucus.....	47
Figure 2-2. <i>E. coli</i> O157:H7 24 hour counts in mucus	48
Figure 2-3. <i>E. coli</i> O157:H7 replication in fresh and stored rumen contents	49
Figure 2-4. <i>E. coli</i> O157:H7 attachment to BTRE cells from mucus	51
Figure 2-5. Progression of <i>E. coli</i> O157:H7 colonisation on BTRE cells	52

Chapter 3

Figure 3-1 Faecal counts and environmental levels of <i>E. coli</i> O157:H7 Pen 1	69
Figure 3-2 Faecal counts and environmental levels of <i>E. coli</i> O157:H7 Pen 2	70
Figure 3-3 Daily faecal counts of PT 21/28 <i>E. coli</i> O157:H7 in Room 1 calves.....	72
Figure 3-4 Daily faecal counts of PT 32 <i>E. coli</i> O157:H7 in Room 2 calves.....	72
Figure 3-5 Comparison of AUCs and peak counts of calves by PT and challenge type	73

Chapter 4

Figure 4-1 Growth of Stx ⁺ and Stx ⁻ <i>E. coli</i> O157:H7 strains in fresh rumen fluid with and without protozoa 1	86
Figure 4-2 Growth of Stx ⁺ and Stx ⁻ <i>E. coli</i> O157:H7 strains in fresh rumen fluid with and without protozoa 2.....	87
Figure 4-3 Replication rates of PT 21/28, PT 32 and W3 strains in mucus.....	88
Figure 4-4 Mean 24 hour counts for PT 21/28, PT 32 and W3 in mucus.....	89
Figure 4-5. Replication of W1, W3 Stx ⁻ and W3 Stx ⁺ strains in mucus	89
Figure 4-6. Mean 24 hour counts of W1, W3 Stx ⁻ and W3 Stx ⁺ in mucus	90
Figure 4-7 Rates of attachment of PT 21/28, PT 32 and W3 strains to BTRE cells..	91
Figure 4-8 Progression of PT 21/28, PT 32 and W3 colonisation on BTRE cells.....	92

Acknowledgements

Firstly I would like to thank my supervisors Mike Hutchings, Piran White and Chris Low for their cheerful and down-to-earth guidance and for buoying me up when I felt like I was sinking. Thank you also to David Gally, Tom McNeilly and Arvind Mahajan for their ‘unofficial’ supervision and for sharing their time and expertise with me in this project.

I gratefully acknowledge The University of York, Scotland’s Rural College and the different laboratories at the ERI, the Roslin Institute, Moredun and Moredun Scientific which have hosted me for my research and DEFRA for funding it.

Particular thanks goes to Sean McAteer and Edith Paxton for answering all my lab-based queries and for running PCRs, isolating strains and harvesting and feeding BTRE cells among other things. I would also like to thank Chris Low, Tom McNeilly, and especially Dave Anderson and Alex Corbishley and the Moredun farm staff for their help with animal studies. Thanks are also due to John Wallace, Bert Tolcamp and Jan Dijkstra for their advice on rumen protozoa and to Ian Nevison and Iain McKendrick at BIOSS for their advice on statistics.

Thank you also to all the wonderful people I have met in the various labs and offices over the past few years for the help and advice, coffee breaks, lab banter and general hilarity. In particular thank you to Liza who has been there through the ups and downs with advice, tea and many fun times and who pointed out that ‘theses’ rhymes with ‘faeces’; an apt observation given our PhD topics.

Special thanks are owed to my friends and family for their love and support during the past 4 years particularly my parents who have put up with all my years of studying and stood by me through it all. Particular mention also goes to my uncle Murray and my gran Agnes, both of whom did not live to see me finish my PhD but who were, I know, proud of my achievements.

Finally I owe my deepest thanks to my husband Fraser who has been there with me from the beginning, from negotiating flat buying and wedding planning during the first year of my PhD to feeding me and making me tea while I write up; always supporting me, looking after me and making me laugh.

Author's Declaration

I declare that the work contained in this thesis is my own and has not been submitted for any other degree or award.

Kirsty J. Hughes

Chapter 1

Introduction

1.1 An Introduction to *Escherichia coli* O157:H7

1.1.1 *Escherichia coli* (*E. coli*) and its basic properties

Escherichia coli is a facultative anaerobic bacterium which typically colonises the gastrointestinal tract of humans and animals within hours of birth to mutual benefit. In the healthy host, the majority of *E. coli* strains are non-pathogenic and form a substantial part of the host's commensal population in the gut where they typically colonise the mucous layer of the colon (Kaper et al, 2004). Due to changes in the physiological state of the host such as immunosuppression however, some *E. coli* strains can cause opportunistic infections of the host. There are also some highly adapted strains that are inherently pathogenic and cause severe disease typically in the form of gastrointestinal disease, urinary tract infections or sepsis/meningitis. As well as being adapted to specific hosts *E. coli* can also survive for extended periods in the environment and has been recovered from soil and water sources (Winfield and Groisman, 2003).

E. coli are Gram-negative rod-shaped bacteria within the family *Enterobacteriaceae*. They can be grown readily on general or selective agar at 37°C in aerobic conditions on which they form circular non-pigmented colonies that produce indole. They are generally motile, non acid-fast, do not form spores, are oxidase negative and catalase positive and can reduce nitrate to nitrite. Identification of specific strains of *E. coli* has been principally based on serotyping according to their O (somatic), H (flagellar) and and/or K (capsular) and F (fimbriae) antigen profiles (Nataro and Kaper, 1998). There are also a number of other molecular typing methods to distinguish between different *E. coli* strains including PCR (polymerase chain reaction) and PFGE (pulsed field gel electrophoresis).

1.1.2 Intestinal Pathotypes of *E. coli*

Pathogenic *E. coli* strains have acquired various virulence factors, frequently from mobile genetic elements, which allow them to cause disease. They are mucosal pathogens which are adept at overcoming host defences and cause disease through colonisation of host mucosa and damage to host tissues. A variety of combinations of expressed or secreted virulence factors have been described which cause a number of distinct pathologies (Nataro and Kaper, 1998). There are six major human intestinal pathotypes of *E. coli* as defined by the pathology they cause and their

interactions with eukaryotic cells: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC). An extra-intestinal pathotype (ExPEC) has also been described for strains that cause urinary disease (uropathogenic *E. coli*, UPEC) and meningitis and sepsis (meningitis-associated *E. coli*, MNEC) (Kaper et al, 2004). Small intestinal pathotypes include EPEC which adhere tightly to enterocytes via attaching and effacing lesions damaging the microvilli leading to inflammation; ETEC which also adheres to enterocytes and secretes heat-labile (LT) and/or heat-stable (ST) enterotoxins and DAEC which adhere diffusely to enterocytes and cause elongation of the microvilli (Kaper et al, 2004). Large intestinal pathotypes include EHEC which attaches in the colon creating attaching and effacing lesions like EPEC but in addition secreting Shiga toxin (Stx). These pathotypes also include EIEC strains that invade colonic epithelia and can move laterally through the epithelium, and EAEC, that adhere to epithelia in both the small and large intestine aggregating in a thick biofilm and secreting enterotoxins and cytotoxins (Kaper et al, 2004).

All the intestinal pathotypes cause watery diarrhoea, often in young infants and children and the severity of disease varies with host factors and the combinations of virulence factors of the strain involved. The watery diarrhoea of EHEC can progress to haemorrhagic colitis and potential systemic complications due to the action of Stx. Of the EHEC pathotypes, the most common serotype is *E. coli* O157:H7, which is a significant pathogen of humans (Kaper et al, 2004).

1.1.3 Human disease and pathogenesis

E. coli O157:H7 was first associated with human disease in the 1980s when it was linked to haemorrhagic colitis and then to haemolytic uraemic syndrome (HUS), (Karmali et al, 1983; Riley et al, 1983). This particular serotype had not been previously linked to human disease (Besser et al, 1999) but since then *E. coli* O157:H7 has been increasingly implicated in sporadic cases of human diarrhoeal disease, as well as in major outbreaks in up to 30 countries including the UK, North America and Japan (Besser et al, 1999; Kaper et al, 2004). There are around 1000 cases per year in the UK and 73,000 cases and 60 deaths in the U.S.A. though many more cases may go unreported (Callaway et al, 2009; DEFRA, 2011). Scotland has

one of the highest incidences of human disease at an average of 4.6 cases per 100,000 people, much higher than the prevalence in England and Wales where only 1.35 and 1.1 cases in 100,000 are seen respectively (Chase-Topping et al, 2007). The high mean prevalence in Scotland is skewed by high prevalence in certain areas however with the highest annual prevalence in Grampian at 8 – 10 cases per 100,000 (Locking et al, 2006; Strachan et al, 2006). Cases of disease in humans are also seasonal with the majority occurring between June and September (Besser et al, 1999).

It is estimated that ingestion of less than 100 *E. coli* O157:H7 bacteria may be sufficient to cause human infection and can result in a range of symptoms. The incubation period is 3 - 4 days and initial symptoms include watery diarrhoea with abdominal cramps which can progress to haemorrhagic colitis with fresh blood in stools on the 2nd or 3rd day. The infection normally resolves itself after 1 week but in more susceptible people including children under 5, the elderly and the immunocompromised it can progress to more severe disease in the form of haemolytic uraemic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP). HUS, comprising haemolytic anaemia, acute kidney failure and thrombocytopenia, develops in 10-15 % of cases (Tarr et al, 2005) primarily children, and can be fatal. TTP is similar to HUS but generally does not include diarrhoea and is more common in adults (Besser et al, 1999; Pennington, 2010). Though relatively uncommon in incidence, the potential severity of this pathogen, especially for children, makes *E. coli* O157:H7 a major concern for the general public and the UK health authorities, both in terms of public health and the costs of treating and investigating cases. It is estimated that a single outbreak in the UK may cost upwards of £15 million, with additional legal settlements to affected people (Pennington, 2010).

The pathogenesis of *E. coli* O157:H7 in humans is driven by a number of encoded factors which facilitate attachment and persistence of the bacteria, of which the phage-encoded Stx is key (Pennington, 2010). The toxin is transported from the intestine into the blood stream where it binds to the glycolipid globotriaosylceramide (Gb3) receptor on the surface of vascular cells in different tissues including the intestine, kidney and nervous system (Engedal et al, 2011; Smith et al, 2002). Once the attached Stx is internalised, it cleaves ribosomal RNA which prevents protein

synthesis leading to cell death and consequential haemorrhage (Kaper et al, 2004). There are two main antigenically distinct types of Shiga toxin; Stx1, which is closely related to the Shiga toxin from *Shigella dysenteriae*, and Stx2 which shares 50-60 % homology with Stx 1 and of which there are a number of subvariations, named Stx2a-h (Chibani-Chennoufi et al, 2004; Fuller et al, 2011; Pennington, 2010). Each Stx toxin type is encoded by different lambdoid bacteriophages inserted as prophages into the host bacterial chromosome and different strains can have more than one Stx-encoding phage allowing for multiple combinations and differing levels of virulence (Mauro and Koudelka, 2011).

Bacteriophages are bacterial viruses which are found wherever there is abundance of bacteria such as in aquatic and terrestrial environments or the intestinal tracts of humans and animals (Chibani-Chennoufi et al, 2004). Stx-encoding lambdoid bacteriophages have been found in urban and municipal wastewater, river and beach water and human and bovine faeces with an estimated 1-10/ml of Stx2-encoding phage in sewage (Muniesa and Jofre, 1998). When a bacteriophage infects a bacterial cell, the phage can either go into a lytic cycle to produce more phage which lyses the host cell releasing the fresh phage or alternatively insert its genome into the bacterial chromosome so as to be replicated along with the host. Lysis provides the short-term advantage of producing more phage to infect further hosts but as release of fresh phage kills the host cell, there needs to be a large enough population of host cells to sustain this cycle (Chibani-Chennoufi et al, 2004). Instead lysogeny provides a more long-term approach to survival as the prophage is replicated along with the host genome whilst retaining the ability to produce new phage to infect further hosts if the current host is damaged (Campbell, 1994). Just as lysogeny provides an advantage to Stx phages allowing them to replicate along with the host, it can also help the host bacteria as lysogenic conversion of prophage genes provides bacteria a selective advantage through enhanced immunity and metabolism and faster and longer growth (Chibani-Chennoufi et al, 2004). It can also make bacteria competitive in specific environments or niches through provision of bacterial virulence factors (Chibani-Chennoufi et al, 2004). The lytic phase of growth can then still be induced either spontaneously or due to bacterial stress which triggers the SOS response, lysing the cell to release infectious phage and toxins like Stx (Mauro and Koudelka, 2011).

A large number of different phage types (PT) of *E. coli* O157:H7 have been described and these have also been classified into one of 9 clades based on their genetic origins and relatedness (Manning et al, 2008). Recent work in the U.S.A. reported an increasing incidence of clade 8 strains in human disease which are more virulent and more likely to have both Stx2 and Stx2c prophages than strains in other clades (Manning et al, 2008). *E. coli* O157:H7 strains isolated from humans tend to produce the more virulent Stx2, which is 1000 times more toxic to human kidney cells than Stx1 in purified form (Baker et al, 2007; Ritchie et al, 2003).

Apart from its toxicity, Stx2 has also been shown to play a role in adherence to intestinal epithelium by increasing the expression of nucleolin receptors for intimin on the surface of eukaryotic cells (Robinson et al, 2006). During human infection, H7 flagella of *E. coli* O157:H7 are involved in initial adherence to host cells which is then superseded by more intimate attachment through the type III secretion system (T3SS) encoded by the LEE pathogenicity island (Mahajan et al, 2009; Tree et al, 2009). The T3SS allows the bacterium to inject a receptor for itself (the translocated intimin receptor; Tir) into the host cell which then migrates to the surface where it binds to intimin on the bacterial cell surface to form close attachment to the host cell (Roe et al, 2003). Stx up-regulation of nucleolin also occurs where Stx is present as an intermediate step between flagellar and T3SS mediated attachment (Robinson et al, 2006). Re-arrangement of the host actin to assist binding leads to the characteristic attaching and effacing (A/E) lesion and disruption of the surface microvilli (Kaper et al, 2004; Pennington, 2010). *E. coli* O157:H7 strains also contain a large plasmid (pO157) which encodes enterohaemolysin and a number of other putative virulence factors including an *E. coli* secreted protein (EspP) (Dziva et al, 2007; Nataro and Kaper, 1998).

1.1.4 Sources of human infection

Humans are considered to be an incidental host of *E. coli* O157:H7, although infection may also be spread between cases due to the low infectious dose (Kaper et al, 2004). Cattle and sheep have been most often linked to human disease and cattle in particular are considered the primary reservoir host (Caprioli et al, 2005; Pennington, 2010). Early outbreaks of *E. coli* O157:H7 were traced to undercooked ground beef and consumption of unpasteurised milk and the majority of *E. coli*

O157:H7 outbreaks are traced to cattle products or vegetable products contaminated with cattle waste (Borczyk et al, 1987; Chapman et al, 1997; Riley et al, 1983). Between 1982 and 2002 there were 350 reported outbreaks in the U.S.A. and the sources of infection included: foodborne from ground beef or produce, person-person spread, waterborne contamination and animal contact (Rangel et al, 2005). Clustering of human cases occurs in areas with highest cattle density and there is a significant association between the numbers of human cases and the ratio of beef cattle to humans (Chase-Topping et al, 2008; Valcour et al, 2002). This clustering is evident in Scotland where the majority of cases are seen in the Grampian area where there is a high beef cattle to human ratio and the risk of human infection is higher in rural areas than urban areas (Strachan et al, 2006). Outbreaks of *E. coli* O157:H7 have also been associated with other food products due to contamination with animal manure including: fresh-pressed apple cider, yoghurt, and vegetables like lettuce, radish sprouts, alfalfa sprouts, and tomatoes (Kaper et al, 2004).

In an effort to reduce risk of foodborne transmission to humans, tighter regulations for slaughter practices and food preparation aimed at reducing cross-contamination have been introduced in a number of countries including the U.S.A. and the UK. These regulations involve screening and recalling contaminated meat and other products at considerable cost to the industries involved (Pennington, 2010) but only target foodborne transmission and are unlikely to reduce the risk of infection from animal contact or the environment (Strachan et al, 2006). While foodborne outbreaks can affect large numbers of people at once and tend to be high profile they actually account for a low percentage of individual cases of *E. coli* O157:H7 in the UK (Locking et al, 2006). In Scotland it is estimated that only 40% of human cases are foodborne while 54% are from environmental sources including animal contact (O'Brien and Adak, 2002; Strachan et al, 2006). One outbreak in Scotland was caused by environmental exposure to contaminated sheep faeces at a Scout camp (Howie et al, 2003) and a number of other outbreaks across the UK have resulted from direct contact or environmental exposure including in recent years in children who had visited petting farms (Ihekweazu et al, 2012). These outbreaks have increased the public profile of *E. coli* O157:H7 in the UK as not only a foodborne pathogen but also an environmental one and increased the impetus to control the organism in the animal reservoir.

1.2 Cattle as reservoirs of *E. coli* O157:H7

1.2.1 Reservoirs of disease

A reservoir of disease is an environment or population in which a disease-causing organism persists indefinitely (Ashford, 1997) and there are a number of ways in which it can do this. Some pathogens can survive in the environment, either in soil or water and may use free-living stages or form spores which help them to persist and remain infectious for long periods (Boom and Sheath, 2008; Phillips et al, 2003). Others can cause latent infection, have long incubation periods or infect vector species via which they can transmit to the target host (Haydon et al, 2002; Judge et al, 2006). Animal populations can also be reservoirs of infection and pathogens can be maintained in a single host species or a combination of hosts and environments (Haydon et al, 2002). Bovine tuberculosis caused by *Mycobacterium bovis* is one pathogen for which at least two main animal species, cattle and badgers, appear to be reservoirs involved in maintenance of the disease (Phillips et al, 2003).

Pathogens that are able to persist in the environment or within a host population have a selective advantage and *E. coli* O157:H7 is capable of both. It can persist in a range of conditions in the environment including water sources and soil for up to 91 and 105 days respectively whilst still remaining viable (Ogden et al, 2002; Wang and Doyle, 1998). Counts of *E. coli* O157:H7 can also increase in bovine faeces for 2 days at 37 °C and 3 days at 22 °C and it can survive for over 40 days at those temperatures and for up to 70 days at 5 °C (Wang et al, 1996). *E. coli* O157:H7 can replicate and remain viable in treated and untreated wastes from sewage works, abattoirs, dairies and creameries for at least 2 months (Avery et al, 2005). Long-term storage of these products reduces, but does not eliminate, the bacterial load therefore opening up the potential to contaminate the environment if any of these are spread on land as fertiliser (Avery et al, 2005).

Cattle are considered the primary reservoir host of *E. coli* O157:H7, due to the association with human disease, and the organism has been shown to have a specific tropism for adherence to the mucosal epithelium at the terminal rectum of cattle (Naylor et al, 2003). *E. coli* O157:H7 has also been isolated from a large variety of other species including: sheep, goats, pigs, deer, rabbits, dogs, cats, rodents and wild birds such as pigeons and gulls, all of which could contribute to maintenance and

transmission of the organism in the farm environment (Hogg et al, 2009; La Ragione et al, 2009; Nielsen et al, 2004; Shere et al, 1998). The ability of *E. coli* O157:H7 to survive and replicate in faeces and the environment as well as a number of host species, including cattle, presents the possibility for it to persist through passive shedding by in-contact animals and environmental replication without the need for a specific host.

1.2.2 The bovine gut as a reservoir of *E. coli* O157:H7

The bovine gut could act as a reservoir for *E. coli* O157:H7 both through passive transit and colonisation. Levels of shedding of *E. coli* O157:H7 by cattle will be a function of dose ingested, survival during gut transit and rates of colonisation at the terminal rectum and survival and replication in the gut will have a large impact on both passive shedding and the chances of colonisation. The following paragraphs present a summary of some of the many studies of *E. coli* O157:H7 survival in the bovine gut, which serve to highlight the variation that occurs in biological processes. Many theories have been proposed and refuted that certain conditions or diets are responsible for increased shedding and the search is still ongoing for the main determinants of *E. coli* O157:H7 survival in the gut.

Up to 90% of digesta transit time in the bovine gut is spent in the rumen (Huhtanen et al, 2008) and it could be argued that survival and passage through this compartment presents the greatest challenge for *E. coli* O157:H7 in the host. Early studies suggested that the rumen was a primary site of colonisation of *E. coli* O157:H7 in calves (Brown et al, 1997; Dean-Nystrom et al, 1997) and it has been isolated from both the rumen and abomasum at slaughter and several days after experimental inoculation (Cray and Moon, 1995; Laven et al, 2003). Grauke et al (2002) found that *E. coli* O157:H7 levels in the rumen and duodenum decreased rapidly after ruminal challenge in cannulated steers, isolating it in duodenal contents 1 hour and in faeces 6 hours after challenge. *In vitro* studies by the same group measured an increase of 1 Log₁₀ C.F.U. over 6 hours in rumen fluid and survival in rumen contents for over 10 hours has also been recorded (Chaucheyras-Durand et al, 2010). A number of studies have looked at the factors which affect *E. coli* O157:H7 proliferation in the rumen as this site could be a potential target for control through manipulation of the rumen environment with diet or drug treatment (Fox et al, 2009;

Stanford et al, 2010; Thran et al, 2003; Zhao et al, 1998). High levels of volatile fatty acids (VFA) in the rumen of well-fed animals have been linked to suppression of *E. coli* O157:H7 growth in the rumen of cattle (Rasmussen et al, 1993) and the decline of 2 Log₁₀ over 24 hours in rumen fluid seen by Thran et al. (2003) was independent of pH and instead attributed to competitive exclusion by other micro-organisms. These studies and others suggest that a variety of rumen conditions may affect *E. coli* O157:H7 survival in the bovine gut and that further study of this complex organ is merited.

Acidic conditions in the abomasum and human stomach have both been purported to have an inhibitory effect on *E. coli* O157:H7 in cattle and perhaps in response to this *E. coli* O157:H7 strains often show a high level of acid resistance (Arnold and Kaspar, 1995; Chaucheyras-Durand et al, 2010). Acid-resistant strains can persist for 4 or more hours in abomasal fluid while acid-susceptible strains are undetectable after 1.5 hours in abomasal fluid (Chaucheyras-Durand et al, 2010). Survival in the small intestine also appears to be variable as *E. coli* O157:H7 counts in duodenal fluid decreased by half a Log₁₀ C.F.U. in 6 hours in one study (Grauke et al, 2002) and increased by 1 Log₁₀ in jejunal content over 2 hours and in caecal contents by 2 Log₁₀ over 8 hours in another (Chaucheyras-Durand et al, 2010).

E. coli O157:H7 is most often isolated from the contents of the caecum or colon rather than upper GI sites however and its ability to replicate in faeces along with its specific tropism for attachment at the terminal rectum suggests that conditions are more favourable for the bacteria in the large intestine as seen in humans (Cray and Moon, 1995; Diez-Gonzalez et al, 1998; Grauke et al, 2002; Laven et al, 2003; Naylor et al, 2003; Van Baale et al, 2004; Wang et al, 1996). Colonisation of *E. coli* O157:H7 at the bovine terminal rectum is typified by large numbers of bacteria closely adhered through attaching and effacing (A/E) lesions on the mucosa up to 5 cm from the recto-anal junction (Naylor et al, 2003). Although A/E lesions have occasionally been found in other parts of the gut suggesting that *E. coli* O157:H7 is capable of low level colonisation in other locations, they are far more common in the terminal rectum (Brown et al, 1997; Dean-Nystrom et al, 1997; Naylor et al, 2003). Cattle that are colonised with *E. coli* O157:H7 are asymptomatic and appear less sensitive to the toxic effects of Stx, due at least in part to the different distribution of Gb3 receptors between cattle and humans (Smith et al, 2002). In cattle, Gb3

receptors are located mainly on intestinal epithelial cells and not endothelium where the local action of Stx enhances expression of receptors for intimin and aids attachment (Naylor et al, 2005; Robinson et al, 2006). Stx is not essential for colonisation of cattle, unlike intimin which is required to form intimate attachment with the host cell (Cornick et al, 2002; Sheng et al, 2006b). A number of other virulence factors are also important for colonisation, including the pO157 plasmid which encodes factors like the secreted protease EspP (Bai et al, 2011; Bridger et al, 2010; Dziva et al, 2007; Kudva and Dean-Nystrom, 2011; Mahajan et al, 2009). Although cattle which are colonised with *E. coli* O157:H7 are asymptomatic, they do experience low level pathology and inflammation at the site of colonisation in the terminal rectum which leads to a temporary immune response (Nart et al, 2008),

A number of reasons have been proposed for the *E. coli* O157:H7's tropism for this particular niche which has not been demonstrated in other bacteria (Naylor et al, 2003). The tissue of the terminal rectum has a high concentration of lymphoid follicles, similar to Peyer's patches in other parts of the gut, and *E. coli* O157:H7 has been found attached to both absorptive epithelium and follicle-associated epithelium at the terminal rectum (Nart et al, 2008; Naylor et al, 2003). Other pathogenic species such as *Salmonella typhimurium* are known to have a tropism for Peyer's patches (Jensen et al, 1998), which also seem to be a target for *E. coli* O157:H7 in humans (Phillips et al, 2000). The physical proximity to the host cells at the terminal rectum has also been proposed as a reason for *E. coli* O157:H7's tropism for the particular site due to the thinner protective mucous layer and the potential for close contact to the cell wall for attachment during defecation (Nart et al, 2008; Roe et al, 2003). *E. coli* O157:H7 flagella have been shown to bind to bovine colonic mucus (Erdem et al, 2007) and cell contact induces the LEE and Tir *in vitro* (Roe et al, 2003). Another possible reason may be the ability of *E. coli* O157:H7 to make use of nutrients at that site as blocking of *E. coli* O157:H7's ability to utilise nutritional components of mucus reduces shedding in cattle (Snider et al, 2009), and terminal rectum mucus and mucus components stimulate *E. coli* O157:H7 growth (Bai et al, 2011; Fox et al, 2009).

1.2.3 Prevalence of *E. coli* O157:H7 in cattle

Accurate estimates of *E. coli* O157:H7 prevalence in cattle are difficult to obtain because shedding is transient and shedding cattle show no clinical signs meaning active faecal sampling is necessary to identify positive farms (Bach et al, 2002). A vast number of studies have collected either single-point or longitudinal data to measure prevalence rates in cattle in different settings and countries (Duffy, 2003). It is difficult to compare across prevalence studies however due to differences in sampling and enumeration techniques and estimates of *E. coli* O157:H7 prevalence vary widely. Slaughter studies have identified shedding in 4.7 – 15.7 % of individual cattle in the UK and up to 28 % of cattle in the U.S.A. (Chapman et al, 1997; Gansheroff and O'Brien, 2000; Low et al, 2005; Omisakin et al, 2003; Paiba et al, 2002), while recorded prevalence rates for UK farms vary from 4.2 - 8.6 % of cattle and 23 - 100 % of farms (Gunn et al, 2007; Gyles, 2007; Paiba et al, 2003; Smith et al, 2002). In fact serological studies suggest that the majority of cattle herds have been exposed to *E. coli* O157:H7 at some point and farms frequently change status from positive to negative or vice versa between samplings (Renter and Sargeant, 2002).

Apart from differences in study design there are a number of potential reasons for the variation in reported prevalence rates. As in humans, *E. coli* O157:H7 shedding in cattle tends to be seasonal with prevalence highest in spring and late summer or autumn, although year-round shedding has also been identified (Chapman et al, 1997; Lahti et al, 2003; Smith et al, 2010). This seasonality could be explained by the predominance of warmer, wetter weather providing optimal conditions for *E. coli* O157:H7 growth in the environment or because these times represent periods of increased animal movement between housing and pasture and changes in diet which may lead to stress (Gunn et al, 2007; Vidovic et al, 2007). Several risk factors have been identified for farms in Scotland being positive for *E. coli* O157:H7 which include cattle stress along with: large numbers of finishing cattle, pigs on the farm, younger ages of cattle and female breeding cattle (Chase-Topping et al, 2007; Gunn et al, 2007). Young weaned calves shed at higher levels for longer than adult cattle in experimental studies, which could be due to dietary stress during weaning and because they do not yet have a fully formed rumen to help suppress *E. coli* O157:H7 growth (Cray and Moon, 1995). Finishing cattle and female breeding cattle are also

often under dietary stress and finishing cattle are normally housed in mixed groups from multiple farms which could lead to increased transmission (Gunn et al, 2007). Finally, although pigs are rarely found to be positive in prevalence studies, they can become persistently infected with *E. coli* O157:H7 for up to 2 months and effectively transmit by aerosol to naïve animals and so could potentially play a role in transmission to other species on farms (Booher et al, 2002; Cornick and VuKhac, 2008).

The reasons for regional variation in prevalence of *E. coli* O157:H7 such as in seen in Scotland are still largely unknown but could be due to some as yet unconfirmed environmental or wildlife reservoir (Renter and Sargeant, 2002). Specific *E. coli* O157:H7 clonal types or phage types (PT) have been found to persist over time in different farming systems in a number of countries suggesting a role for environmental sources in maintenance of *E. coli* O157:H7 populations (Lahti et al, 2003; LeJeune et al, 2004; Shere et al, 1998; Van Donkersgoed et al, 2001). In the 1990s in the UK 58 % of human and 18.5 % of cattle isolates were PT 2 but since the early 2000s the most common phage type in humans and cattle is PT 21/28 (Allison et al, 2000; Lynn et al, 2005). In Scotland, the two most common phage types in cattle are PT 21/28 and PT 32 accounting for 46 % and 19 % of cattle isolates, respectively (Chase-Topping et al, 2007; Pearce et al, 2009). PT 21/28 is associated with a much higher proportion of high-shedding animals than PT 32 and has therefore been predicted to have a basic reproduction number (the number of naïve individuals an infectious animal can infect; R_0) in cattle three times that of PT 32 (Matthews et al, 2009). These differences could be due to more successful passive shedding or colonisation and greater understanding of these two phenomena with different strains could help to establish why certain strains are more prevalent.

1.2.4 Shedding patterns of *E. coli* O157:H7 in cattle

Though prevalence studies provide an estimate of how common *E. coli* O157:H7 is in the cattle population, they do not generally provide information on shedding patterns. Experimental and some longitudinal studies instead provide insight into the duration and level of shedding of animals exposed to *E. coli* O157:H7. Following experimental challenge with 10^9 - 10^{10} C.F.U. a typical shedding curve sees a peak of faecal shedding at around 6 - 7 days post challenge followed by lower level

shedding for anything from a few days to a number of weeks in colonised animals (Naylor et al, 2003; Robinson et al, 2004). Although there is some variation in methodology, the majority of studies report durations of *E. coli* O157:H7 shedding between 1 and 2 months with occasional animals shedding for longer and all animals eventually stop shedding at detectable levels (Cornick et al, 2002; Cray and Moon, 1995; Rice et al, 2003; Sanderson et al, 1999). The reduction in shedding and eventual clearance is thought to be due to the immune response mobilising to remove attached bacteria and modelling by Tildesley et al. (2012) suggests that immune-mediated changes in rates of replication in terminal rectal mucus and mucosal attachment are important in clearing the infection. Both mucosal and systemic antibodies to *E. coli* O157:H7 have been detected in animals shedding *E. coli* O157:H7 and short-term immunity to re-infection of up to 3 weeks experimentally demonstrated in calves (Johnson et al, 1996; Naylor et al, 2007a). Long-term immunity is not achieved however and animals which have previously cleared the infection can later begin shedding again (Shere et al, 1998). As well as colonisation leading to persistent shedding over a period of weeks or months followed by clearance, intermittent faecal shedding is also observed in some animals, which could be due to repeat exposure to small doses and passive shedding (Lahti et al, 2003; Shere et al, 1998; Smith et al, 2010).

Studies that report on magnitude of shedding have found large variation both within groups and within animals highlighting the need to understand why some animals shed at higher levels than others. Within groups of positive animals and within individual positive animals the concentration of *E. coli* O157:H7 in faeces can range from $<10 - 10^6$ C.F.U./g between sampling occasions (Shere et al, 1998). A typical pattern within groups is for the majority of positive animals to shed low levels of bacteria ($<10^2$ C.F.U./g faeces) while only a small proportion (3 – 9 %) of animals shed high numbers (Cobbald et al, 2007; Gally et al, 2003; Low et al, 2005; Naylor et al, 2005; Omisakin et al, 2003). Slaughter studies have found cattle that are colonised at the terminal rectum also tend to have higher levels of *E. coli* O157:H7 in their faeces, usually $>10^3$ C.F.U./g, compared to animals which are not colonised (Low et al, 2005; Omisakin et al, 2003). This has led to high-shedding animals being defined as colonised ‘super-shedders’ and shedding above 10^3 C.F.U./g has been considered a measure of colonisation (Naylor et al, 2003). A more recent definition

of colonisation proposed that animals shedding $>10^4$ C.F.U./g in faeces and animals that persistently shed *E. coli* O157:H7 over a period of weeks can be considered 'super-shedders' (Chase-Topping et al, 2008; Davis et al, 2006). Modelling studies have predicted that super-shedders pose the majority of the risk for transmission on farms (Matthews et al, 2006a). These studies identify super-shedders as key determinants in transmission and assume that high-level shedding and colonisation are synonymous yet few experimental and field studies have actually measured the potential for high-level passive shedding without colonisation. Additionally, most studies of super-shedding have not actively confirmed the presence of *E. coli* O157:H7 on the terminal rectum mucosa of high-shedding animals and instead use positive rectal swab samples and high-level shedding as proof of colonisation (Cobbold et al, 2007; Davis et al, 2006; Rice et al, 2003).

Current definitions of colonisation do not account for the variation in faecal counts that may occur; for example in a recently colonised animal where lower bacterial numbers may be considered likely or when an animal is developing an immune response and is reaching the end of its shedding cycle. This has been observed in one study using rectal swabs where some animals were culture positive from rectal swabs at the same time as their faeces were negative suggesting that faecal sampling alone may miss low-level colonisation and misclassify an animal as not colonised (Rice et al, 2003). Conversely, if an animal ingested a high enough dose of *E. coli* O157:H7 which then replicated in the gut, a single sample occasion could classify a passive shedding animal as colonised. Low-level shedders tend to be identified in pens with high-shedding animal and negative animals are more often found in pens without a high-shedding animal which does suggest a role for at least low-level passive shedding (Cobbold et al, 2007; Stephens et al, 2009). Further experimental work is required to determine the potential for passive shedding and rates of colonisation of *E. coli* O157:H7 and whether passive shedding can account for high-level shedding.

1.3 Transmission of *E. coli* O157:H7 in cattle

1.3.1 Routes of disease transmission in animals

In order to persist in a population, a pathogen must be able to survive in the host and/or the environment long-term and have the means to transmit to its target host. There are two broad categories for describing the ways in which pathogens are

transmitted between hosts; vertical and horizontal. Vertical transmission utilises the reproductive physiology of the host to directly transmit pathogens to the offspring in utero such as occurs in Bovine Virus Diarrhoea (BVD) leading to persistently infected animals which shed high levels of virus over their lifetime (Cray and Moon, 1995; Goens, 2002). Pseudo-vertical transmission, which combines aspects of vertical and horizontal transmission, utilises maternal behaviours to transmit directly through milk or other excreta fed to neonates such as the spread of *Mycobacterium avium* subsp. *paratuberculosis* to young rabbits through coprophagy of maternal faecal pellets (Judge et al, 2006).

Horizontal transmission of pathogens can occur through either direct or indirect routes. Direct transmission can occur through the respiratory system via contact with droplets or aerosols as in, for example, Foot-and-Mouth disease which is highly infectious and can spread by aerosol to farms miles away (Sellers and Gloster, 2008); direct contact with infected lesions on the skin such as in ringworm (Chermette et al, 2008); sexual contact, another route by which BVD spreads in the semen of infected bulls (Philpott, 1993) and faecal-oral transmission, through ingestion of infected faeces as occurs in paratuberculosis when cattle ingest infected rabbit faeces (Judge et al, 2005). Indirect transmission occurs when another organism such as a vector or intermediate host is the means of transmission from one infected host to another. An example of indirect transmission is liver fluke, a disease of mainly ruminants caused by the trematode *Fasciola hepatica* which requires a freshwater snail as an intermediate host to complete its life cycle (Taraschewski, 2006).

E. coli O157:H7 is transmitted through the faecal oral route which relies on faecal shedding of the organism into the environment by previously exposed animals and oral exposure of naïve animals. Contact with faecal contamination and hence risk of infection by the faecal-oral route by animals in a herd is not homogenous however and depends on a number of internal and external factors. In general, grazing animals use visual cues such as sward height and colour as well as olfactory cues to select the best grazing and will avoid faecal contaminated swards over those without faeces (Dohi et al, 1999; Hutchings et al, 1998; Hutchings et al, 2001a; Hutchings and Harris, 1996; Hutchings and Harris, 1997). Livestock will graze parasite larvae in the absence of faeces suggesting that faecal avoidance is a general protective behaviour designed to minimise contact with a range of pathogens (Cooper et al, 2000). The

olfactory stimulus is highest in fresh faeces and thus avoidance is highest when faeces are youngest, with avoidance waning after a number of weeks (Dohi et al, 1999). Bacteria and viruses are most prevalent in fresh faeces before dying out relatively quickly and so faecal avoidance may reduce contact with them however parasites some more hardy bacteria that survive longer may be prevalent in older faeces leading to increased risk of ingestion once faecal avoidance has waned (Hutchings et al, 1998).

Faecal avoidance leads to swards in areas with faecal contamination growing taller and more attractive due to lack of grazing and nutrients from the faeces and grazing animals then have to make a choice between better nutrition and increased disease risk (Hutchings et al, 2001a). All things being equal, livestock will still choose to avoid grazing faecal contamination however a range of different factors can force them to choose higher risk, better quality grazing over poorer grazing with lower disease risk (Hutchings et al, 2001a). Internal factors such as genetics, physiological state, disease status, hunger motivation and external factors like grazing and farm management practices influence the choices animals make to optimise their nutrition and health leading to increased or decreased contact with faeces and faecal pathogens in their environment (Hutchings et al, 2001b; Hutchings et al, 2002). Such factors must be taken into account when considering potential routes of disease transmission and control strategies for a specific pathogen such as *E. coli* O157:H7.

1.3.2 Faecal-oral transmission of *E. coli* O157:H7 in cattle

Numbers of *E. coli* O157:H7 excreted by cattle and its ability to survive in and replicate in faeces will determine the density of the organism in the environment, while exposure rates to naïve cattle and will depend on the degree of host contact and dose ingested. Contact with actively shedding animals poses one main risk to naïve cattle of exposure to *E. coli* O157:H7. Faecal excretion of *E. coli* O157:H7 poses the risk of hide contamination and ingestion through self or allogrooming and specific contamination of animal hides may have a greater impact on transmission than environmental contamination alone (Mcgee et al, 2004; Stanford et al, 2011). *E. coli* O157:H7 has been isolated from the oral cavity, hide surfaces and around the anus of cattle in feedlot and experimental systems, in some cases in cattle that were negative in their faeces indicating the potential for transmission through

allogrooming (Keen and Elder, 2002; Mcgee et al, 2004; Stephens et al, 2009). Cattle that are actively colonised at the terminal rectum can have high concentrations of *E. coli* O157:H7 in their faeces, in particularly on the faecal surface due to sloughing off of attached bacteria during defecation (Naylor et al, 2007b; Pearce et al, 2004a). Colonisation at the terminal rectum may therefore increase the risk of hide contamination and transmission to in-contact animals (Arthur et al, 2009; Elder et al, 2000).

Higher levels of shedding are also generally observed in housed animals where they are in closer proximity to one another than when on pasture (Gunn et al, 2007). In larger herds such as beef finishing systems where a large number of animals are bought in from different places and mixed, this also increases the risk of bringing in a currently-shedding animal (Zhang et al, 2010). There is also more potential in a larger herd for a continuous cycle of transmission to occur where by the time the last animal has been exposed to *E. coli* O157:H7 and gained short-term immunity, the first exposed animal's immunity may have waned allowing it to begin shedding again.

Apart from exposure to *E. coli* O157:H7 through shedding cattle and their faeces, a number of other potential sources of *E. coli* O157:H7 on cattle farms have been identified. *E. coli* O157:H7 has been isolated from farm surfaces (Lahti et al, 2003) as well as in water and feed on farms and there is evidence that it can disseminate in cattle exposed to contaminated drinking water or feed (LeJeune et al, 2001; Mcgee et al, 2004; Shere et al, 1998; Shere et al, 2002; Van Donkersgoed et al, 2001). Flies have also been found to carry *E. coli* O157:H7 and can be responsible for transmission both in experimental settings and on farms (Ahmad et al, 2007; Alam and Zurek, 2004; Shere et al, 1998). Other potential means of spread include birds and other wildlife on farms either by contact with cattle or contamination of the environment, feed or water (Ahmad et al, 2007; Shere et al, 1998).

Long-term survival in the environment could provide *E. coli* O157:H7 the opportunity to re-infect a previously exposed and immune population once immunity has worn off while the ability to replicate in faeces following defecation poses an increased immediate risk of exposure to other cattle and people (Bolton et al, 1999; Wang et al, 1996). Risk of ingestion by naive animals is thus especially dependent

on whether positive animals are shedding large numbers or low numbers of bacteria in their faeces. It is as yet unclear as to how much transmission of *E. coli* O157:H7 in cattle is due to direct contact with shedding animals and how much comes from the farm environment and more research is required to unpack the importance of different shedding patterns and routes of transmission.

1.3.3 Experimental studies of transmission of *E. coli* O157:H7

Experimental challenge of cattle with *E. coli* O157:H7 has demonstrated high level faecal shedding in a proportion of calves that is self-limiting, lasting typically >9 days but often well over a month as described in Section 1.2.4 (Brown et al, 1997; Cray and Moon, 1995; Naylor et al, 2003). Rates of shedding in experimental situations can be variable and oral doses of 10^9 or 10^{10} C.F.U. are necessary to ensure high level-shedding in the majority of animals and some animals may only shed at enrichment level ($<10^2$ C.F.U. /g) or not at all (Grauke et al, 2002; Naylor et al, 2003; Sheng et al, 2004). Some experimental studies have reported natural occurrence of *E. coli* O157:H7 transmission in calves following low level exposure from pen mates with shedding at similar levels to challenged animals (Besser et al, 2001). Yet experimental challenge doses of less than 10^9 C.F.U. generally achieve low rates of shedding (Besser et al, 2001; Cray and Moon, 1995; Sheng et al, 2004)(Besser et al, 2001; Cray and Moon, 1995; Sheng et al, 2004). This discrepancy between the high level challenge needed to create experimentally colonised animals and apparent colonisation due to natural exposure to low level challenge presents a dilemma to those studying the transmission dynamics of *E. coli* O157:H7. There is only so much that can be learned from studying shedding by animals that have received an unrealistically high challenge dose. Instead, more valuable are models of transmission which more closely emulate the natural situation on farms. Most transmission studies attempt this by experimentally challenging a number of individuals and introduce them to naïve animals as a ‘Trojan horse’ from which ‘natural’ transmission can occur (Table 1.1).

Table 1.1 Experimental *E. coli* O157:H7 transmission studies

Age of animal	Mode of transmission	Number of naïve animals that shed	Level of shedding (C.F.U./g faeces)	Study
10 weeks	1 shedding animal mixed with 5 naïve 1 shedding animal mixed with 2 naïve	5/5	<30 – 10 ⁶ for up to 70 days	(Besser et al, 2001)
10 weeks	Calves given access to <i>E. coli</i> O157:H7 spiked water	4/4	<10 ⁶ for up to 87 days	(LeJeune et al, 2001)
Weaned calves	Calves given access to <i>E. coli</i> O157:H7 spiked water	10/12	10 ² – 10 ⁶ for 18 - >43 days	(Shere et al, 2002)
5-8 months	3 shedding animals mixed with 15 naïve 2 shedding animals mixed with 15 naïve	6/15 15/15	E+ - 10 ² once or twice E+ - 10 ⁴ for several days	(Sheng et al, 2004)
6-9 months	1 shedding animal mixed with 5 naïve in each of 3 pens	15 of 30	<50 in 13/15 10 ³ - 10 ⁴ 1-2 times in 2/15	(Mcgee et al, 2004)
8 calves	Calves exposed to <i>E. coli</i> O157:H7 contaminated house flies	8/8	<10 ² - 1.1 x 10 ⁶ for 11 days	(Ahmad et al, 2007)
Yearlings	Pats containing 10 ² C.F.U. added to pens of naïve animals Pats containing 10 ⁵ C.F.U. added to pen of naïve animals	0/16 13/16	N/A E+ only	(Stephens et al, 2008)
Pre-weaned calves	2 shedding animals mixed with 1 naïve	2/2	1 calf; <10 ⁴ for >7 days 1 calf; E+ only	(Jonsson et al, 2009)
12-15 weeks	5 shedding animals mixed with 5 naïve in each of 2 pens	10/10	E+ only measured – positive for >15 days	(Schouten et al, 2009)

E+; Enrichment positive only = <10² C.F.U./g faeces

Transmission rates from such studies as described in the table are variable though shedding in 5/5 and 12/12 calves exposed to shedding animals and 5/8 and 10/12 calves exposed to contaminated water have been reported (Besser et al, 2001; Mcgee et al, 2004; Schouten et al, 2009; Shere et al, 2002). One study proposed an R_0 of 7.3 based on rates of shedding in a group of cattle exposed to colonised calves (Schouten et al, 2009). Most transmission studies use Stx^+ strains however one study with a Stx^- strain detected shedding at enrichment level in 13/30 animals and shedding at levels of 10^3 or 10^4 once or twice in 2 animals (Mcgee et al, 2004). The level and duration of shedding in animals in studies of *E. coli* O157:H7 transmission varies widely however from single or occasional enrichment level ($<10^2$ C.F.U./g) counts only to persistent shedding of up to 10^6 C.F.U./g in some animals. Few, if any of these studies specifically confirm the colonisation status of the animals, instead assuming that high-level shedding on a number of occasions constitutes colonisation or ‘super-shedding’. This could be inaccurate however, given *E. coli* O157:H7’s ability to replicate in the environment which could lead to ingestion of a high dose and higher level passive shedding. Additionally, where studies have considered transmission to mean finding a single positive sample from an animal they run the risk of labelling passive shedding due to passive transit through the gut as a transmission event which may give a false impression of transmission rates.

1.3.4 The importance of correctly defining colonisation and transmission of *E. coli* O157:H7 in cattle

Some of the key questions still to be answered in understanding *E. coli* O157:H7 shedding and transmission relate to the potential for passive shedding, what actually constitutes a colonised animal and how easily an animal can be colonised. *E. coli* O157:H7 can survive for extended periods in the environment and low-level intermittent shedding is seen, often in animals that are grouped with a high-shedding animal, which could be due to passive transit through the gut (Cobbold et al, 2007). Passive shedding could not only account for low-level shedding but if sufficient replication in gut contents was achieved it could theoretically be responsible for high-level shedding, although whether this would be sustained would depend on the rate of ingestion of the organism. Related to that, the natural infectious dose required to colonise an animal at the terminal rectum is still unknown. Experimental studies normally use high doses ($10^9 - 10^{10}$ C.F.U.) to achieve this and lower challenge

doses have poor success in causing high-level shedding. Oral doses of 10^4 C.F.U. are insufficient to cause detectable shedding and doses of up to 10^7 or 10^8 C.F.U. still do not lead to consistent high-level shedding (Besser et al, 2001; Cray and Moon, 1995). Given that very high experimental doses are needed to guarantee colonisation in most calves and that cattle are unlikely to be exposed to that level of dose on farms it is possible that a large proportion of observed *E. coli* O157:H7 shedding could be due to passive shedding. Further work is needed to investigate rates of passive shedding and colonisation of *E. coli* O157:H7 following a variety of doses and through contact with a shedding animal versus a contaminated environment.

The importance of correctly classifying animals as colonised or passively shedding is also highlighted by modelling studies which suggest that 80 % of disease transmission arises from the 20 % most infectious individuals and that preventing high-level shedding in the top 5 % infectious animals could reduce the R_0 enough to prevent further transmission (Matthews et al, 2006a; Woolhouse et al, 2005). This approach depends greatly on the presence and significance of high-shedding animals for maintenance of infection and what is causing the high-shedding (Matthews et al, 2006a). If high-level shedding is principally due to passive transit and not colonisation, for example, then this would have a major effect on determining where to target control measures. Current targets for control are aimed at reducing the number of colonised animals through, for example vaccines, a method of control that relies on there being host interaction which can trigger an immune response (McNeilly et al, 2008). If animals are passively shedding a vaccine is unlikely to affect them and knowing more about how much *E. coli* O157:H7 shedding in cattle is passive would help to predict whether vaccines could work in the field. Even if colonisation of cattle was prevented by vaccination, passive shedding could still be possible especially if there was a viable external source of *E. coli* O157:H7. In that case, targeting growth in the rumen or increasing gastrointestinal killing of *E. coli* O157:H7 through diet manipulation or probiotics and environmental control could be necessary to prevent both passive and active shedding (Bach et al, 2005; Zhao et al, 1998). Further understanding of rates of passive shedding and colonisation are therefore necessary to help identify whether control measures should focus more on passive shedding or colonisation or a combination of the two.

1.4 The Thesis

1.4.1 Aims

The overall aim of this work is to investigate patterns of shedding and colonisation of *Escherichia coli* O157:H7 in cattle and consider how they impact upon transmission within cattle populations. Firstly rates of *E. coli* O157:H7 replication and colonisation in the bovine gut will be measured and the contributions of passive and active shedding to shedding patterns seen in epidemiological studies discussed. Next the importance of dose ingested and host contact on shedding patterns and rates of transmission of different *E. coli* O157:H7 strains in calves is explored. Finally the role of strain differences in replication and colonisation in the bovine gut is investigated. It is intended that findings from this work will improve current understanding of how *E. coli* O157:H7 persists both within the individual host and within cattle populations and will inform future work on control strategies.

1.4.2 Thesis structure

This Chapter has provided an overview of *E. coli* O157:H7 and its importance as a human pathogen; discussed what is currently known about shedding and transmission in cattle and identified a number of unanswered questions, some of which will be dealt with in later chapters.

Chapter 2 investigates potential levels of passive and active shedding of *E. coli* O157:H7. Firstly survival and replication in gut contents are measured and used to calculate the potential dose of *E. coli* O157:H7 reaching the terminal rectum and the potential for passive carriage. Rates of colonisation at the terminal rectum are also measured and the potential contributions of passive and active shedding to explaining observed shedding patterns discussed.

In Chapter 3 the shedding and transmission dynamics of *E. coli* O157:H7 are explored through a number of animal challenge experiments. These experiments consider transmission of *E. coli* O157:H7 in calves in the presence or absence of shedding animals and following a range of doses as well as strain differences in shedding and transmission.

In Chapter 4, the replication and colonisation rates of different *E. coli* O157:H7 strains are measured. A comparison of growth in rumen contents in the presence and

absence of protozoa and replication and colonisation at the terminal rectum provide insight into the mechanisms behind strain differences in shedding and transmission.

Finally Chapter 5 revisits the aims in light of the key findings and discusses the impact of passive and active shedding on *E. coli* O157:H7 transmission, the importance of strain differences in shedding and transmission, and the implications of this work for development of control strategies.

Chapter 2

Impact of *E. coli* O157:H7 replication and colonisation in the bovine gut on shedding patterns in cattle

2.1 Preface

For pathogens that transmit via the faecal-oral route, faecal shedding by infected animals determines the risk of exposure of naive animals within the population. Exposure occurs primarily through ingestion of the organism and subsequent shedding rates are determined by the organism's ability to survive in and colonise the gut as it passes through the gastrointestinal tract. Survival and replication of *E. coli* O157:H7 during gut transit will therefore determine passive shedding rates and challenge at the terminal rectum, the site of specific tropism for colonisation in cattle. In this Chapter, rates of *E. coli* O157:H7 replication and colonisation in the bovine gut are measured and used to calculate potential rates of passive shedding and challenge at the terminal rectum to help explain observed shedding patterns in cattle.

The results presented in this Chapter will be prepared by the author for publication with a view to submitting to Applied Environmental Microbiology.

2.2 Introduction

Cattle that are shedding *E. coli* O157:H7 show no clinical signs and therefore random faecal sampling is required to identify positive farms and in most studies animals are sampled only once or at intervals. Additionally, most farm studies test only the presence or absence of the organism and the colonisation status of sampled animals is unknown. Heterogeneous patterns of shedding are common at both farm and individual cattle level with up to 78 % of farms having no detectable shedding but up to 100 % of faecal pats may be positive on farms where shedding is found (Matthews et al, 2006b). Shedding of *E. coli* O157:H7 by individual animals can be either intermittent or persistent lasting several weeks or months with eventual clearance (Besser et al, 1997; Robinson et al, 2004). Where magnitude of shedding is known the majority of shedding animals in a group shed $<10^2$ C.F.U./g while a small proportion (3-8%) shed high levels, usually over 10^3 C.F.U./g faeces (Chase-Topping et al, 2007). This variation is also seen at slaughter in groups of animals close together on the line where a few animals have high level carriage both at the rectum and in faeces and others lower level carriage only in the faeces (Low et al, 2005; Omisakin et al, 2003). Such heterogeneities in shedding of *E. coli* O157:H7 could have a large impact on transmission as animals shedding high levels up to 10^7 C.F.U./g faeces will pose a greater risk to naïve animals than animals shedding 10^2 C.F.U./g (Matthews et al, 2006a).

Observed heterogeneities in shedding level within groups have been attributed to some animals being colonised by the organism leading to shedding $>10^3 - 10^4$ C.F.U./g into the environment, followed by low level exposure and passive shedding in in-contact animals (Cobbold et al, 2007; Low et al, 2005). In many cases the colonisation status is not confirmed however but is instead assumed based on one of a number of definitions of colonisation that have arisen to explain the differences in shedding level. Definitions of a colonised animal or 'super-shedder' include measures of magnitude or duration of shedding (Chase-Topping et al, 2008). Typically the concentration cut-off for classifying an animal as colonised or a 'super-shedder' is 10^3 or 10^4 C.F.U./g faeces on a single occasion as it is considered that shedding above that level is unlikely by passive carriage (Chase-Topping et al, 2008). In terms of persistence, an animal has been considered a 'super-shedder' if it is positive via rectal swab or sheds in its faeces consistently at multiple samplings (Davis et al, 2006).

These definitions are based on experimental data and some farm studies where animals that are actively colonised at the terminal rectum shed high levels of *E. coli* O157:H7 in their faeces (Naylor et al, 2003) and higher faecal concentrations of *E. coli* O157:H7 in faeces lead to longer term shedding (Davis et al, 2006). They do not necessarily distinguish between, for example, an animal that has been colonised but is now at the end of its shedding curve and an un-colonised animal persistently shedding low levels through passive carriage. There is evidence of low level persistent shedding for up to 20 weeks in experimentally challenged animals which could be due to passive shedding for example (Cray and Moon, 1995). Additionally, while experimentally challenged animals have been shown to shed high levels of *E. coli* O157:H7 for a number of days or weeks in their faeces, even after high challenge doses the level and duration of shedding are highly variable and some animals may not shed the organism at all (Besser et al, 2001; Cray and Moon, 1995). This coupled with the low rates of high level shedding observed in prevalence studies, suggests that colonisation at the terminal rectum may be an uncommon event and raises the possibility that much of the shedding observed is due to passive shedding alone.

E. coli O157:H7 can survive in the environment and the persistence of specific clonal types on farms suggests the possibility for environmental or other reservoirs

for maintaining populations on farms (LeJeune et al, 2004; Liebana et al, 2005). Passive shedding through exposure to an environmental reservoir could account for intermittent shedding (Smith et al, 2010; Stanford et al, 2005). *E. coli* O157:H7 can grow successfully in the bovine gut contents including the rumen, duodenal contents and faeces which could contribute to passive shedding (Bridger et al, 2010; Grauke et al, 2002; Rasmussen et al, 1993; Thran et al, 2003; Wang et al, 1996). The rumen has been looked at in greater detail both as a potential colonisation site and as a point of control as rumen-cannulated animals inoculated with *E. coli* O157:H7 showed a decline in counts over time (Grauke et al, 2002; Zhao et al, 1998). The effects of different diets, rumen conditions and constituents such as protozoa have all been conjectured as having an impact on *E. coli* O157:H7 growth rates in the rumen which would in turn affect the numbers shed in faeces and the chances of colonisation (Chaucheyras-Durand et al, 2010; Stanford et al, 2010; Thran et al, 2003).

Since *E. coli* O157:H7 may be acquired from the environment and replicate in the gut contents this provides the opportunity for passive carriage, with no mucosal attachment, to cause shedding in cattle. Low level shedding seen on farms could be due to ingestion of small numbers of bacteria leading to passive shedding with or without replication in the gut and sufficient replication in gut contents could lead to higher level shedding in un-colonised animals and determine challenge at the terminal rectum and rates of colonisation.

2.2.1 Aim

The aim of this chapter is to investigate the potential contributions of passive and active shedding due to colonisation of *E. coli* O157:H7 by cattle to shedding patterns seen in epidemiological studies. The potential for passive shedding will be quantified by measuring survival and replication rates of *E. coli* O157:H7 in bovine gut contents and terminal rectum mucus and estimating the levels of faecal shedding that may derive from passive shedding alone. Additionally attachment and replication rates on bovine primary terminal rectum epithelial (BTRE) cells will be measured and the contribution of colonisation and active shedding to shedding patterns discussed.

2.3 Materials and Methods

2.3.1 Culture and storage of bacteria

The *E. coli* O157:H7 strains used in this study are shown in Table 2.1. Strains were stored at -70°C in Luria-Bertani (LB) broth (Oxoid, Basingstoke, UK) with 25 % glycerol. For use, strains were revived on an LB agar plate (Oxoid) and incubated statically at 37°C for 16 hours (h). For liquid culture, a single colony from a streak plate was added to LB broth and incubated at 37°C shaking at 200 rpm for 16 h.

Table 2.1 *E. coli* O157:H7 strains used in Chapter 2

Strain	Characteristics	Source
Walla 3 (W3)	Stx ⁻	Lab stock, originally from (Ostroff et al, 1990)
W3 NalR	Stx ⁻ , Nalidixic acid-resistant (NalR)	This study, derived from W3

2.3.2 Derivation of spontaneous antibiotic-resistant strains

A spontaneously Nalidixic Acid-resistant (NalR) isolate W3 was isolated by inoculating a colony of W3 into LB broth and incubating statically at 37°C for 16 h. Samples were centrifuged briefly at 17000 x g, the supernatant poured off and the bacteria re-suspended in the remaining medium and plated onto an LB plate containing 15 µg/ml Nalidixic Acid (Sigma-Aldrich, Gillingham, UK). Plates were then incubated statically at 37°C for 24-48 hours until large colonies were present. The large colonies were streaked out individually onto fresh LB-Nal plates and incubated statically at 37°C for 16 h to purify the isolates. Colonies from these plates were then inoculated into LB broth and incubated statically at 37°C for 16 h and frozen down at -70°C in 25 % glycerol.

2.3.3 Collection and storage of samples

Ruminal and small intestinal contents were collected from freshly killed animals at a local abattoir and returned to the laboratory within 2 hours. Samples were diluted for use with sterile distilled H₂O (dH₂O) 1:1 or 1:2 and vortexed vigorously to homogenise the solution. Samples were aliquoted and used either immediately to create overnight cultures, chilled at 4°C for use the next day or frozen at -70°C for

later use. Mucus samples were collected from the terminal recta of slaughtered cattle as part of the process of culturing primary bovine terminal rectum cells (see Section 2.3.4). Mucus samples were diluted 1:10 in sterile dH₂O and stored at -20°C for later use. Fresh rumen samples were collected by stomach tube from live animals, pooled into pre-warmed pots and transported back to the laboratory where they were stored at 37°C prior to use within 2 h of collection. Faecal samples were collected fresh from cattle on a local finishing farm. Faecal samples were diluted 1:2 in warm sterile dH₂O and vortexed vigorously to homogenise and used within 2 h of collection. All samples were checked for the presence of confounding background flora prior to use by spreading a sample onto a sorbitol MacConkey (Oxoid) plate supplemented with 15 mg/ml Nalidixic Acid (SMAC-Nal).

2.3.4 Isolation of primary bovine terminal rectum epithelial (BTRE) cells

Bovine recta were collected from slaughtered animals at an abattoir. The canals were opened longitudinally and the mucosa washed in H₂O. Approximately 5 cm of tissue proximal to the recto-anal junction was cut away from the connective tissue underneath. Harvested tissue was washed in H₂O and then placed in washing media (Hank's Balanced Salt Solution (HBSS), Sigma-Aldrich,) containing 25 µg/ml Gentamicin (Sigma-Aldrich), 5 µg/ml Fungizone (Invitrogen (Now Life Technologies), Paisley, UK), 100 U/ml Penicillin/Streptomycin (Life Technologies)) and incubated at room temperature (RT) for 30 min. Epithelial mucus was then gently scraped away from the tissue surface using sterile glass slides. On occasions where mucus was being collected for use in other experiments the tissues were first placed in HBSS media and then transferred to the washing media once the mucus had been removed. The epithelial layers were then removed from the sub-mucosa by scraping the tissue firmly with sterile glass slides. The removed cells were added to HBSS and shaken vigorously to break up the tissue. Cells were then centrifuged at 300 x g for 2 min at RT, removing the supernatant each time, until the supernatants were clear.

Cells were re-suspended in digestion medium (Dulbecco's Minimum Essential Medium (DMEM, Sigma-Aldrich), 1% (v/v) fetal bovine serum (FBS, Sigma-Aldrich), 25 µg/ml Gentamicin (Sigma-Aldrich), 100 U/ml Penicillin/Streptomycin) and then 75 U/ml Collagenase (Sigma-Aldrich) and 20 µg/ml Dispase I (Roche,

Welwyn Garden City, UK) were added. Following vigorous mixing the cells were incubated at 37°C shaking at 200 rpm for 1 h 20 min to digest the connective tissue and release the crypts.

Following digestion tissues were centrifuged at 300 x g for 2 min at RT and the pellets re-suspended in HBSS. This process was repeated until the top portions of the supernatants were free of crypts. From then on, one fifth less HBSS was added with each re-suspension until the whole of the supernatants were clear. Pellets were then washed with HBSS and re-suspended in primary cell culture medium (DMEM, 2% (v/v) FBS, 2 mM L-glutamine (Life Technologies), 25 µg/ml Gentamicin (Sigma-Aldrich), 100 U/ml Penicillin/Streptomycin (Life Technologies), 10 ng/ml epidermal growth factor (Sigma-Aldrich), 0.25 U Insulin (Sigma-Aldrich)) to a concentration of 5000 crypts/ml.

2.3.5 Primary BTRE growth

Collagen (Nutacon, Leimuiden, The Netherlands) coated plates were seeded with 500 - 700 crypts/well in primary cell culture medium. Plates were incubated at 37°C, 5 % CO₂, 80 % humidity for 1-2 days, at which point half of the medium was replaced with feeding medium (as primary cell culture medium but with 5 % FBS). Medium was removed from the wells and replaced every 2 days until cells were confluent.

2.3.6 *E. coli* O157:H7 replication in bovine gut contents, faeces and terminal rectum mucus

To gain a greater understanding of *E. coli* O157:H7 survival in the bovine gut, luminal contents from the rumen, small intestine, faeces and mucus from the terminal rectum were inoculated with an *E. coli* O157:H7 strain and incubated statically at 37°C before taking counts at a number of time points. A single colony of Walla 3 NalR from an LB streak plate was inoculated into a 1ml aliquot of the material to be tested and incubated statically at 37°C for 16 h to allow acclimatisation to the test media. The overnight culture was then diluted to the required level with sterile dH₂O warmed to 37°C. Distilled H₂O was used as a dilution medium in order that the test materials be kept in as natural a state as possible without the addition of chemicals. *E. coli* O157:H7 survival in water and PBS was compared prior to the study and were comparable over 6 hours and

therefore experiments were conducted using dH₂O. The test solution was then used to inoculate multiple aliquots of each test sample which were then incubated statically at 37°C along with control samples which had received no inoculum. At set time points a sub-set of the aliquots were serially diluted in sterile dH₂O and plated in triplicate onto SMAC-Nal agar plates. The initial inoculum was also serially diluted and plated. Plates were incubated statically at 37°C for 16 h and the number of colonies on each plate counted up to a maximum of 500, counts above which were considered too many to count. The limit of detection for this enumeration method using marked strains is 10² C.F.U./g faeces (LeJeune et al, 2006).

2.3.7 *E. coli* O157:H7 replication rates in fresh rumen fluid

The aim of this experiment was to test the effects of storage and removal of rumen microflora on *E. coli* O157:H7 replication rates in bovine rumen contents. Samples of frozen rumen fluid were thawed and split into 1 ml aliquots to which an inoculum of W3 NalR which had been cultured overnight in the same rumen fluid was added. Samples were incubated statically at 37°C and plating at set time points up to 24 hours as described in Section 2.3.6.

The fresh rumen fluid was split into two halves. One half was kept unaltered and the other half was centrifuged at 300 x g for 5 minutes to remove protozoa. To confirm absence or presence of protozoa a sub-sample of each half was diluted 1:4 with 4 % formalin (Fisher-Scientific, Loughborough, UK) and 10 µl aliquots added to a haemocytometer and protozoa counted. The centrifuged and original rumen fluids were then split into 9 ml aliquots. 1 ml of W3 NalR overnight culture (1 colony in LB incubated with shaking at 200 rpm for 16 h at 37°C) diluted in dH₂O was added to the aliquots to give a final concentration of 10⁴ C.F.U./ml. Samples were incubated at 37°C with shaking at 80 rpm to prevent settling. At set time points a 1 ml aliquot from each sample was collected, some of which was fixed for storage by diluting it 1:4 with 4 % formalin and to the remainder 0.2 % Triton X-100 (Sigma-Aldrich) in Dulbecco's Phosphate Buffered Saline (PBS, Oxoid) was added to lyse any living cells in the fluid. Following incubation at RT for 5 min, lysed samples were serially diluted and spread on SMAC-Nal plates as previously described (Section 2.3.6). Counts in fresh rumen fluid were taken up to 8 hours only because protozoa were unlikely to survive past this time point without addition of nutrients

which would have affected the pH of the samples (John Wallace, personal communication, October 4, 2012).

2.3.8 Estimation of potential challenge dose at terminal rectum and rates of passive shedding

Based on gut transit information and the measured rates from the previous experiments, the potential terminal rectum challenge and rates of passive shedding were calculated. Compartmental mean retention times (CMRT) for major locations in the gut were identified. The CMRT for the rumen is split into the solid and liquid phases as these phases spend differing lengths of time in the rumen, an effect which is lost in the digesta in the small and large intestines as contents are mixed together (Huhtanen et al, 2008; Pelikaan, 2004). Next the replication rates measured in Sections 2.3.6 and 2.3.7 (in generations per hour) for fresh rumen without centrifugation, small intestine and faeces were multiplied with the CMRT for each compartment. This gave the number of generations of bacteria gained or lost during transit through the compartment per unit entering. The number of generations was then used to calculate the C.F.U. gained or lost when leaving the compartment by multiplying the number of generations by Ln 2 to get the Ln (C.F.U.) and then back transforming the calculated value. The C.F.U. values represent the potential change in counts of *E. coli* O157:H7 achieved in that location per unit entering the compartment. Based on a typical ingested dose of 10^4 C.F.U. (Wood et al, 2006) and assuming that all *E. coli* O157:H7 would move through the gut at the same rate the potential count leaving the rumen in the solid or liquid phase was calculated by multiplying the dose of 10^4 C.F.U. by the potential change in each phase. Counts leaving the rumen were then multiplied by the potential change in the small intestine to estimate the range of counts leaving that compartment. Finally counts leaving the small intestine were multiplied by the potential change in the large intestine to calculate the potential terminal rectum challenge and total faecal output following gut transit.

2.3.9 Attachment rates on primary BTRE cells from mucus

The aim of this experiment was to measure attachment rates of *E. coli* O157:H7 on BTRE cells using mucus as the growth medium. Cultures of BTRE cells were obtained as previously described (Sections 2.3.4, 2.3.5). Following harvest the 12-

well plates of BTRE were fed every 2 days until confluent at around 5-6 days after harvest. A colony of NalR Walla 3 was inoculated into 1 ml of 10 % mucus and incubated statically for 16 h at 37°C. The overnight culture was then diluted further with 10 % mucus and incubated at 37°C for 3 hours to obtain mid-log growth bacteria (determined from previous growth rates in mucus). Prior to adding the bacterial inoculum, medium was removed from wells of BTRE cells and the cells washed 3 times with warmed PBS. 450 µl of 10 % mucus was then added to each well along with 50 µl of the inoculum to give an MOI of ~ 10 bacteria to 1 BTRE cell. Plates were incubated at 37°C, with 5 % CO₂ and 80 % humidity. At set time points the mucus mixture was removed and the cells washed three times with warmed PBS. Cells were then lysed by adding 0.1 % Triton X-100 in PBS to each well and incubating at RT for 5 min. Each well was then scraped using the end of a pipette tip to fully remove all cells from the well and the lysates from multiple wells (6 per time point) collected and pooled. The initial inoculum and cell lysates were then serially diluted and spread on SMAC-Nal plates as previously described (Section 2.3.6).

2.3.10 Progression of colonisation on primary BTRE cells

The aim of this experiment was to study the process of colonisation of *E. coli* O157:H7 on BTRE cells once attached. Cultures of BTRE cells were obtained as previously described (Sections 2.3.4, 2.3.5). A colony of NalR Walla 3 was inoculated into 25 ml LB in a conical flask and incubated for 16 h at 37 °C with shaking at 200 rpm. The overnight culture was then split 1:100 with Minimal Essential Medium with HEPES buffer (MEM-HEPES, Sigma-Aldich) supplemented with 250 nM Fe(NO₃)₂ and 0.2 % glucose. This medium promotes production of type III secretory proteins necessary for cell adherence (Flockhart et al, 2012; Roe et al, 2003). Cultures were then incubated at 37°C for 2 hours with shaking at 200 rpm to obtain an OD at 600 nm of 0.3. Wells of cells were washed gently 3 times with warmed PBS and 400 µl medium added to each well. The bacterial culture, once it had reached an OD of 0.3, was then split 1:20 with the supplemented MEM-HEPES and 100 µl added to each well of cells to give an MOI of ~ 10 bacteria to 1 BTRE cell. Plates were then incubated at 37°C, with 5 % CO₂ and 80 % humidity for 1 h to allow attachment. The wells were washed 3 times with warmed PBS to remove unattached bacteria and 500 µl fresh medium added. Plates were incubated again and

at set time points the cells from multiple wells (3 per time point) were washed three times with PBS, harvested and plated as previously described to enumerate attached bacteria (Section 2.3.6).

2.3.11 Statistical analysis

Counts from plates were recorded and those from multiple dilutions used to calculate the most probable number of C.F.U. by fitting generalized linear models with a Poisson error distribution and logarithmic link function incorporating the logarithm of dilution as an offset variable. Final counts were then log-transformed using the formula $\text{Log}_{10}(\text{C.F.U.}+1)$ and statistical analysis performed on the Log_{10} counts. Statistical analysis was performed using the statistics package Genstat (15th ed).

In all gut locations, *E. coli* O157:H7 growth was linear until a peak at 9 hours post inoculation and the final 24 hour counts were normally the same as or lower than the 9 hour counts. Curves were therefore analysed in two parts. Firstly the linear phase of each growth curve up to the 9 h time point was compared using random coefficient regression with Animal_Location as subject and a treatment structure of Location*Time. As rumen and small intestinal samples were collected from the same animals while other samples were from different animals, the factors Animal and Location were combined and analysed as a single factor Animal_Location. The final 24 h count for each location was also compared using REML with Location as the fixed model and Animal_Location as the random model. The linear phase of growth curves in stored rumen and fresh rumen with and without centrifugation up to 9 h were also compared using random coefficient regression using a combined factor of Animal and Rep as subject and Rumen Type*Time as treatment structure. Levels of attachment and colonisation on BTRE cells were analysed using REML to compare the Log_{10} transformed counts with Time as the fixed model and Animal as the random model.

2.4 Results

2.4.1 *E. coli* O157:H7 survival and replication in the bovine gut

Figure 2-1 shows the fitted regression lines for the linear phase of growth up to 9 hours for *E. coli* O157:H7 in rumen and small intestinal contents, faeces and terminal rectal mucus. Counts in mucus increased by nearly 5 log₁₀ C.F.U. over 9 hours compared to just over 1 log₁₀ C.F.U. at the other locations. Counts were significantly different over time (*wald* = 106.49, d.f. = 3, *P* < 0.001).

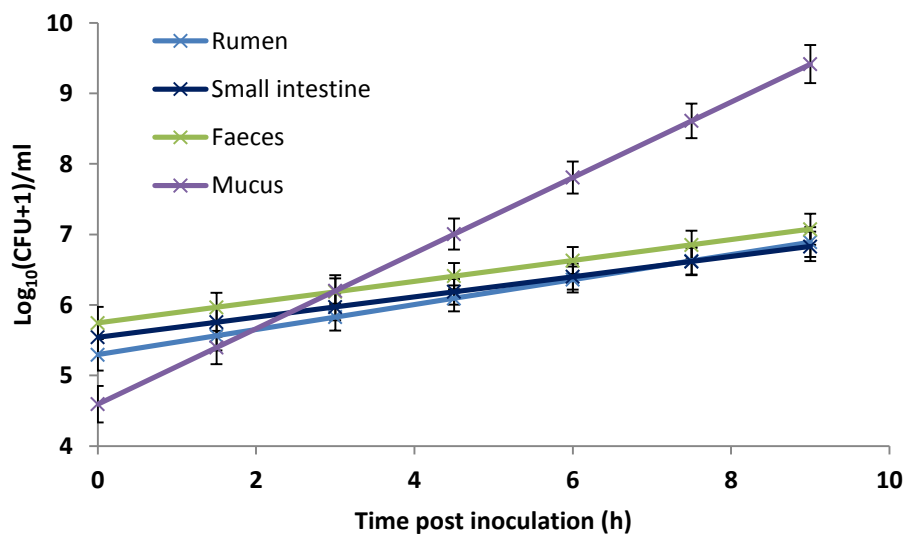


Figure 2-1. *E. coli* O157:H7 replication in gut contents and mucus

Mean growth curves with standard errors (SED of location*time interaction = 0.281) for the linear phase of *E. coli* O157:H7 replication in rumen and small intestinal contents, faeces and TR mucus in log₁₀ C.F.U./ml (4 biological replicates for rumen, small intestine and faeces and 3 biological replicates for mucus).

The growth curves were used to calculate the average doubling time for each location over the linear phase (Table 2.2). Doubling times for rumen, small intestine and faeces were 102 min, 126 min and 122 min respectively, all at least 3 times slower than that of mucus for which the doubling time was 34 min.

Table 2.2 Generation rate and doubling time of *E. coli* O157:H7 in different gut locations

	Rumen	Small Intestine	Faeces	Mucus
Generations/h	0.59	0.48	0.49	1.78
Doubling time (min)	102	126	122	34

The final 24 h results counts were also significantly different (Figure 2-2; $wald = 39.38$, d.f. = 3, $P < 0.005$).

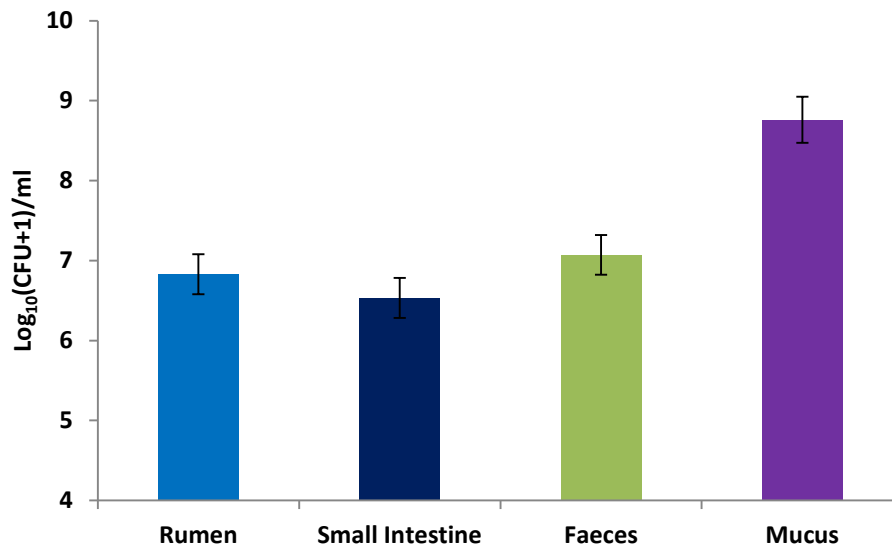


Figure 2-2. *E. coli* O157:H7 24 hour counts in mucus

Mean 24 hour counts with standard errors (SED for location = 0.334) for *E. coli* O157:H7 in each gut location in \log_{10} C.F.U./ml (4 independent biological replicates for rumen, small intestine and faeces and 3 independent biological replicates for mucus).

2.4.2 Investigation of *E. coli* O157:H7 growth in fresh rumen contents

Counts of *E. coli* O157:H7 in untreated fresh rumen fluid decreased by 0.5 \log_{10} while counts in fresh rumen fluid from which the protozoa had been removed decreased only slightly and stored rumen fluid in which the protozoa and other microorganisms should have died increased over 9 hours by 3 \log_{10} C.F.U./ml (Figure 2-3). There was a significant difference for each main effect of time ($wald = 60.41$, d.f. = 1, $P < 0.001$) and rumen treatment ($wald = 476.15$, d.f. = 2, $P < 0.001$) and for the interaction between time and rumen treatment ($wald = 274.04$, d.f. = 2, $P < 0.001$).

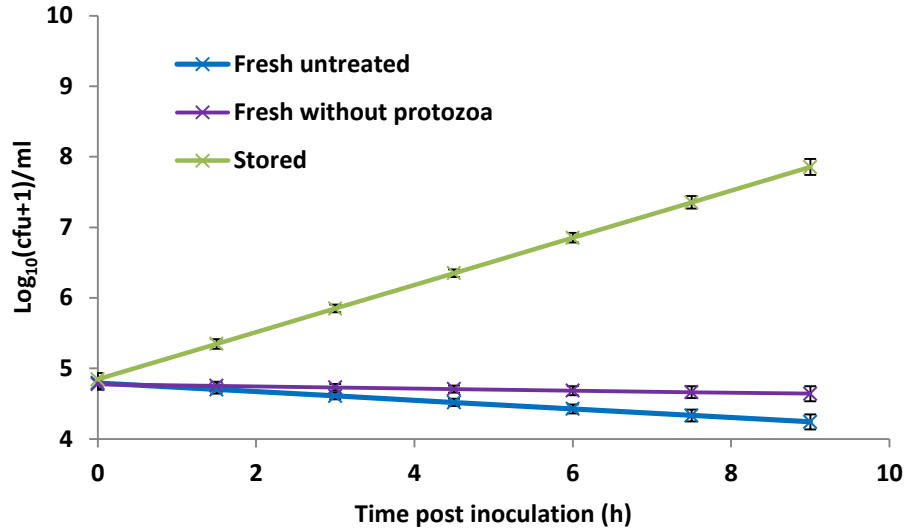


Figure 2-3. *E. coli* O157:H7 replication in fresh and stored rumen contents

Mean growth curves with standard errors (SED of rumen type*time interaction = 0.099) for *E. coli* O157:H7 replication in untreated fresh rumen, centrifuged fresh rumen with protozoa removed and stored rumen fluid in log₁₀ C.F.U./ml (4 technical replicates from a single pooled biological sample for stored rumen and 3 technical replicates each from 2 biological samples for fresh rumen).

The fitted growth curves were used to calculate the average doubling time or rate of reduction for each experiment (Table 2.3). *E. coli* O157:H7 counts increased by 1.11 generations per hour over the time period in the stored rumen fluid but there was a decrease of 0.05 and 0.20 generations per hour in fresh centrifuged and fresh uncentrifuged rumen fluid respectively.

Table 2.3. *E. coli* O157:H7 generation rate and doubling time in rumen contents.

Generation rate and doubling time of *E. coli* O157:H7 in stored rumen fluid and fresh rumen contents with and without centrifugation.

	Stored rumen	Fresh with centrifugation	Fresh untreated
Generations/hr	1.11	-0.05	-0.20
Doubling time (min)	54.00	-1207.66	-294.75

2.4.3 Estimation of potential attachment dose at terminal rectum and rates of passive shedding

Table 2.4 and Table 2.5 bring together gut transit data and the replication rates in small intestine and faeces from Section 2.4.1 and fresh rumen without centrifugation from Section 2.4.2 to estimate potential rates of passive shedding and numbers of bacteria available for attachment at the terminal rectum. The potential total C.F.U. reaching the terminal rectum for attachment or shedding in faeces 24 – 36 hours after ingestion of 1×10^4 C.F.U. was calculated as 1.76×10^4 - 1.23×10^5 C.F.U. depending on which rumen phase the bacteria were associated with. Based on a daily faecal output of 26 kg in a beef animal over ~ 12 occasions (Smith and Frost, 2000) the potential C.F.U. per gram of faeces on a single occasion of the total C.F.U. mentioned above could then be 8 – 56 C.F.U./g in each faecal pat over 24 hours. Regular ingestion of 1×10^4 C.F.U. could then lead to sustained low level shedding of the organism.

Table 2.4 Calculation of potential counts of *E. coli* O157:H7 in different gut compartments based on replication rates.

	Rumen (Solids)	Rumen (Liquid)	Small intestine	Large intestine
Compartmental retention time (CMRT) in h^a	24	10	6	6
Rate of replication from this study (h)	-0.20	-0.20	0.48	0.49
No. of generations gained or lost per unit entering	-4.8	-2	+ 2.9	+ 2.9
C.F.U. leaving per unit entering	3.6×10^{-2}	2.5×10^{-1}	0.7×10^1	0.7×10^1
C.F.U. leaving compartment based on 10^4 oral dose^b	360	2500	(S) 2.52×10^3 (L) 1.75×10^4	(S) 1.76×10^4 (L) 1.23×10^5

a. Sources: (Chaucheyras-Durand et al, 2010; Huhtanen et al, 2008; Pelikaan, 2004)

b. Based on a likely ingested dose of 10^4 (Wood et al, 2006). The letters in brackets refer to Liquid (L) or Solid (S) phase of rumen contents.

Table 2.5. Potential *E. coli* O157:H7 faecal output/challenge at TR due to passive shedding.

Total C.F.U. shed/reaching TR following dose of 10^4 C.F.U.	Shedding rate (C.F.U./g of faeces) ^a
$1.76 \times 10^4 - 1.23 \times 10^5$	8 – 56

a. Based on 26 kg faeces over ~ 12 occasions produced by a growing beef animal per day (Smith and Frost, 2000)

2.4.4 Rates of *E. coli* O157:H7 attachment to BTRE cells

Figure 2-4 shows the numbers of attached *E. coli* O157:H7 per well at time points up to 1 hour. Within 15 minutes of inoculation, 1.5×10^5 C.F.U. of *E. coli* O157:H7 had attached to the cells and very similar numbers attached at each time point (*wald* = 3.27, d.f. = 1, $P > 0.1$).

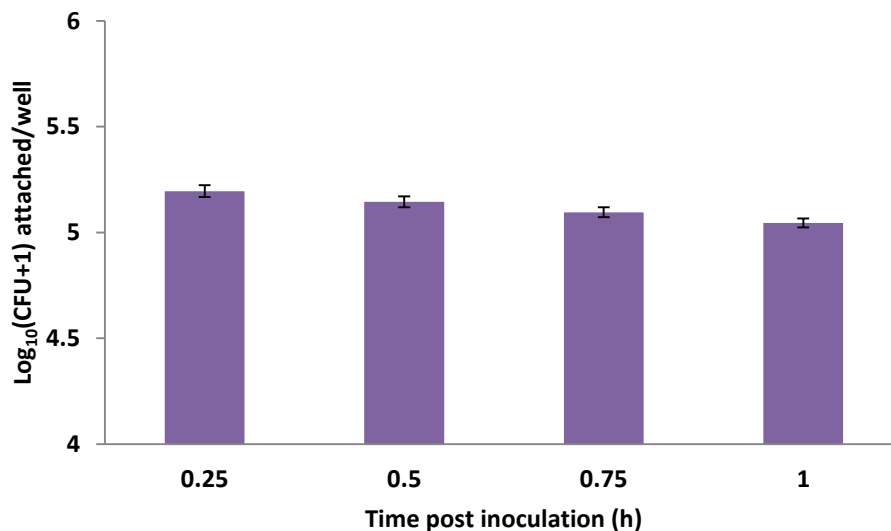


Figure 2-4. *E. coli* O157:H7 attachment to BTRE cells from mucus

Mean levels of attachment with standard errors (SED for time = 0.046) of *E. coli* O157:H7 to BTRE cells at time points up to 1h (\log_{10} C.F.U./well: 3 independent biological replicates).

2.4.5 Progression of *E. coli* O157:H7 colonisation on BTRE cells

Counts of *E. coli* O157:H7 attached to BTRE cells one hour after the infected cells had been washed and fresh medium added were 6.4×10^3 C.F.U. per well (Figure 2-5). Numbers of attached bacteria per well increased by just over 1 \log_{10} C.F.U. in 2 hours to give a generation time of 1.96 generations per hour and doubling time of 30

minutes. There was a significant difference in counts over time ($wald = 13.52$, d.f. = 1, $P < 0.05$).

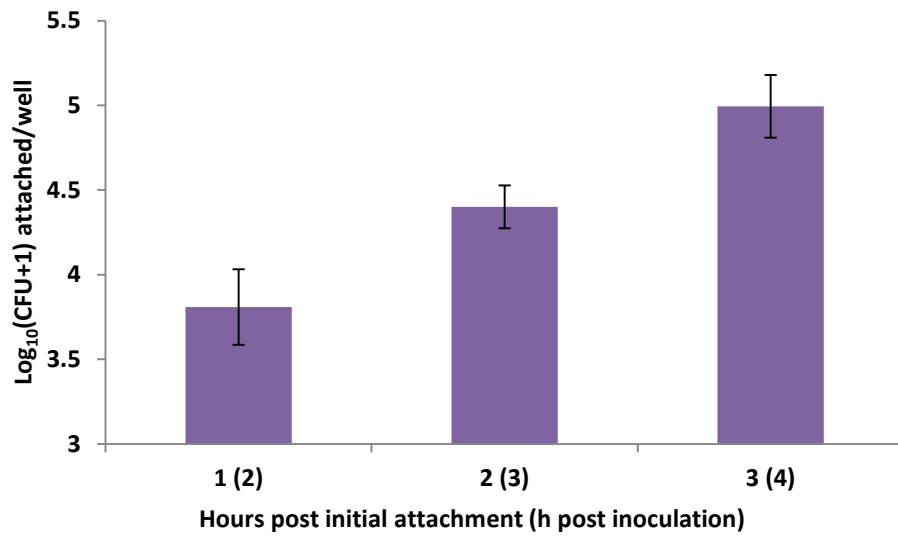


Figure 2-5. Progression of *E. coli* O157:H7 colonisation on BTRE cells

Mean levels of attached bacteria over time with standard errors (SED for time = 0.215) after an initial attachment period of 1 h followed by washing and further incubation with fresh medium (\log_{10} C.F.U. /well; 3 independent biological replicates).

2.5 Discussion

The aim of this chapter was to investigate rates of passive shedding and colonisation of *E. coli* O157:H7 in cattle and consider their contribution to observed shedding patterns. It was demonstrated that *E. coli* O157:H7 can replicate in different parts of the gut and the accumulative effect of this, despite a potential point of die off in the rumen, was that faecal shedding without terminal rectal colonisation is possible at low levels. These results have implications for understanding shedding patterns as passive transit and replication in the gut contents could account for the low level shedding ($<10^2$ C.F.U./g) seen in a large proportion of positive animals in epidemiological studies (Cobbold et al, 2007; Low et al, 2005). The calculated potential total C.F.U. shed in faeces following an oral dose of 10^4 C.F.U. was also the same or higher than the ingested dose suggesting that passive carriage without colonisation alone could be sufficient to maintain *E. coli* O157:H7 populations on farms.

Rates of potential shedding following passive carriage calculated in this Chapter are conservative as they do not include the exceptionally high rate of replication in terminal rectum mucus. Replication rates in TR mucus were so high it is feasible that passive transit of *E. coli* O157:H7 with replication in gut contents and TR mucus could lead to high levels of shedding in cattle without colonisation of the mucosa. This study did not look at replication in mucus from other parts of the gut but there is growing evidence for the importance of mucus to *E. coli* O157:H7 growth (Bai et al, 2011; Bertin et al, 2013; Fox et al, 2009; Snider et al, 2009) and replication in mucus from other locations in the gut could also add to the potential passive shedding rates. Replication in the terminal rectum mucus in particular could also account for the high proportion of bacteria found on the faecal surface compared to inside the pat (Naylor et al, 2007b; Pearce et al, 2004b). Observed shedding patterns in cattle could therefore theoretically be due to passive shedding alone with replication in gut contents accounting for low level shedding and replication in gut contents and mucus leading to higher level shedding in the faeces.

While theoretically *E. coli* O157:H7 replication in TR mucus could account for high shedding animals, at the point where the organism enters the mucus it would be difficult to distinguish between passive shedding without colonisation and active shedding caused by colonisation of the mucosa. The high rate of attachment seen on

BTRE cells in the presence of mucus in 15 minutes makes it likely that once *E. coli* O157:H7 bacteria reach the mucus, colonisation of the mucosa occurs quickly. The number of attached *E. coli* O157:H7 at 30, 45 and 60 minutes were almost identical to that at 15 minutes which is evidenced by the very small SED of 0.046. Adhered EPEC and EHEC bacteria attempt to deter incoming rival bacteria from attaching by preventing effector translocation necessary for binding (Berger et al, 2012; Mills et al, 2008). As the typical number of BTRE cells per well is 5×10^5 it is possible the assay reached saturation once all cells had a single bacterium attached. If this was the case and the assay was saturated after 15 minutes then it is possible that hourly rates of attachment from mucus would be even higher had there been more cells available to the bacteria. Saturation at the first time point was not a problem in the colonisation experiment as the different growth media resulted in a lower initial attachment. Following initial attachment, colonisation progressed at a remarkable rate and numbers attached on the cells increased by half a \log_{10} or 1.96 generations per hour, similar to the generation rate seen in mucus. These results indicate that once attached, *E. coli* O157:H7 can rapidly increase its numbers on the mucosal epithelium. At the final time point, levels of attached bacteria had reached 10^5 C.F.U. which could again indicate saturation of the assay.

The combined attachment and colonisation rates demonstrate how quickly an animal could become colonised once *E. coli* O157:H7 reach the mucus. With final counts in mucus of 10^9 C.F.U./ml and potential attachment of 10^5 C.F.U. bacteria per 10^5 terminal rectum cells with further replication on cells of ~ 2 generations per hour it can be presumed that active colonisation of the terminal rectum would lead to high level faecal shedding. High rates of attachment and replication on cells at the terminal rectum could therefore account for the high faecal outputs seen in some animals and for the high surface ratio in faeces (Naylor et al, 2007b). Additionally *E. coli* O157:H7 that are sloughed off into the mucus could replicate there further adding to the pool of bacteria for attachment and to numbers shed in faeces.

The chances of *E. coli* O157:H7 colonising at the terminal rectum will depend at least in part on the dose reaching the terminal rectum and therefore its ability to survive and replicate in the gut. The range of *E. coli* O157:H7 growth rates in rumen fluid subjected to different conditions suggests that the rumen is a critical point in determining the dose reaching the terminal rectum. When rumen protozoa were

removed from the rumen fluid, counts of *E. coli* O157:H7 stayed the same or increased but when rumen protozoa were present, counts decreased suggesting that protozoa play a role in *E. coli* O157:H7 survival. The removal of protozoa in fresh rumen did not increase replication of *E. coli* O157:H7 to the same levels as for stored rumen contents suggesting that there are other rumen constituents which affect its survival perhaps even more than protozoa. If differences between the activity of stored and fresh contents are possible in the rumen this could also apply to other parts of the gut and as the small intestinal contents in this study were stored it is possible that different rates would have been achieved in fresh contents which would affect potential shedding rates. This is unlikely to be the case for mucus however as freezing does not adversely affect its composition (Cohen and Laux, 1995; Gastaldi et al, 2000). These results are consistent with those of others in suggesting that the rumen is a potential point of die off for *E. coli* O157:H7 (Grauke et al, 2002; Zhao et al, 1998). They also confirm the hypothesis that rumen protozoa impact *E. coli* O157:H7 survival in rumen contents (Stanford et al, 2010; Thran et al, 2003) although it must be considered that other mechanisms may have greater effects. The findings support work into exploiting rumen conditions for *E. coli* O157:H7 control through for example diet and probiotics (Chaucheyras-Durand et al, 2010).

In conclusion, *E. coli* O157:H7 can replicate in gut contents and faeces in the bovine gut sufficient to cause low level passive shedding in cattle at rates seen in the field, despite a potential point of die off in the rumen. Replication in gut contents followed by replication in mucus at the terminal rectum could also account for high level shedding without colonisation and for the high proportion of bacteria on the faecal surface though it would be difficult to distinguish between passive and active shedding at that point. While proliferation in mucus could increase passive shedding the high rates of attachment and colonisation on BTRE cells suggest that once the mucus is penetrated most animals will become colonised explaining higher levels of shedding seen in the field. These results are therefore consistent with current cut-offs of shedding $>10^3 - 10^4$ C.F.U./g faeces to describe an actively colonised animal which is shedding high levels while shedding of anything below that would relate to animals which are either passively shedding or are colonised but which are increasing towards or decreasing from super-shedding in their faeces.

Chapter 3

Rates of shedding and colonisation in calves following experimental or natural challenge with *E. coli* O157:H7

3.1 Preface

Many elements of the transmission of *E. coli* O157:H7 are still poorly understood, including the risk of exposure and rates of host contact with the organism on farms. In Chapter 2 survival and replication of *E. coli* O157:H7 in the bovine gut were investigated to determine how passive transit might explain observed shedding patterns and rates of colonisation. The results showed that passive transit with replication in the gut could be responsible for low level shedding and when coupled with the exceptional replication rates in terminal rectum mucus could also account for high level shedding. High rates of attachment and colonisation on BTRE cells were also observed making it more likely that high level shedding is due to colonisation not passive shedding. A combination of low level passive shedding and high level shedding from colonised animals could present a considerable risk of exposure to in-contact animals. In this Chapter, rates of host contact with *E. coli* O157:H7 are explored through transmission experiment in which colonised and naïve calves are housed together and rates of shedding and colonisation quantified. The importance to transmission of contact with shedding animals versus a contaminated environment and the level of exposure required to cause shedding and colonisation are also investigated.

3.2 Introduction

Pathogen transmission via the faecal-oral route relies on faecal shedding of an organism into the environment by infected hosts and sufficient oral exposure of naïve hosts to infectious faeces to cause further infection and colonisation. Individual shedding rates of infected animals are critical in determining levels of the pathogen in the environment and risk of exposure to in-contact hosts. Most information on *E. coli* O157:H7 shedding rates comes from experimentally colonised animals where challenge doses of 10^9 or 10^{10} C.F.U. administered are required to achieve colonisation and persistent shedding in the majority of animals (Naylor et al, 2003; Sheng et al, 2004). In these animals shedding reaches a peak over the first few days before reducing dramatically after 14 - 21 days though shedding often continues beyond 28 days and in some cases much longer (Brown et al, 1997; Naylor et al, 2003).

Challenge doses of 10^9 or 10^{10} C.F.U. are unrealistic in terms of natural challenge as the most that has been detected in a shedding animal is 10^7 C.F.U./g faeces meaning

an animal would have to ingest 1 kg of faeces to receive a dose of 10^{10} C.F.U. of *E. coli* O157:H7 (LeJeune et al, 2001; Naylor et al, 2007b). This is substantially greater than the predicted 'typical' dose of 10^4 C.F.U. based on numbers found in the farm environment (Wood et al, 2006). It is difficult to determine the likely dose ingested by individual cattle in farm situations because host contact with faeces is heterogeneous and affected by a combination of internal factors such as behaviour and physiological status and external factors like grazing and housing management (Hutchings et al, 2001b; Smith et al, 2009). Management decisions which lead to faecal contamination on animal hides presents a risk for grazing and housed animals of exposure to pathogens through allogrooming (Mcgee et al, 2004). Additionally, when livestock are housed, faecal contamination builds up in the bedding and can also contaminate feed and water troughs and both housing and hide contamination have been linked to *E. coli* O157:H7 prevalence and transmission (Gunn et al, 2007; Stanford et al, 2011).

Dose response experiments have characterised to some extent the rate of shedding and colonisation in cattle following lower levels of exposure which would be more realistic in a farm situation. In general lower challenge doses reduce the probability of shedding and colonisation of *E. coli* O157:H7 (Besser et al, 2001; Cray and Moon, 1995; Sheng et al, 2004). None of five animals challenged with 10^4 C.F.U. shed *E. coli* O157:H7 at all in one study, while only 2 of 5 challenged with 10^7 C.F.U. shed at less than 10^2 C.F.U./g (Cray and Moon, 1995). Besser et al (2001), found that doses of <300 C.F.U. caused shedding in only 2 of 17 calves, 4 of which did not shed after 4 doses at that level. In the 2 calves that did shed, shedding lasted over 20 days reaching 10^5 C.F.U./g at one point and in-contact calves began to shed the same strain, at up to 10^7 C.F.U./g. In the same study however, challenge doses of 10^8 C.F.U. were shown to be insufficient to infect all calves (Besser et al, 2001). In another study, 18 out of 18 calves were positive on Day 1 following challenge with 10^7 C.F.U., but shedding was at lower levels ($<10^2 - 10^4$ C.F.U./g) and of shorter duration (1 positive at Day 23) than animals challenged with 10^{10} C.F.U., all of which shed at 10^4 C.F.U./g for up to 5 days or more and 8 of 10 were still positive at Day 22 (Sheng et al, 2004).

Experimental transmission studies which try to replicate the natural exposure routes either through shedding animals, contaminated water or feed also typically result in

variable detection rates and low colonisation success. Trojan animal studies result in low rates of intermittent shedding at 10^2 C.F.U./g or less although some have observed low level shedding in all in-contact animals (Besser et al, 2001; Schouten et al, 2009; Sheng et al, 2004). Colonisation and shedding at up to 10^7 C.F.U./g for up to 87 days has also been recorded in calves exposed to water which had been previously inoculated with *E. coli* O157:H7 (LeJeune et al, 2001). Addition of *E. coli* O157:H7 spiked faecal pats to pens of naïve animals at levels of 10^2 and 10^5 C.F.U./g has also been shown to cause shedding at low levels for a short period in some animals (Stephens et al, 2008). Experimental challenges normally use a mixture of strains from human or bovine origin and most are Stx⁺. At least one Trojan animal study using a Stx⁻ strain of *E. coli* O157:H7 has achieved low level shedding (<50 C.F.U./g) in 13 of 30 animals and shedding of 10^3 - 10^4 C.F.U./g by a further 2 animals (Mcgee et al, 2004). Stx is non-essential for terminal rectum colonisation in experimentally challenged animals (Sheng et al, 2006b) and similar shedding curves have been observed for animals experimentally challenged with Stx⁻ strains as with Stx⁺ ones (Naylor et al, 2003).

Contact rates of naïve cattle with *E. coli* O157:H7 either from shedding animals or contaminated environments are crucial to determining transmission dynamics. Understanding rates of passive shedding versus colonisation following ‘natural’ exposure at a range of doses and exposure routes could shed light on contact rates and important drivers of *E. coli* O157:H7 transmission.

3.2.1 Aims

The aim of this chapter is to measure rates of shedding and colonisation of *E. coli* O157:H7 in naïve calves exposed to ‘natural’ levels of challenge and compare them to those of experimentally challenged animals. Firstly rates of shedding and colonisation of *E. coli* O157:H7 in calves in the presence or absence of shedding animals will be measured. The dose-response rates in naïve calves after challenge with *E. coli* O157:H7 in the faeces of colonised animals will also be quantified. Finally shedding curves and transmission rates will be measured in calves following experimental and natural challenge with *E. coli* O157:H7 strains of two different phage types which differ in their number of Stx phages and level of Stx expression.

3.3 Methods

3.3.1 Culture and storage of bacteria

E. coli O157:H7 strains (Table 3.1) were stored and cultured as described in Section 2.3.1.

Table 3.1 *E. coli* O157:H7 strains used in Chapter 3

Strain	Characteristics	Source
Walla 3 (W3)	Stx ⁻	Lab stock, originally from (Ostroff et al, 1990)
W3 NalR	Stx ⁻ , NalR	Lab stock, derived from W3 in Chapter 2
W3 NalR, Lac ⁻	Stx ⁻ , NalR, non-lactose fermenting	This study, derived from W3 NalR
85-170	Stx ⁻	Lab stock, originally from (Tzipori et al, 1987)
85-170 NalR	Stx ⁻ , NalR	This study, derived from 85-170
85-170 NalR, Lac ⁻	Stx ⁻ , NalR, non-lactose fermenting	This study, derived from 85-170 NalR
PT21/28 9000 Strain	From high shedding animal (10 ⁵). Low T3S. Contains Stx2 and Stx2c.	Lab stock, originally from (Xu et al, 2012)
PT21/28 9000 NalR Strain	From high shedding animal (10 ⁵). Low T3S. Contains Stx2 and Stx2c. NalR	This study, derived from 9000
PT32 10671 Strain	From low shedding animal (<50). High T3S. Contains Stx2c only.	Lab stock, originally from (Xu et al, 2012)
PT32 10671 NalR Strain	From low shedding animal (<50). High T3S. Contains Stx2c only. NalR	This study, derived from 10671

3.3.2 Derivation of antibiotic-resistant strains

Spontaneously Nalidixic Acid-resistant (NalR) isolates of strains were obtained as described in Section 2.3.2. To obtain non-lactose fermenting isolates of NalR strains overnight cultures were centrifuged briefly at 17,000 x g, the pellet re-suspended in the remaining medium and plated onto minimal arabinose plates (400 ml distilled H₂O (dH₂O) containing 7.5 g agar (Melford, Chelworth, Ipswich, UK), 100 ml 5X M9 salts (Formedium, Hunstanton, UK), 1 ml 1 M MgSO₄ (Sigma-Aldrich), 10 ml 20 % arabinose (Melford), 2.5 ml 1 M IPTG (Biolone, London, UK)) and 0.5 ml

Nalidixic Acid at 15 mg/ml). Minimal arabinose plates were incubated statically at 37°C for 48 h until a number of large colonies had grown. These colonies were then streaked individually onto further minimal arabinose plates and incubated statically at 37°C for 24 hours. Finally purified colonies from these plates were streaked onto MacConkey agar (Oxoid) plates with 15 µg/ml Nalidixic Acid and incubated statically at 37°C for 16 h. Those colonies that appeared pale on MacConkey agar, instead of purple/red like the parent strain, were kept and frozen down as described in Section 3.3.1. The parent lactose-positive and the derived lactose-negative strains were then streaked on either half of an LB plate and incubated statically at 37°C for 16 h to check that the growth rates and morphologies were comparable.

3.3.3 Polymerase chain reactions (PCR) to confirm presence or absence of Stx phage-encoding genes

All strains used in the experiments were screened using PCR prior to use to confirm either the presence of Stx phage-encoding genes in Stx⁺ strains or the absence of these genes in Stx⁻ strains. Briefly, 1 colony of each strain was suspended in 100 µl dH₂O and boiled for 10 min at 95°C to produce crude lysates. Reaction mixtures contained 1X DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Loughborough, UK), 0.2 µM primer, made up to 20 µl with molecular water (Sigma-Aldrich). Primers used are shown in Table 3.2. PCR reactions were undertaken in a Thermo-Hybrid PCR Express thermocycler (Thermo Fischer Scientific), with the following program: 30 cycles of the sequence 94 °C for 2 min; 94 °C for 30s; 50 °C for 30s and 68 °C for 1 min followed by holding at 4 °C. PCR products were then electrophoresed on a 1.5 % agar gel at 100 V for 60 min with a DNA ladder to check the PCR products.

Table 3.2 Primers used in this study

Gene	Primer	Source
eaeAF	gaccggcacaagcataagc	(Paton and Paton, 1998)
eaeAR	ccacctgcagcaacaagagg	(Paton and Paton, 1998)
stx1F	ataaatcgccattcgttgactac	(Paton and Paton, 1998)
stx1R	agaacgcccactgagatcatc	(Paton and Paton, 1998)
stx2 5'	gcggttttattgcattagc	(Wang et al, 2002)
stx2 3'	tcccgtaaccttactgta	(Wang et al, 2002)
stx2c 5'	gcggttttattgcattagt	(Wang et al, 2002)
stx2c 3'	agtactctttccggccact	(Wang et al, 2002)

3.3.4 Screening of calves for wild-type *E. coli* O157:H7 by Immunomagnetic Separation (IMS)

IMS was conducted as has been described previously (Low et al, 2005). Briefly, 1 g samples of faeces were added to bottles of 20 ml Buffered Peptone Water (Oxoid) and shaken vigorously before being incubated for 6 hours at 37°C. A positive control sample (known *E. coli* O157:H7 strain) and a negative control (no added material) were included as well. Following incubation 1 ml from each bottle was added to a screw-top 1.5 ml tube containing 20 µl of para-magnetic beads (Lab-M, Heywood, UK). With lids firmly attached tubes were gently inverted 3-5 times to re-suspend the beads. The tubes were then fixed to a rotating mixer and mixed for 30 minutes at RT. Following mixing tubes were placed in a magnetic separator for 5 minutes to concentrate the beads on one side of the tube. The tubes were then rotated gently 3 times to further concentrate the beads. The liquid in the tube was then removed carefully using a pastette without disturbing the beads. The magnet was removed from the separator and 1 ml of sterile PBS (10 mM PBS pH 7.4) containing Tween 20 0.05 % v/v (Sigma) added. The magnet was replaced and the beads again attracted on to the side of the tube for 5 min. The wash step was repeated twice more, the liquid removed and the beads re-suspended in 50 µl PBS-T. The bead suspension was then spread onto a pre-prepared Sorbitol MacConkey agar plate supplemented with 0.050 µg/ml cefiximine and 2.5 µg/ml tellurite (CT-SMAC) plate (E&O Laboratories, Falkirk, UK) and incubated statically at 37°C for 16 h. Colonies on the plate were then tested with latex agglutination to confirm they were *E. coli* O157:H7. The limit of detection of this technique is between 10¹ and 10² C.F.U./g (Besser et al, 2001).

3.3.5 Experiments 1 and 2: Transmission and Dose-Response of Stx⁻ strains

3.3.5.1 Stx⁻ strains

The Stx⁻ strains used in this experiment were Walla 3 (W3), a PT 32 strain isolated from a human case of *E. coli* O157:H7 (Ostroff et al, 1990), which had spontaneously lost its Stx phage and 85-170, which was also isolated from a human and then spontaneously lost its Stx phage during laboratory storage (Tzipori et al, 1987). Previous work by this group and others have demonstrated that Stx⁻ strains can colonise calves at the terminal rectum following experimental challenge and these were chosen for use as the lower containment requirements allow for larger

experiments and are considerably less expensive (McNeilly et al, 2008; Sheng et al, 2006b).

3.3.5.2 Calves

42 dairy calves aged between 4 and 5 months old were purchased and housed together for 7 days to acclimatise. Calves were tested for wild-type *E. coli* O157:H7 using Immunomagnetic Separation (Section 3.3.4) and any positives removed. Calves that were IMS negative on two occasions were randomised by age and allocated into test pens for the experiment.

3.3.5.3 Preparation of Stx⁻ strains for challenge

Firstly the four challenge strains (lactose positive and negative Nal^R isolates of the W3 and 85-170 strains, Table 3.1) were streaked out from frozen stocks onto LB plates and incubated statically at 37°C for 16 h. Starter cultures were created by adding 4 colonies from each strain into individual universals of 5 ml LB broth. Colonies were confirmed to be *E. coli* O157:H7 by latex agglutination. Cultures were incubated at 37°C with shaking at 200 rpm for 6 h. Following the 6 h incubation 1 ml of each sub-culture from a single strain was incubated in 350 ml LB at 37°C for 18 h with shaking at 80 rpm. These cultures formed the challenge inocula. A sub-sample was frozen down in 25% glycerol at -70°C and 5 ml aliquots produced for oral calf challenge and 1 ml for rectal challenge. Each challenged calf was given per os a total of 10 ml of culture containing 1 – 2 x 10¹⁰ bacteria. To confirm this challenge cultures were serially diluted and plated as described previously using LB and pre-prepared MAC-Nal plates (E&O Laboratories).

3.3.5.4 Calf challenge

To challenge calves, the calf was restrained and a stomach tube passed into the oesophagus. 10 ml of inoculum was then added to the stomach tube bag and followed with 500 ml sterile PBS. Challenged calves were dosed with a mixture of either the lactose fermenting W3 and 85-170 cultures or the non-lactose fermenting W3 and 85-170 cultures. As well as oral dosing, a rectal swab dipped in 1 ml of the challenge culture was rubbed onto the mucosal surface of the rectum of each challenged calf (Naylor et al, 2007b; Sheng et al, 2004).

3.3.5.5 Collection of faecal and environmental samples

To collect faecal samples, defecation was stimulated by digital manipulation of the anal sphincter. Samples were initially placed in a sterile plastic bag and mulched together to homogenise the faeces so that bacteria both on the surface and inside the pat could be detected. 10 g of faeces from the bag was then weighed into 90 ml pots of sterile PBS and shaken to mix. After 1 minute standing time, a sub-sample of ~ 5 ml was transferred into labelled bijoux. Environmental samples were taken using strips of sterile tubular bandage placed over clean Wellington boot covers which were worn while walking around the pen (Cobbaut et al, 2008; Widgren et al, 2013). The two pieces of tubular bandage for each floor sample were then placed into 90 ml PBS and shaken to mix. A sub-sample of ~ 5 ml of each mix was then transferred into labelled bijoux. Samples in bijoux were taken to the laboratory for enumeration of *E. coli* O157:H7.

3.3.5.6 Enumeration of *E. coli* O157:H7 in samples

To enumerate *E. coli* O157:H7 the faecal and environmental samples from Experiment 1 and faecal samples from Experiment 2 were serially diluted and 100 µl of each dilution spread onto pre-prepared MAC-Nal plates (E & O Laboratories), in triplicate per dilution, and plates incubated statically at 37°C for 16 h. Colonies on each plate were counted and latex agglutination used to confirm that colonies detected on the plates were *E. coli* O157:H7. Plates with up to a maximum of 500 colonies were recorded, but in some situations if further dilutions were unavailable then higher counts were recorded. The limit of detection for this enumeration method using marked strains is 10² C.F.U./g faeces (LeJeune et al, 2006). In Experiment 2, samples were also subjected to broth enrichment (BE). This was done by adding the remaining contents of the bijoux (5 ml of 1:10 faecal suspension) to 10 ml of Tryptone Soya Broth (TSB) (Oxoid) containing 15 µg/ml Nalidixic Acid and incubating statically at 37°C for 16 h. Samples were then streaked onto SMAC-Nal plates and incubated 37°C for 16 h. Any resulting colonies were tested using an *E. coli* O157 Latex Test Kit and a positive BE test was recorded as 10 C.F.U./g faeces for the purposes of analysis. The limit of detection for broth enrichment techniques is between 10¹ and 10² C.F.U./g and is more sensitive when using marked strains than IMS (Besser et al, 2001).

3.3.5.7 Experiment 1 – Transmission of Stx⁻ strains

The objective of this experiment was to study the shedding curves of calves ‘naturally’ challenged with *E. coli* O157:H7 through exposure to experimentally colonised pen mates and/or contaminated environments. First ‘donor’ animals were created by orally and rectally challenging 4 animals in Pen 1 with Lac⁺ NalR variants of *E. coli* O157:H7 strains W3 and 85-170 and 4 animals in Pen 2 with Lac⁻ derivatives of the same strains. These calves were left in these pens for 5 days to become colonised and shed bacteria into the environment. Faecal samples were taken daily from Day 3 post challenge; after natural flow-through of the challenge dose had occurred. On Day 5 post challenge the 3 highest shedding animals in each pen were exchanged with the calves in the other pen and 3 naïve ‘sentinel’ animals added to each pen. For the Lac⁻ challenged animals, only 2 of 4 calves were shedding *E. coli* O157:H7 on Day 5 so one of the 2 non-shedding animals was transferred with the 2 shedding animals.

Daily faecal samples were taken from all calves, from the day of mixing, for 8 days for enumeration of *E. coli* O157:H7 by direct plating. Pens were left to accumulate faecal matter to a level that would be normal on a farm and environmental samples were collected daily to estimate the bacterial load in the environment. Any sentinel calves that began shedding the organism during the sampling period were removed from the pen and replaced with a naïve calf to maintain the ratio of shedding to non-shedding calves. The positive sentinel animals were faecal sampled on the day of removal and on Days 3 and 7 after removal to determine whether they had become colonised or if the faecal bacteria were more likely to be from passive shedding.

3.3.5.8 Experiment 2 – Dose response with Stx⁻ strains

To provide further information on the natural challenge dose required to cause shedding and colonisation in cattle, a dose response experiment was conducted. This was done by challenging calves with one of 3 doses of *E. coli* O157:H7 collected from the faeces of colonised animals. To provide the challenge dose 2 ‘donor’ calves were experimentally challenged rectally and orally with Lac⁺ NalR *E. coli* O157:H7 strains W3 and 85-170. The challenged calves were sampled daily until peak shedding at around 5 days post challenge. On Day 5 the calves were fitted with faecal collection bags for 24 hours to collect *E. coli* O157:H7 contaminated faeces. The collected faeces was mixed together, weighed and using the previous day’s

counts then diluted with sterile PBS to create 3 dose levels of approximately 10^7 , 10^5 and 10^3 C.F.U. per 500 ml. For the experiment, 18 calves were randomly allocated into one of three different groups. The six allocated calves for each group were then dosed with the appropriate challenge dose faecal suspension via stomach tube. In the case of the higher dose extra PBS and water was required to move the faecal suspension down the tube. Faecal samples were collected from calves daily for 14 days for enumeration of *E. coli* O157:H7 in the faeces by both direct culture and broth enrichment.

3.3.6 Experiment 3: Transmission of Stx⁺ strains

3.3.6.1 Stx⁺ strains

The Stx⁺ strains used were originally isolated from bovine faecal pats collected in a field study (Chase-Topping et al, 2007). One strain was known to be PT 21/28, the most commonly seen PT in cattle and humans in Scotland, and the other was PT 32, a less commonly seen PT in cattle which is also less frequently isolated from humans (Table 3.1). While both have similar single-nucleotide polymorphisms (SNPs), these strains show differences in levels of Stx and T3S production and in their likelihood to shed at high levels (Chase-Topping et al, 2007; Xu et al, 2012). PT 21/28 strain 9000 contains the Stx2 and Stx2c phages, has a low T3S secretor profile, and was collected from the pat of a high shedding animal (686,400 C.F.U./g from pat). PT 32 strain 10671 contains only Stx2c, has a high T3S profile, and was isolated from a low shedding animal (<50 C.F.U./g from pat) (Chase-Topping et al, 2007; Xu et al, 2012).

3.3.6.2 Calves

16 dairy calves aged between 8 and 10 weeks old were purchased and housed together for 1 week to acclimatise. Faecal samples were collected from the calves and IMS performed to detect any animals shedding wild-type *E. coli* O157:H7 which were then removed (Section 3.3.4). Once the animals had been confirmed negative by IMS, the calves were split into 2 groups of 6 randomised by age and moved into one of two high containment rooms in the Containment Level 3 (CL3) facility.

3.3.6.3 Preparation of Stx⁺ strains for challenge

Preparations were made of the wild-type and NaIR derivatives of PT 21/28 strain 9000 and PT 32 strain 10671 (Table 3.1). Colonies of the challenge strains were

inoculated into flasks of LB broth and incubated at 37°C with shaking at 200 rpm for 16 h before being split into 10 ml aliquots for challenge.

3.3.6.4 Calf challenge

Calf challenge was undertaken only by oral dosing as described in Section 3.3.5.4. Each calf received a mixture of both the wild-type and the Nal^R versions of either the PT 21/28 strain (Room1) or the PT 32 strain (Room 2) in a final dose of 1×10^{10} C.F.U.

3.3.6.5 Collection of faecal samples

Samples were collected from the rectum and laid on clean paper towels. 10 g of surface faeces was weighed out by scraping the surface (S) with wooden spatulas. If the faeces could not be separated, a 10 g sample of whole (W) faeces was weighed. Samples were then transferred from weigh boats to 150 ml pots containing 90 ml sterile PBS. After shaking vigorously, pots were left to stand for 1 min and a sub-sample transferred to a labelled bijoux. Samples were then transferred to the laboratory for enumeration of *E. coli* O157:H7.

3.3.6.6 Enumeration of *E. coli* O157:H7 bacteria from faecal samples

Enumeration of bacteria from faecal samples was undertaken in Containment Level 3 facilities and methods were as previously described (Section 3.3.5.6) except that pre-prepared CT-SMAC and SMAC-Nal plates (E & O laboratories) were used. If a sample was negative by direct plating on both sets of plates, the subsequent sample from the same animal was subjected to broth enrichment as previously described (Section 3.3.5.6).

3.3.6.7 Experimental design

The aim of this experiment was to determine rates of shedding and transmission of PT21/28 and PT3 *E. coli* O157:H7 strains. The experiment was undertaken under Containment Level 3 (CL3) conditions in 2 rooms with 6 calves in each room. Once the calves had been confirmed negative by IMS, they were randomly sorted into 2 groups of 6, each of which was moved into a sealed room in the CL3 facility. After a day of acclimatisation, 4 calves in Room 1 were challenged with both the wild-type and a Nal^R derivative of the PT 21/28 strain and 4 calves in Room 2 were challenged with the wild-type and a Nal^R derivative of the PT 32 strain (Table 3.1). Each challenge calf received 1×10^{10} C.F.U of the bacteria. The remaining 2 calves in

each pen were sham-challenged with PBS only. Faecal sampling commenced on Day 3 post challenge to give time for natural flow-through of challenge dose. Animals were sampled daily from Day 3 to Day 11 and then every other day until Day 27 post challenge. The NaIR strains were used in case contaminant bacteria made it too difficult to obtain counts of the wild-type strain on CT-SMAC plates. However, counts on CT-SMAC plates were possible and as they included both variants of each strain only these counts were used for the results and analyses.

3.3.7 Ethics

All challenges and transmission experiments were performed at the Moredun Research Institute, authorised by Home Office licence 60/3179. Ethical approval was obtained from the Moredun Research Institute Animal Experiments Committee.

3.3.8 Statistical analysis

Counts from plates were recorded and those from multiple dilutions used to calculate the most probable number of C.F.U. by fitting generalised linear models with a Poisson error distribution and logarithmic link function incorporating the logarithm of dilution as an offset variable. Final counts were then log-transformed using the formula $\text{Log}_{10}(\text{C.F.U.}+1)$ and statistical analysis performed on the Log_{10} counts. Statistical analysis was performed, where possible, using the statistics package Genstat (15th ed). For Experiment 3, faecal shedding data from the 12 calves were used to calculate the total area under the curve (AUC) for each calf and these, along with the peak count for each calf, were analysed. Both the Log_{10} total AUC and peak counts for each calf were compared in separate two-way ANOVAs with the treatment factors Phage Type (PT) and Challenge type, and Animal as blocks.

3.4 Results

3.4.1 Experiment 1: Transmission of Stx⁻ *E. coli* O157:H7 strains

Faecal shedding curves for calves in Pens 1 and 2 and level of environmental contamination in each pen are shown in Figures 3-1 and 3-2. In Pen 1 three calves were experimentally challenged on Day 0 with Lac⁺ strains to seed the environment. All challenged calves were shedding >10³ C.F.U./g faeces and the Lac⁺ strains were detected in the environment from the first day of sampling (Figure 3-1). On Day 5 the Lac⁺ calves were replaced with 3 calves from Pen 2 that had been challenged with Lac⁻ *E. coli* O157:H7 and 3 naïve calves. Following the day of transfer the 2 calves that were shedding Lac⁻ *E. coli* O157:H7 shed the strains in their faeces daily for up to 8 days and the strains were detected in the environment one day after the calves had been introduced. Neither the 3rd challenged calf nor any of the 3 naïve sentinel animals shed detectable levels of *E. coli* O157:H7 during the experiment.

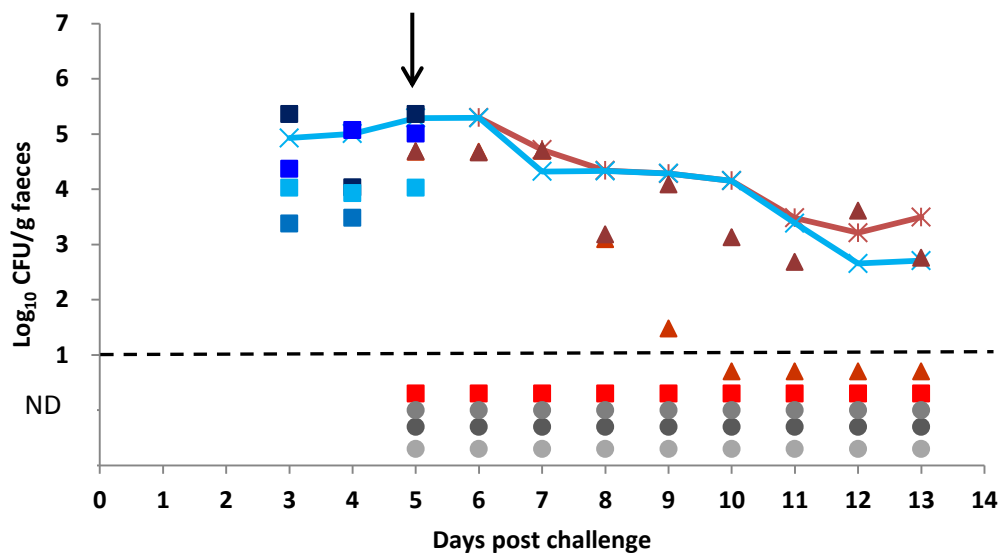


Figure 3-1 Faecal counts and environmental levels of *E. coli* O157:H7 Pen 1

Faecal shedding curves from calves in Pen 1 during Experiment 1 (log₁₀ C.F.U./g). The blue squares represent calves that were challenged on Day 0 with Lac⁺ *E. coli* O157:H7, with the different shades representing different calves. On Day 5 (represented by the arrow), the 3 highest shedding challenged calves were moved to Pen 2 and replaced with 3 calves that had been challenged with Lac⁻ *E. coli* O157:H7 in Pen 2 (red triangles). 3 naïve animals were also added as sentinels for transmission (grey circles). Environmental levels of Lac⁺ (blue line) and Lac⁻ *E. coli* O157:H7 (red line) over the period of the experiment are also shown. Points below the dotted line represent samples where no *E. coli* O157:H7 was detected.

Environmental levels of both the original Lac⁺ and introduced Lac⁻ *E. coli* O157:H7 strains were highest when shedding was highest in the calves in Pen 1 and declined steadily over the sampling period along with shedding in the calves.

In Pen 2, only 2 of the 4 calves challenged with Lac⁻ strains of *E. coli* O157:H7 on Day 0 began shedding the bacteria in their faeces (Figure 3-2). Both calves shed at levels of between 10³ and 10⁵ C.F.U./g faeces up to Day 5 when they were moved to Pen 1 and the Lac⁻ strains were detected in the environment from Day 3. Following their transfer into Pen 2 the 3 calves shedding Lac⁺ strains shed at levels up to 10⁶ C.F.U./g faeces for several days and strains were detectable in the environment one day after mixing. One of the in-contact sentinel calves shed the Lac⁺ strains from in-contact calves, on the third day after mixing at a level of 10² C.F.U./g. This animal was removed to an individual pen to monitor for colonisation and a new naïve animal added to Pen 2. The positive calf was sampled again on the 1st, 3rd and 7th day following its removal and was negative on all occasions. The other sentinel calves did not shed the organism at a detectable level during the study.

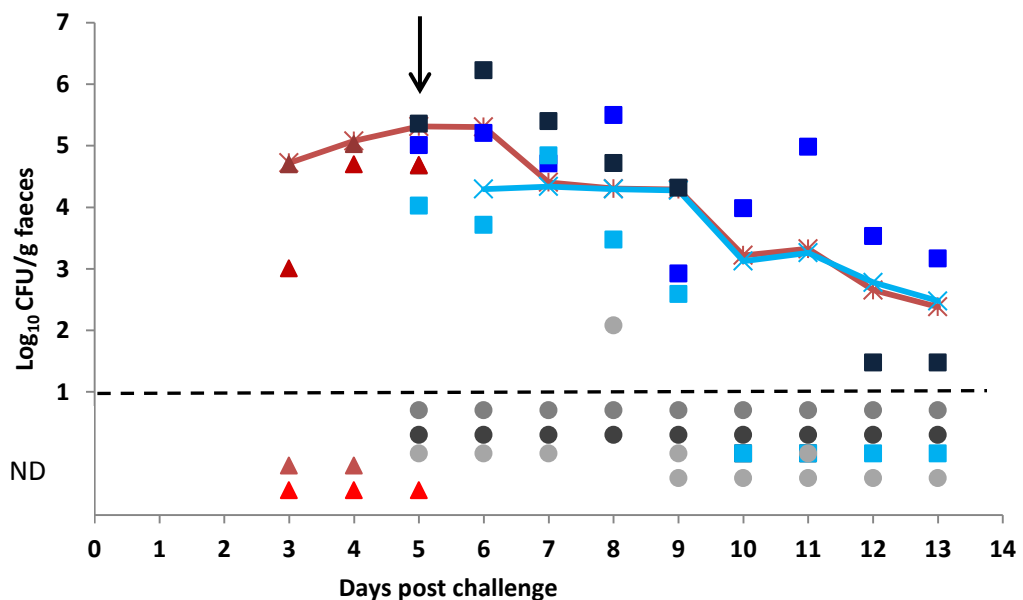


Figure 3-2 Faecal counts and environmental levels of *E. coli* O157:H7 Pen 2

Faecal shedding curves from calves in Pen 2 during Experiment 1 (log₁₀ C.F.U./g). The red triangles represent calves that were challenged on Day 0 with Lac⁺ *E. coli* O157:H7, with the different shades representing different calves. On Day 5 (represented by the arrow), the 2 shedding calves and one other were moved to Pen 1 and replaced with 3 calves that had been challenged with Lac⁻ *E. coli* O157:H7 in Pen 1 (blue squares). 3 naïve animals were also added as sentinels for transmission (grey circles). Environmental levels of Lac⁺ (blue line) and Lac⁻ *E. coli* O157:H7 (red line) over the period of the experiment are also shown. Points below the dotted line represent samples where no *E. coli* O157:H7 was detected.

As in Pen 1, environmental levels of both the original Lac⁻ and introduced Lac⁺ strains peaked at the same time as shedding in the calves and then steadily decreased over the study.

3.4.2 Experiment 2: Dose response with Stx⁻ *E. coli* O157:H7 strains shed by colonised animals

No calves in any of the three groups dosed with contaminated faeces shed *E. coli* O157:H7 at levels detectable by direct plating or enrichment over 14 days.

3.4.3 Experiment 3: Transmission of Stx⁺ *E. coli* O157:H7 strains

The shedding curves from counts on CT-SMAC plates of the calves in Rooms 1 and 2 are shown in Figure 3-3 and Figure 3-4 respectively. In Room 1 (Figure 3-3), the 4 challenged calves (C) shed *E. coli* O157:H7 at levels above 10⁴ C.F.U./g faeces for at least 7 days post challenge. After Day 10 post-challenge, shedding levels in two calves declined to undetectable levels but one calf began shedding again and continued to do so until the final sampling day. The other two calves shed *E. coli* O157:H7 every day until the last day of sampling. The sentinel calves in Room 1 were both shedding on the first day of sampling at around 10⁴ C.F.U./g faeces and followed a similar shedding pattern to the challenged calves with a peak of shedding at 7 days before declining. In one sentinel calf shedding declined to undetectable levels while in the other, shedding began to rise again towards the end of the study.

In Room 2 (Figure 3-4), the 4 challenged calves (C) also shed above 10⁴ C.F.U./g faeces for at least 7 days post challenge. Shedding by all 4 calves declined to undetectable levels around Day 15 before increasing again for a second peak around Day 20. As in Room 1, both sentinel calves began to shed positive for *E. coli* O157:H7 however at much lower levels. One sentinel calf shed consistently over the whole sampling period while the other was only positive by direct count once and positive by enrichment on 3 other occasions.

The results from the sentinel calves in Room 2 may be slightly misleading as background flora on the CT-SMAC plates for both calves caused problems with counting. For one calf the CT-SMAC plates tended to contain, as well as *E. coli* O157:H7 colonies, other pale colonies which could be mistaken for *E. coli* O157:H7 but which when tested were not. The other sentinel calf had a considerable background flora which made it difficult to count colonies and it is likely that on Days 4 and 5, where this calf was positive by enrichment only, that these counts were higher. The NaIR results for this calf showed a peak of shedding over Day 5 to Day 8 before dropping to 0 as seen on the CT-SMAC plates (data not shown).

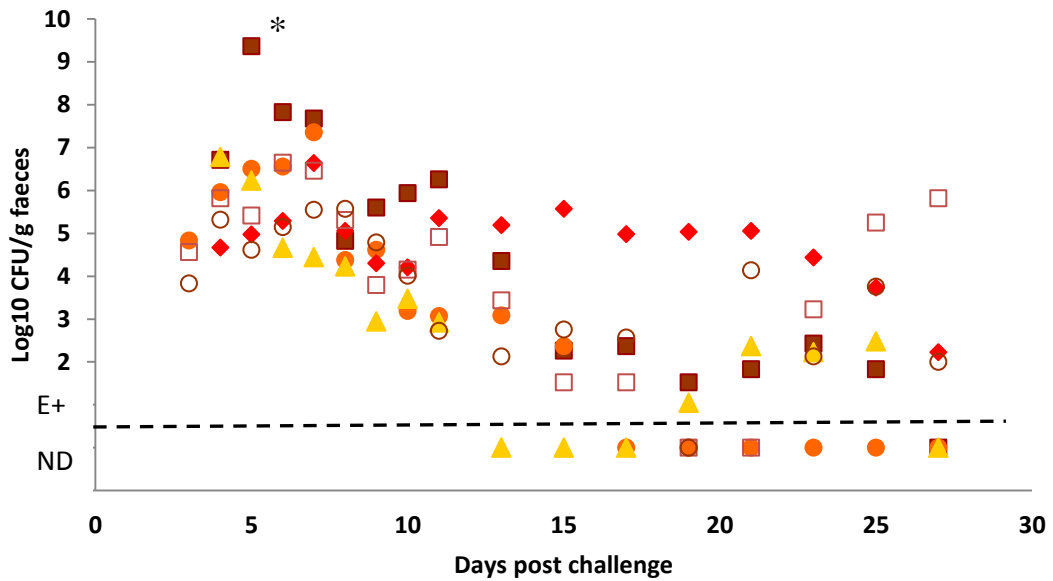


Figure 3-3 Daily faecal counts of PT 21/28 *E. coli* O157:H7 in Room 1 calves

Shedding curves of PT 21/28 *E. coli* O157:H7 in Room 1 calves (\log_{10} C.F.U./g). Solid points represent challenged calves and un-filled points represent sentinel calves. The unusually high data point at * was from an animal which had no faeces present in its rectum and only a small volume of rectal mucus could be obtained for culture. Some Day 3 counts were not possible due to high numbers on plates. On Day 7 there was a missing value for one calf (yellow triangles) and the average of the previous and next days' counts was used. E+ counts were enrichment positive only ($<10^2$ C.F.U./g) and ND stands for 'Not detected'.

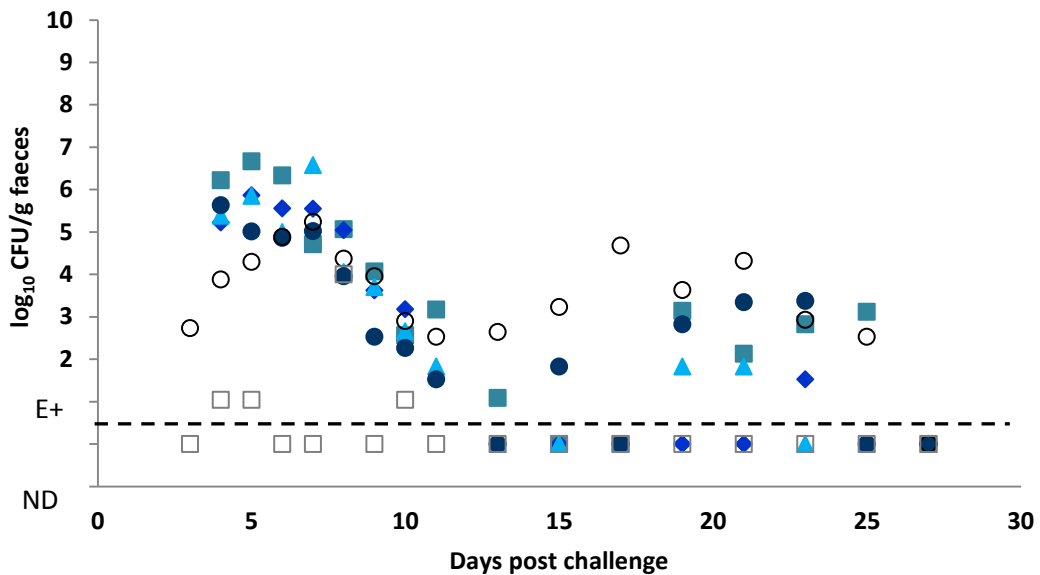


Figure 3-4 Daily faecal counts of PT 32 *E. coli* O157:H7 in Room 2 calves

Shedding curves of PT 32 *E. coli* O157:H7 in Room 2 calves (\log_{10} C.F.U./g). Solid points represent challenged calves and un-filled points represent naïve sentinel calves. Some Day 3 counts were not possible due to high numbers on plates. On Day 13 there was a missing value for one calf (blue squares) and the average of the previous and next days' counts was used. E+ counts were enrichment positive only ($<10^2$ C.F.U./g) and ND stands for 'Not detected'.

3.4.4 Comparison of shedding level between PT 21/28 and PT32 strains and between experimentally and naturally infected calves in Experiment 3

The total AUC and peak count for each calf in Experiment 3 were calculated and the mean of each for the groups of challenged and sentinel calves of each phage type were compared (Figure 3-5). There was a significant difference between total AUCs for each main effect of phage type ($F 1, 8 = 7.24, P < 0.05$) and challenge type ($F 1, 8 = 7.37, P < 0.05$) but not the phage type*challenge interaction ($P > 0.5$). There was also a significant difference between peak counts for phage type ($F 1, 8 = 10.26, P < 0.01$) and challenge type ($F 1, 8 = 11.94, P < 0.01$) but not phage type*challenge interaction ($P > 0.5$).

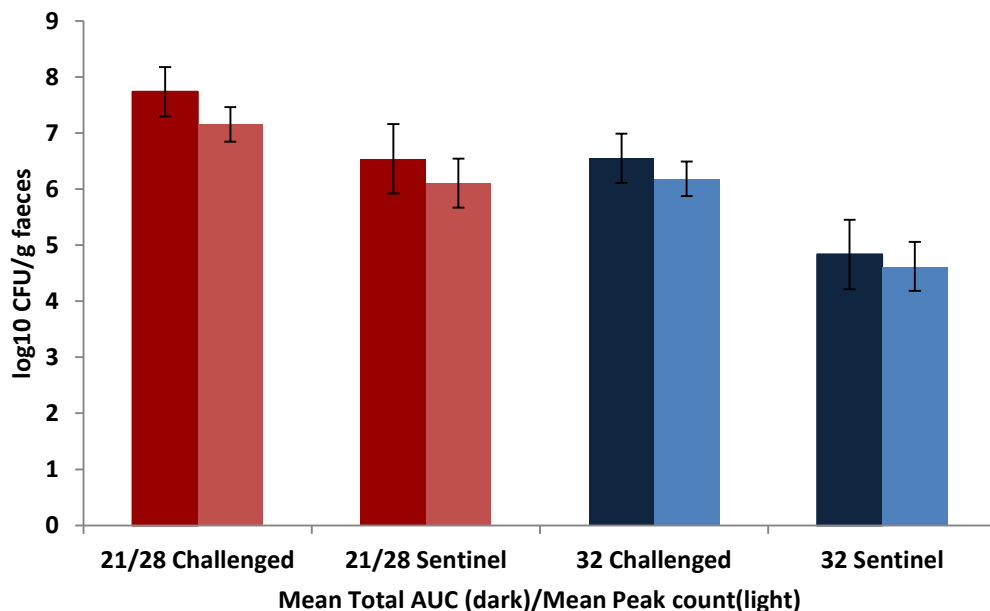


Figure 3-5 Comparison of AUCs and peak counts of calves by PT and challenge type

Mean total AUC and peak count with standard errors for each calves challenged or naturally exposed to (sentinel) PT 21/28 strains (dark and light red columns respectively, SED of PT*challenge interaction = 0.755) and PT32 strains (dark and light blue columns respectively, SED of PT*challenge interaction = 0.531) in Experiment 3 in \log_{10} C.F.U./g faeces.

3.5 Discussion

This Chapter aimed to investigate the transmission dynamics of *E. coli* O157:H7 in cattle by measuring rates of faecal shedding and colonisation in calves following experimental or ‘natural’ challenge. In Experiment 1, 5 of 6 experimentally challenged ‘donor’ calves shed at high levels ($<10^4$ C.F.U./g faeces) for several days and can be considered colonised; yet only 1 in-contact naïve calf shed 10^2 C.F.U./g on a single occasion, most likely the result of passive shedding. The sensitivity of direct plating is 10^2 C.F.U./g faeces and it is possible that there was passive shedding below this level in the other naïve calves which would not have been detected. In Experiment 2, faecal samples were subjected to both direct plating and enrichment to detect *E. coli* O157:H7 shedding down to between 10^1 and 10^2 C.F.U./g faeces, yet not even this level of shedding was observed following oral dosing of calves with faeces containing up to 10^7 C.F.U. of the organism. Previous studies in which doses of $<10^7$ C.F.U. of Stx⁺ *E. coli* O157:H7 strains were given have generally seen some level of shedding in a proportion of animals and at times high level shedding (Besser et al, 2001; Cray and Moon, 1995; Sheng et al, 2004). The absence of even low level faecal shedding, except in one animal, in Experiments 1 and 2 in this Chapter using Stx⁻ strains suggests that strains without Stx are at a disadvantage to Stx⁺ strains.

In Experiment 3 Stx⁺ strains of 2 different phage types were detected 3 days post challenge in the faeces of all in-contact naïve calves, which is in contrast to the Stx⁻ strains used in Experiments 1 and 2. In three in-contact calves shedding continued until the end of the study and rose to a second peak at the same time as challenged animals’ shedding was reducing suggesting that they had become colonised, rather than being passive shedders of the organism. Shedding in the 4th calf was of shorter duration as it cleared the organism within 10 days and counts were confounded by background flora. This animal still shed on a number of days at levels up to 10^5 C.F.U./g suggesting that it may also have been colonised at the terminal rectum. For both strains used in Experiment 3 faecal shedding was significantly higher, measured as overall levels and by peak shedding, in the experimentally challenged animals than in those animals that naturally acquired the organism. However the duration, in all but one of the naturally challenged calves, matched the experimentally challenged ones.

Experimental challenges typically use doses of 10^9 or 10^{10} C.F.U. to obtain a high rate of colonisation in challenged animals (Cray and Moon, 1995; Naylor et al, 2007b; Sheng et al, 2004). Such doses of *E. coli* O157:H7 are unrealistic in terms of what cattle are likely to be exposed to on farms. While the dose ingested by the in-contact calves in either Experiment 1 or Experiment 3 cannot be determined, this study shows that transmission of Stx⁺ strains leading to colonisation and persistent shedding with was possible from natural exposure routes compared to Stx⁻ strains where there was no appreciable shedding at all.

In Experiment 3, a significant difference was observed in faecal shedding between the phage types with animals shedding PT 21/28 strains at higher overall and peak levels than those shedding PT 32 strains. This is consistent with the observed prevalence of these strains in Scotland as PT 21/28 is the most common accounting for 46 % of strains in cattle and 58 % of human cases and PT 32 strains are the next most common in cattle accounting for 19 % of cattle strains, though they are uncommon in humans (Chase-Topping et al, 2007). Experiment 3 represents the first characterisation of shedding curves for these phage types and confirms bacterial differences that are consistent with the level of shedding observed in the field where PT 21/28 are more likely to be associated with high counts of bacteria in faecal pats than PT 32 (Chase-Topping et al, 2007).

Up to 99 % of *E. coli* O157:H7 strains are Stx positive and strains without Stx are rarely seen in cattle (Chase-Topping et al, 2007). Various hypotheses have been put forward to explain the advantage to *E. coli* O157:H7 that having a Stx phage provides to colonisation of cattle. For example the toxin may enhance survival and replication in the gut, or aid in the evasion or subversion of the immune system (Baines et al, 2008; Robinson et al, 2006; Xu et al, 2012). A key difference between phage types 21/28 and 32, is that 21/28 strains have both the Stx2 and Stx2c genes while 32 strains only carry the Stx2c gene. Level of Stx2 present has been associated with virulence in gnotobiotic pigs and Stx2 is considered one of the most important virulence factors in causing HUS in humans (Baker et al, 2007; Boerlin et al, 1999). The presence of Stx2 could explain why PT 21/28 strains are associated with more severe human disease leading to HUS more often than other phage types (Lynn et al, 2005). Further analysis of PT 21/28 and 32 strains of *E. coli* O157:H7 have shown

that the presence of Stx2 in 21/28 affects the expression of the T3S system which could aid colonisation both in cattle and humans (Xu et al, 2012).

It may be concluded from these experiments that Stx⁻ strains, while capable of colonising after high level oral or rectal challenge, are not effective at transmitting to naïve animals at lower doses that would reflect normal farm conditions. Stx⁻ strains, while suitable for use in models for vaccine trials, are less suitable for exploring natural transmission of *E. coli* O157:H7. In comparison transmission of Stx⁺ strains occurred quickly and effectively suggesting that possession of the Stx phage provides a benefit for transmission to *E. coli* O157:H7 in cattle. Different types and combinations of Stx may have varying effects and these differences may be due to regulatory aspects as much as the immediate impact of the toxin.

Chapter 4

Rates of replication and colonisation in the bovine gut of *E. coli* O157:H7 strains with and without Stx

4.1 Preface

Successful transmission of a pathogen relies on a combination of exposure of a naïve host to a sufficient dose to cause a new infection and the ability of the pathogen to survive in and infect the new host. Chapter 3 examined aspects of host contact with *E. coli* O157:H7 in a housed environment through transmission and dose-response experiments and also shed light on strain differences which could be important for understanding transmission. Calves in contact with high levels of Stx⁻ *E. coli* O157:H7 in the environment and shed by pen-mates as well as calves experimentally challenged with up to 10⁷ C.F.U. of Stx⁻ strains in the faeces of colonised animals shed no bacteria in their faeces. This was in stark contrast to high level shedding within 3 days by in-contact calves housed with animals shedding Stx⁺ *E. coli* O157:H7 strains indicating that possession of Stx contributes to the transmission success of *E. coli* O157:H7 in cattle. In this Chapter, survival and colonisation in the bovine gut by Stx⁺ and Stx⁻ strains is compared to determine the advantages conferred by Stx phage possession for *E. coli* O157:H7 strains in cattle.

4.2 Introduction

E. coli O157:H7 in humans has a low infectious dose with less than 100 organisms needed to cause disease (Karmali, 2004) and Shiga toxin (Stx) is considered an essential virulence factor in humans (Mauro and Koudelka, 2011). In contrast cattle, the main reservoir for human infection, shed *E. coli* O157:H7 asymptotically and Stx is not essential for terminal rectum colonisation (Sheng et al, 2006b). Despite not being essential for colonisation, the genes for Stx are present in almost all cattle strains of *E. coli* O157:H7 and therefore possession of a Stx phage presumably provides some advantage to maintenance of these strains (Chibani-Chennoufi et al, 2004; Paiba et al, 2002). Stx-encoding phage and bacteria which carry them are ubiquitous and found in places lacking the ‘assumed’ mammalian host of the bacteria and many Stx⁺ bacterial strains are non – pathogenic suggesting that the phage provides other advantages not related to virulence (Baker et al, 2007; Ritchie et al, 2003). In situations of predation or competition, lysis of a sub-set of lysogenized bacteria can have a two-fold benefit for the remaining population. Firstly release of Stx toxin kills eukaryotic predators and secondly lysis releases fresh infectious phage to attack bacterial competitors (Mauro and Koudelka, 2011). Inoculation of phage to phage susceptible bacterial cultures produces more toxin than a pure culture of

lysogens showing that phage infection leads to lysis of susceptible bacteria thereby reducing competition for lysogenized populations (Gamage et al, 2006). *E. coli* O157:H7, through the action of Stx toxin, is also able to both kill and survive ingestion by environmental protozoa such as *Tetrahymena pyriformis* and *Acanthamoeba polyphaga* and can replicate inside them (Barker et al, 1999; Lainhart et al, 2009; Steinberg and Levin, 2007). Killing of *Tetrahymena* by *E. coli* O157:H7 is reduced by either blocking the SOS response or removal of H₂O₂ release by protozoa which may act as a trigger for release of Stx (Lainhart et al, 2009).

These general survival advantages may have relevance for colonisation of mammalian hosts. Rumen protozoa, as seen in Chapter 2, present a similar challenge to survival as environmental ones and protection from them would increase chances of *E. coli* O157:H7 survival and colonisation in cattle. There is some evidence that certain protozoa populations can either suppress or increase *E. coli* O157:H7 numbers in a defaunated sheep model (Stanford et al, 2010). Evolution of mechanisms to combat protozoan predators could also help bacteria protect themselves against other eukaryotic cells for example human leukocytes and neutrophils which release H₂O₂ to combat bacterial pathogens in a similar way to protozoa (Lainhart et al, 2009). Release of Stx toxins in humans in response to unfavourable growth conditions such as when iron supply is low leads to killing of host cells and haemorrhage into the lumen, thus increasing iron supply and down-regulating Stx production (Chibani-Chennoufi et al, 2004). To increase Stx production, phage susceptible non-pathogenic *E. coli* in the gut may be infected and lysogenized by Stx phages; commensal *E. coli* in sheep have been shown to acquire Stx phages during gut passage (Cornick et al, 2006; Gamage et al, 2006). In contrast, phage resistant resident *E. coli* reduce the amount of toxin produced in mouse models suggesting that phage resistance or susceptibility of commensal bacteria in the gut can have implications for disease severity in humans and potentially shedding and colonisation in cattle and sheep (Gamage et al, 2006).

In Scotland *E. coli* O157:H7 phage type (PT) 21/28 strains are most prevalent in both cattle and as a cause of human disease and in particular are responsible for more of the serious consequences, including HUS, than other PTs (Chase-Topping et al, 2007; Lynn et al, 2005). Recent work has shown that 90% of 21/28 strains have both the Stx2 and Stx2c phages compared to only 28% of PT 32 strains, the second most

common PT in Scotland in cattle which is rare in man (Xu et al, 2012). Possession of Stx2 along with intimin is highly associated with strains causing disease and in particular HUS in humans whereas Stx2c is less potent than Stx2 in mouse models and using human kidney cell lines (Baker et al, 2007; Boerlin et al, 1999; Fuller et al, 2011). Bacterial strains with Stx2 with or without other phages have been shown to produce 40-fold more toxin than those with Stx2c and not Stx2 (Kawano et al, 2012). Along with increased toxin production, PT 21/28 strains produce lower levels of T3S proteins than PT 32 strains, an effect attributed to Stx2 which appears to exert control over the T3S system to co-ordinate Stx and effector protein action to its advantage and producing increased adherence (Xu et al, 2012).

Possession of one or more Stx phage could therefore provide an advantage to survival of *E. coli* O157:H7 in the rumen and other gut compartments but also in colonisation of the bovine terminal rectum. Understanding more about the role of Stx in *E. coli* O157:H7 survival and colonisation in the bovine gut could help to further explain the prevalence of certain strains in cattle populations.

4.2.1 Aims

The aim of this chapter is to determine whether possession of Stx bacteriophage provides an advantage to *E. coli* O157:H7 infectivity by enhancing replication and colonisation in the bovine gut and consider how this might impact shedding patterns and transmission in cattle. Survival and replication rates will be measured for Stx⁻ and Stx⁺ *E. coli* O157:H7 strains in rumen contents in the presence and absence of rumen protozoa to investigate whether Stx protects against predation in the rumen. Differences in rates of colonisation of Stx⁻ and Stx⁺ *E. coli* O157:H7 strains will also be investigated through comparison of replication in terminal rectum mucus, and by examining attachment to and colonisation of BTRE cells.

4.3 Methods

4.3.1 Culture and storage of bacteria

E. coli O157:H7 strains (Table 4.1) were stored and cultured as described in Section 2.3.1.

Table 4.1 *E. coli* O157:H7 strains used in Chapter 4

Strain	Characteristics	Source
Walla 3 (W3)	Stx ⁻	Lab stock, originally from (Ostroff et al, 1990)
W3 NalR	Stx ⁻ , NalR	Lab stock, derived from W3 in Chapter 2
W3 NalR Stx ⁺	NalR, acquired Stx2 after bovine passage of a NalR W3 strain	Lab stock (collected during a previous study)
Walla 1 (W1) NalR	Stx2, NalR	Lab stock, originally from (Ostroff et al, 1990)
PT21/28 Strain 9000	From high shedding animal (10 ⁵). Low T3S. Contains Stx2 and Stx2c.	Lab stock, originally from (Xu et al, 2012)
PT21/28 Strain 9000 NalR	From high shedding animal (10 ⁵). Low T3S. Contains Stx2 and Stx2c. NalR	This study, derived from 9000
PT32 Strain 10671	From low shedding animal (<50). High T3S. Contains Stx2c only.	Lab stock, originally from (Xu et al, 2012)
PT32 Strain 10671 NalR	From low shedding animal (<50). High T3S. Contains Stx2c only. NalR	This study, derived from 10671

4.3.2 Collection and storage of samples

Fresh rumen contents were collected by stomach tube from 2 live animals, pooled into pre-warmed pots and transported back to the laboratory where they were stored at 37°C prior to use and used within 2 hours of collection.

Mucus samples were collected from the terminal recta of slaughtered cattle as part of the process of culturing bovine primary terminal rectum cells (see Section 2.3.4). For

this study mucus samples collected from 14 animals on 7 occasions were pooled. The pooled sample was diluted 1:10 in sterile dH₂O and split into 50 ml aliquots and frozen at -20°C for later use.

Samples were checked for confounding background flora prior to use by plating a sample onto a SMAC-Nal plate.

4.3.3 Survival and replication in the rumen of *E. coli* O157:H7 strains with or without Stx bacteriophage

The aim of this experiment was to compare survival and replication rates of a Stx⁺ve PT 21/28 NalR and a Stx⁻ve (NalR W3) *E. coli* O157:H7 strain in fresh rumen contents in the presence and absence of rumen protozoa.

Fresh rumen fluid was split into two halves one of which was centrifuged at 300 x g for 5 minutes to remove protozoa. A sub-sample of both halves was then mixed 1:4 with 4 % formalin to fix and 10 µl aliquots added to a haemocytometer to check for presence or absence of protozoa. To 9 ml aliquots of each fluid type was then added 1 ml of overnight culture of each *E. coli* O157:H7 strain diluted with dH₂O to give a final concentration of 10⁴ C.F.U./ml. Samples were incubated at 37°C with shaking at 80 rpm to prevent settling. At set time points a 1 ml aliquot from multiple samples was collected, some of which was fixed as above and to the remainder 0.2 % Triton X-100 in PBS was added to lyse any living cells. Following incubation at RT for 5 min, lysed samples were serially diluted in sterile dH₂O and plated in triplicate onto SMAC-Nal agar plates. The initial inoculum was also serially diluted and plated. Plates were incubated at 37°C for 16 h and the number of colonies on each plate counted up to maximum of 500, counts above which were considered too many to count. The limit of detection for direct plating using marked strains is 10² C.F.U./g faeces (LeJeune et al, 2006).

4.3.4 Replication rates of *E. coli* O157:H7 strains with and without Stx bacteriophage in terminal rectum mucus

The aim of this experiment was to look for differences in replication rates in terminal rectal mucus of *E. coli* O157:H7 strains of different phage types. The experiment compared the Stx⁺ NalR PT 21/28 and PT 32 strains with Stx⁻ W3 (NalR W3), all of which were used in transmission experiments in the previous chapter. Additionally, to test the Stx effect in presumed isogenic strains, Stx⁻ NalR W3 was compared to

W1, a natural Stx⁺ strain from the same outbreak and a W3 strain that had acquired Stx spontaneously following passage through an animal.

A single colony from an LB streak plate of each strain was inoculated into a 1 ml aliquot of 10 % mucus and incubated for 16 h statically at 37°C to acclimatise. The overnight cultures were then diluted to the required level with sterile dH₂O warmed to 37°C. These solutions were used to inoculate multiple aliquots of 10 % mucus for each strain along with control samples that received no bacteria. Samples were incubated at 37°C and at set times a sub-set of aliquots serially diluted in sterile dH₂O and plated in triplicate onto SMAC-Nal agar plates as described in Section 4.3.3.

4.3.5 Attachment rates of Stx⁻ and Stx⁺ *E. coli* O157:H7 strains from mucus

The aim of this experiment was to look for differences in attachment rates from mucus between Stx⁺ and Stx⁻ strains which could explain the differences in shedding and colonisation seen in Chapter 3.

Cultures of BTRE cells were obtained as previously described (See Sections 2.3.4 and 2.3.5). Following harvest 12-well plates of BTRE cells were fed every 2 days until confluent, around 5-6 days after harvest. A single bacterial colony from a streak plate was inoculated into 1 ml 10 % mucus for each strain to be tested and incubated statically at 37 °C for 16 h. The overnight cultures were diluted further with 10 % mucus and incubated at 37°C for 3 hours to obtain mid-log growth bacteria (determined from previous growth rates in mucus). Prior to adding the bacterial inoculum, medium was removed from wells of BTRE cells and the cells washed 3 times with warmed PBS. 450 µl of 10 % mucus was then added to each well along with 50 µl of each strain in 10 % mucus at a ratio of ~ 10 bacteria to 1 cell. Plates were incubated statically at 37°C with 5 % CO₂ and 80 % humidity. At set time points the mucus mixture was removed and the cells in 4 wells for each strain washed three times with warmed PBS. Cells were lysed by adding 0.1 % Triton X-100 in PBS to each well and incubating at RT for 5 min. Each well was then scraped using the end of a pipette tip to fully remove all cells from the well and the lysates from all 4 wells containing a single strain pooled. The initial inoculum and cell lysates were then serially diluted and plated as described in Section 4.3.3.

4.3.6 Progression of colonisation of Stx⁻ and Stx⁺ *E. coli* O157:H7 strains following attachment to primary BTRE cells

The aim of this experiment was to compare colonisation rates of Stx⁻ and Stx⁺ strains in order to investigate the role of Stx in progression of terminal rectum colonisation in cattle. The strains used were the Stx⁺ PT 21/28 and PT 32 strains and the Stx⁻ W3 (NalR W3) strain.

Cultures of BTRE cells were obtained as previously described (See Sections 2.3.4 and 2.3.5). A colony of each strain from an overnight plate was inoculated into 25 ml LB in a conical flask and incubated for 16 h at 37 °C with shaking at 200 rpm. The overnight culture was then split 1:100 with MEM-HEPES supplemented with 250 nM Fe(NO₃)₂ and 0.2 % glucose. Cultures were incubated at 37°C for 2 hours with shaking at 200 rpm to obtain an OD at 600 nm of 0.3. Cells were washed gently 3 times with warmed PBS and then 400 µl medium added. The bacterial culture, once it had reached an OD of 0.3, was split 1:20 with supplemented MEM-HEPES and 100 µl added to each well of cells to give an MOI of ~ 10 bacteria to 1 cell. Plates were incubated at 37°C, with 5 % CO₂ and 80 % humidity for 1 h to allow attachment. The wells were then washed 3 times with warmed PBS to remove unattached bacteria and 500 µl fresh medium added. Plates were incubated again and at set time points the cells from multiple wells (3 per strain per time point) harvested and plated as previously described to enumerate the attached bacteria (Section 4.3.5).

4.3.7 Statistical analysis

Counts from plates were recorded and those from multiple dilutions used to calculate the most probable number of C.F.U. by fitting generalized linear models with a Poisson error distribution and logarithmic link function incorporating the logarithm of dilution as an offset variable. Final counts were then log-transformed using the formula $\text{Log}_{10}(\text{C.F.U.}+1)$ and statistical analysis performed on the Log_{10} counts. Statistical analysis was performed using the statistics package Genstat (15th ed).

For rumen replication, the growth curves of each strain in fresh rumen with and without centrifugation were compared by random coefficient regression using a combined factor of Animal and Rep (Animal_Rep) as subject and Strain*Protozoa*Time as treatment structure. This experiment was undertaken on 2 separate occasions and on each occasion 3 replicates were run for each

strain/treatment combination at each time point. Due to differences in the results between occasions the two experiments were analysed separately. For both replicates the interaction for Strain and Protozoa was not significant indicating that the pattern was the same for both strains and both treatment types. The full model was used in Replicate 1 to calculate predictions however as Strain*Protozoa and the 3 way interaction of Strain*Protozoa*Time were the only non-significant items. In the second replicate, there was no significant protozoa effect except in interaction with time, which was significant, and so the factor Protozoa was removed from the model and the model re-run with Strain*Time as the treatment structure.

In the mucus, *E. coli* O157:H7 growth for all strains was linear until a peak at 7.5 hours post inoculation and the final 24 hour counts were the same as or lower than the 7.5 hour counts. Curves were therefore analysed in two parts. Firstly the linear phase of each growth curve up to the 7.5 h time point was compared using random coefficient regression with Rep as subject and a treatment structure of Strain*Time. The final 24 h count for each strain was then compared using a one-way ANOVA with Strain as treatment and Rep as block. Levels of attachment and colonisation on BTRE cells were analysed using a REML to compare the Log₁₀ transformed counts with Strain*Time as the fixed model and Animal as the random model.

4.4 Results

4.4.1 Survival and replication of Stx⁺ and Stx⁻ *E. coli* O157:H7 strains in fresh rumen contents

Figure 4-1 shows the fitted regression lines for the first rumen survival experiment. PT 21/28 counts were higher than W3 counts in both untreated (with protozoa, P+) and in centrifuged (without protozoa, P-) rumen fluid. There was a significant difference for each main effect of time ($wald = 14.45$, d.f. = 1, $P < 0.01$), strain ($wald = 25.79$, d.f. = 1, $P < 0.001$) and rumen type ($wald = 17.28$, d.f. = 1, $P < 0.001$) There was also a significant interaction for strain*time ($wald = 11.77$, d.f. = 1, $P < 0.01$) and rumen type*time ($wald = 9.02$, d.f. = 1, $P < 0.05$) but not for strain*protozoa ($P > 0.5$) or strain*protozoa*time ($P > 0.5$).

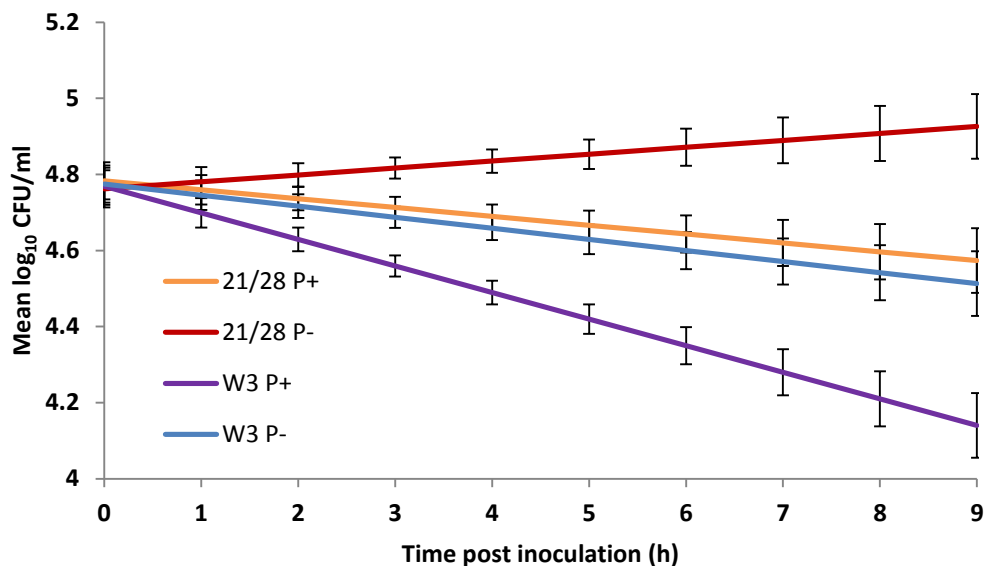


Figure 4-1 Growth of Stx⁺ and Stx⁻ *E. coli* O157:H7 strains in fresh rumen fluid with and without protozoa 1

Mean growth curves with standard errors (SED of strain*protozoa*time = 0.066) of the Stx⁺ PT 21/28 and Stx⁻ W3 strains of *E. coli* O157:H7 strains in fresh rumen fluid from the first experiment in log₁₀ C.F.U./ml (3 technical replicates from a single pooled rumen sample). P+ and P- refer to either untreated (with protozoa) or centrifuged (without protozoa).

Figure 4-2 shows the fitted regression lines for the second experiment. Counts in untreated and protozoa-free rumen fluid for both strains decreased over time. There was a significant effect for the main effects time ($wald = 110.82$, d.f. = 1, $P < 0.001$) and strain ($wald = 15.93$, d.f. = 1, $P < 0.01$) but not rumen type ($P > 0.5$) and the only significant interaction was strain*time ($wald = 21.57$, d.f. = 1, $P < 0.001$).

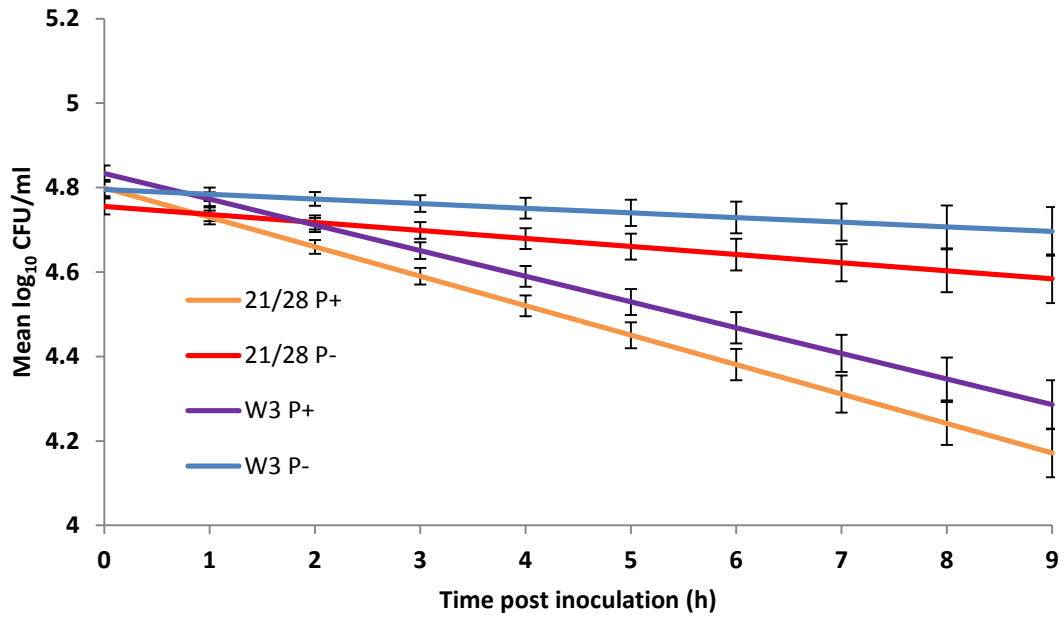


Figure 4-2 Growth of Stx⁺ and Stx⁻ *E. coli* O157:H7 strains in fresh rumen fluid with and without protozoa 2

Mean growth curves with standard errors (SED of strain*protozoa*time = 0.044) the Stx⁺ PT 21/28 and Stx⁻ W3 *E. coli* O157:H7 strains in fresh rumen fluid from the second experiment in log₁₀ C.F.U./ml (3 technical replicates from a single pooled rumen sample). P⁺ and P⁻ refer to either untreated (with protozoa) or centrifuged (without protozoa).

4.4.2 Replication rates of *E. coli* O157:H7 strains in terminal rectum mucus

Figure 4-3 shows the fitted regression lines for the linear phase of growth of Stx⁺ PT 21/28, PT 32 and Stx⁻ W3 *E. coli* O157:H7 strains in mucus. There was a significant difference for each main effect of time ($wald = 864.76$, d.f. = 1, $P < 0.001$) and strain ($wald = 29.22$, d.f. = 2, $P < 0.001$) and the strain*time interaction ($wald = 10.73$, d.f. = 2, $P < 0.01$).

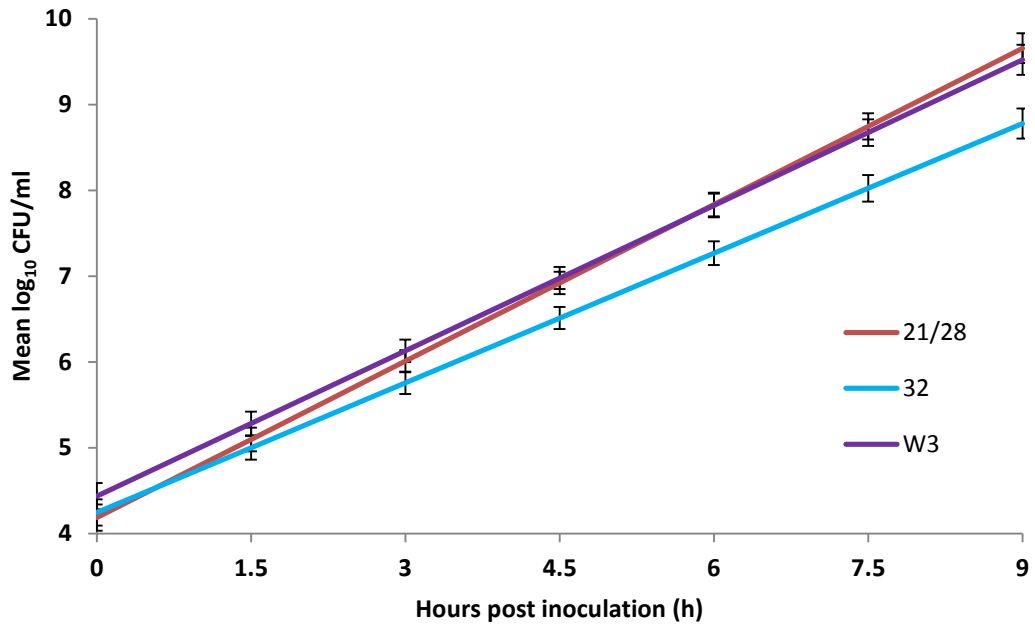


Figure 4-3 Replication rates of PT 21/28, PT 32 and W3 strains in mucus
Mean growth curves with standard errors (SED of strain*time interaction = 0.115) for the linear phase of growth of different strains of *E. coli* O157:H7 in TR mucus in log₁₀ C.F.U./ml (5 independent technical replicates from a single pooled mucus sample).

The generation rates and doubling times in the linear phase for each of the strains are shown in Table 4.2. Phage type 21/28 had a higher generation rate and doubling time than the other strains with a doubling of time of just under 30 minutes, compared with just under 32 and 36 minutes for W3 and PT 32 respectively.

Table 4.2. Generation rate and doubling time of phage types 21/28 and 32 and W3 strains in mucus.

	PT 21/28	PT 32	W3
Generations/h	2.02	1.67	1.88
Doubling time (min)	29.71	35.86	31.98

The 24 hour results for the three strains are shown in Figure 4-4 and were similar at just under 10⁹ C.F.U./ml with no significant difference between the strains (*P* > 0.5).

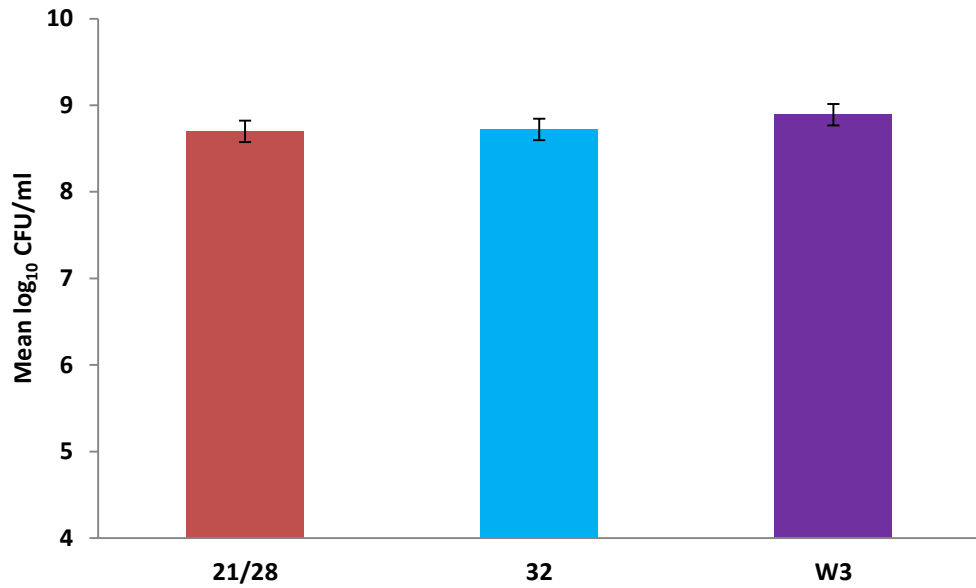


Figure 4-4 Mean 24 hour counts for PT 21/28, PT 32 and W3 in mucus

Mean 24 hour counts with standard errors (SED of strain pairs = 0.177) for different *E. coli* O157:H7 strains in terminal rectum mucus in log₁₀ C.F.U./ml (5 independent technical replicates from a single pooled mucus sample).

Figure 4-5 shows the fitted regression lines for the linear phase of growth of W1, W3 Stx⁺ and W3 Stx⁻ in mucus. There was a significant difference for the main effect of time (*wald* = 908.07, d.f. = 1, *P* < 0.001) but not strain (*P* > 0.5).

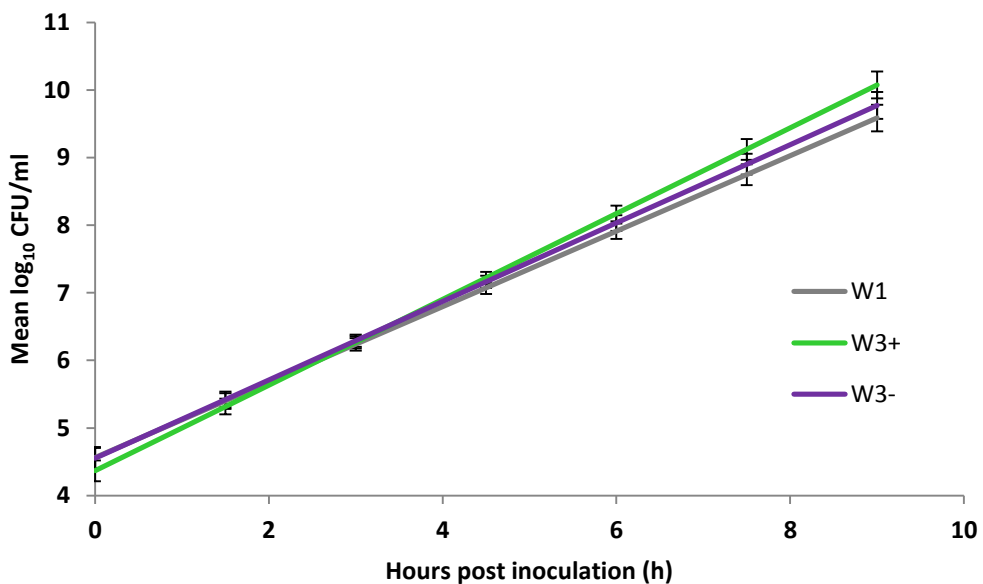


Figure 4-5. Replication of W1, W3 Stx⁻ and W3 Stx⁺ strains in mucus

Mean growth curves with standard errors (SED of strain*time interaction = 0.173) for the linear phase of growth of naturally isogenic strains of *E. coli* O157:H7 in TR mucus in log₁₀ C.F.U./ml (2 technical replicates from a single pooled mucus sample).

There was a slight difference in generation time and doubling time between the strains (Table 4.3).

Table 4.3. Generation rate and doubling time of W1, W3 Stx⁻ and W3 Stx⁺ strains in mucus.

	W1	W3+	W3-
Generations/h	1.85	2.11	1.92
Doubling time (min)	32.37	28.48	31.14

The 24 hour counts for W1, W3 Stx⁻ and W3 Stx⁺ were not significantly different (Figure 4-6, $P > 0.1$).

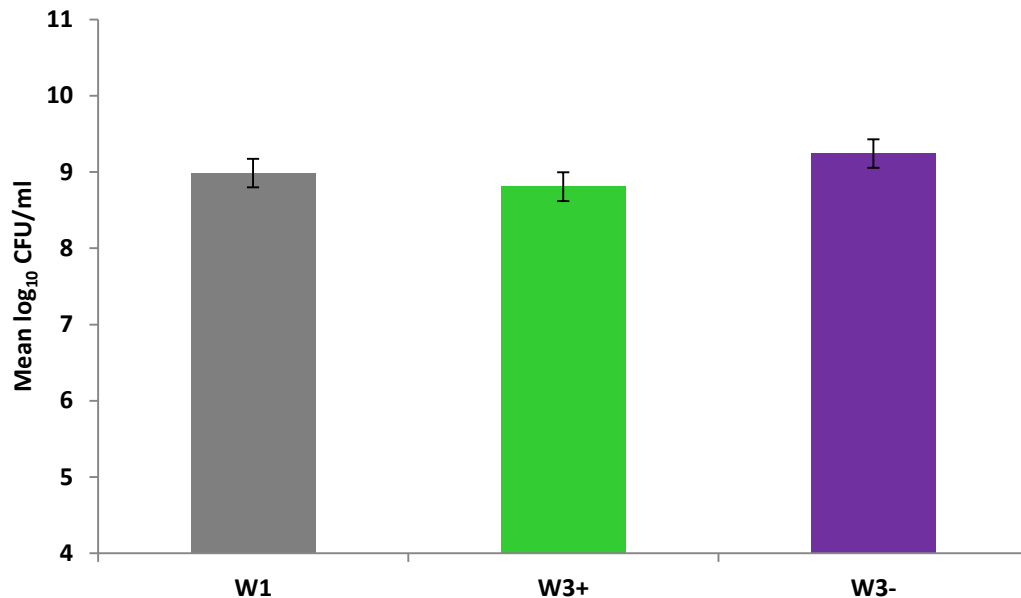


Figure 4-6. Mean 24 hour counts of W1, W3 Stx⁻ and W3 Stx⁺ in mucus

Mean 24 hour counts with standard errors (SED for strain pairs = 0.267) for naturally isogenic strains of *E. coli* O157:H7 in TR mucus in log₁₀ C.F.U./ml (2 technical replicates from a single pooled mucus sample).

4.4.3 Attachment rates of *E. coli* O157:H7 strains on BTRE cells from mucus

The number of attached bacteria per well for each *E. coli* O157:H7 strain is shown in Figure 4-7. Counts of attached PT 21/28 were higher than those of the other 2 strains at all time points with 2.35×10^5 C.F.U./well attached after 30 minutes which changed little over time. Counts of PT 32 attached started from a lower point than the other 2 strains at 5×10^4 C.F.U./well but increased to almost the same level as PT

21/28 over time. Attached W3 counts started at 1.4×10^5 C.F.U./well and increased slightly over time. There was a significant difference for the main effects of time ($wald = 5.90$, d.f. = 1, $P < 0.05$) and strain ($wald = 31.85$, d.f. = 2, $P < 0.001$) but not for the strain*time interaction ($P > 0.1$).

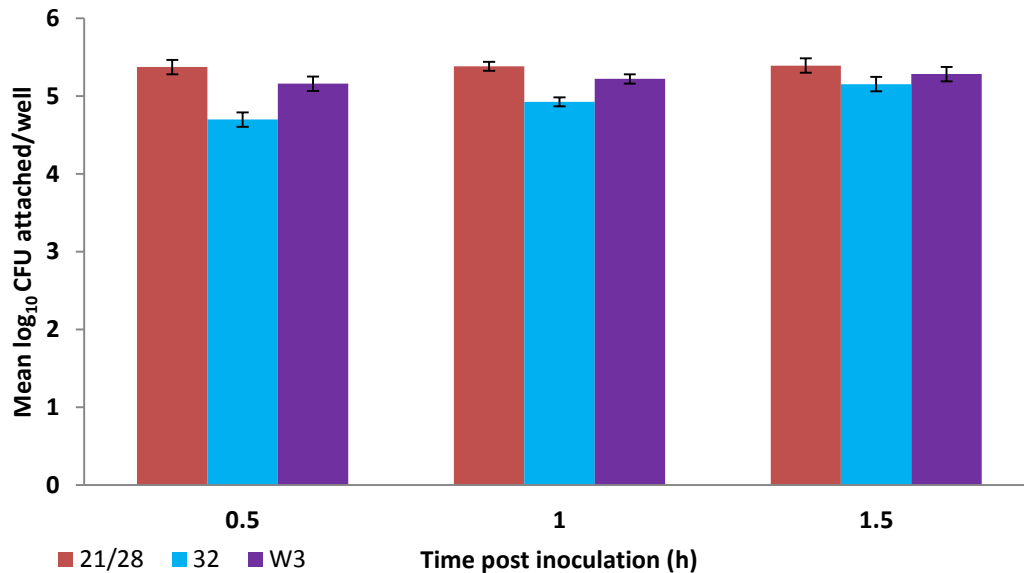


Figure 4-7 Rates of attachment of PT 21/28, PT 32 and W3 strains to BTRE cells
Mean levels of attachment with standard errors (SED of strain*time interaction = 0.110) of *E. coli* O157:H7 strains to BTRE cells at time points up to 1.5h (log₁₀ C.F.U. /well; 3 independent biological replicates).

4.4.4 Progression of colonisation of *E. coli* O157:H7 strains on BTRE cells

Figure 4-8 shows the number of *E. coli* O157:H7 of different strains to BTRE cells after an initial attachment period of 1 hour followed by washing and incubation of fresh medium for an additional 1, 2 or 3 hours. The levels of attached *E. coli* O157:H7 per well increased over time for all strains. Numbers of PT 21/28 were higher than the other strains both at the initial time point at 1.4×10^4 C.F.U./well and at subsequent ones. Numbers of attached PT 32 were over a Log₁₀ lower at the start than PT 21/28 at 1.4×10^3 C.F.U./well and remained almost a Log₁₀ lower at later time points. Numbers of attached W3 started from a point between PT 21/28 and PT 32 at 6.7×10^3 C.F.U./well and remained the second highest throughout. There was significant difference for each main effect of time ($wald = 73.82$, d.f. = 1, $P < 0.001$) and strain ($wald = 18.56$, d.f. = 2, $P < 0.001$) but not for the strain*time interaction ($P > 0.5$).

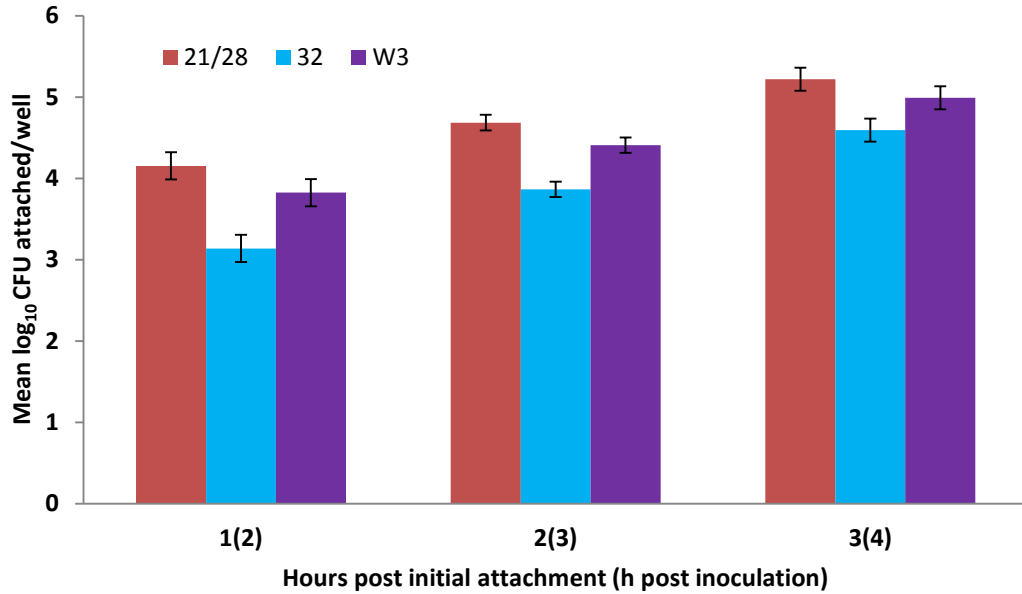


Figure 4-8 Progression of PT 21/28, PT 32 and W3 colonisation on BTRE cells

Mean levels of attached bacteria with standard errors (SED for strain*time interaction = 0.185) of *E. coli* O157:H7 strains at set time points after an initial attachment period of 1 h followed by washing and further incubation with fresh medium (log₁₀ C.F.U. /well; 2 independent biological replicates for 1 hour and 3 independent biological replicates for other time points).

The generation rate and doubling times for each strain over the experiment are shown in Table 4.4. PT 32 had the shortest doubling at 25 minutes while W3 was longer at 31 minutes and PT 21/28 had the longest doubling time at 34 minutes.

Table 4.4 Generation rate and doubling time of *E. coli* O157:H7 strains once attached.

	PT 21/28	PT 32	W3
Generations/h	1.77	2.42	1.94
Doubling time (min)	33.92	24.84	30.98

4.5 Discussion

The aim of this Chapter was to compare replication and colonisation of Stx⁻ and Stx⁺ *E. coli* O157:H7 strains in the bovine gut to identify any advantages possession of a Stx phage may contribute to transmission in cattle. The first location considered was the rumen, which was identified in Chapter 2 as a critical point in determining bacterial numbers reaching the terminal rectum. For both the Stx⁺ and the Stx⁻ strains, the presence of protozoa consistently led to a modest reduction in counts over time, providing further evidence that *E. coli* O157:H7 strains is predated by rumen protozoa. The results do not conclusively indicate an advantage of Stx to survival in the rumen however as in the first experiment the Stx⁺ PT 21/28 strain survived better than Stx⁻ W3 but this advantage was not seen in the second experiment suggesting that any effects of Stx are dependent on individual rumen conditions. Both strains experienced an overall reduction of counts in rumen fluid in the presence and absence of protozoa indicating that other elements can negatively affect *E. coli* O157:H7 replication in the rumen. The variability of these results indicates that fluctuations in rumen conditions can affect the survival dynamics of *E. coli* O157:H7 and are critical in determining the bacterial numbers reaching the terminal rectum, ultimately impacting upon faecal shedding rates.

A potential advantage of Stx was more obvious in the replication rates observed in terminal rectum mucus. PT 21/28, which contains Stx2 and Stx2c bacteriophages increased at a faster rate than the other strains with a doubling time of around 30 minutes compared to 36 minutes for PT 32, which contains Stx2c only and 32 minutes for Stx⁻ W3. Though there was a 6 minute difference in doubling times between the two Stx⁺ strains, Stx⁻ W3 was closer to 21/28 indicating Stx is not the only factor favouring the faster growth rate in these strains. Additionally, after 24 hours all three strains had reached a similar level showing that the advantage to growth is most relevant early on, something that could be important in allowing the organism to reach sufficient numbers to attach prior to defecation and sloughing of the mucus. When a comparison was made of replication rates in mucus of W3 and of W3 that had re-acquired Stx2 during bovine passage, the Stx effect was present as Stx⁺ W3 had a higher replication rate although this was not statistically significant and W1 which is also Stx2 positive was more similar to the Stx⁻ W3. While there

was no consistent Stx effect in either mucus experiment strain differences were particularly evident between PT 21/28 and PT 32.

As discussed in Chapter 2, BTRE binding assays appear to reach saturation point when the ratio of attached bacteria to BTRE cells is around 1:1. This means that saturation occurs at $\sim 5 \times 10^5$ C.F.U./well, possibly due to release of inhibitory factors by already attached bacteria to prevent effector translocation of incoming competitors (Berger et al, 2012; Mills et al, 2008). Attachment from mucus in this chapter was also highly efficient reached saturation within a short space of time but with some key strain differences. Initial attachment from mucus by PT 21/28 was faster and higher numbers were achieved than for the other strains, reaching saturation within the first half an hour. In contrast the PT 32 strain had almost a Log_{10} lower number after initial attachment and these increased over time but did not quite match PT 21/28 numbers after 1.5 hours. As in the mucus experiment the W3 strain behaved more like PT 21/28 than PT 32 and also appeared to reach saturation within half an hour but the counts were still lower overall than those of PT 21/28. The possession of the Stx2 bacteriophage by PT 21/28 has been shown to regulate T3S allowing colonisation at higher numbers through co-ordination of Stx and effector protein action to increase adherence (Xu et al, 2012). The results presented here support those findings as numbers of PT 21/28 attached were higher than for PT 32. Phage type 21/28 therefore has two advantages over PT 32, and to an extent over strain W3, as it not only replicates faster in the terminal rectal mucus but also attaches more readily to terminal rectum cells.

In the BTRE colonisation experiments a different medium was used in which lower levels of initial attachment allowed for the progression of colonisation to be measured prior to saturation. Strain differences in attachment were again evident as initial numbers of attached PT 21/28 were 1 Log_{10} higher than those of PT 32 and 0.5 Log_{10} higher than those of strain W3. The advantage for PT 21/28 continued as counts of PT 21/28 remained higher than those of PT 32 and W3 at subsequent time points. While counts of PT 32 and strain W3 did increase faster than those of PT 21/28, the PT 21/28 maintained its advantage over both strains with higher counts at all time points. The numbers of attached strain W3 were again closer to those of PT 21/28 than PT 32, meaning there was no clear Stx effect apparent however there was a clear strain effect with PT 21/28 being more effective at colonisation of BTRE

cells. Phage type 21/28 therefore has three advantages for terminal rectal colonisation over the other strains from its ability to: replicate faster in terminal rectal mucus, to attach more readily to BTRE epithelial cells and to maintain its colonisation so as to achieve higher numbers than the other strains. These advantages enhance the opportunities for attachment at the terminal rectum and will also contribute to higher levels of faecal shedding in colonised cattle.

In Chapter 3, PT 21/28 colonised more animals and was shed at consistently higher numbers than the other two strains after both experimental and natural challenge. The results from this Chapter suggest how this occurs as PT 21/28 replicated faster in the mucus, attached to BTRE cells faster and at a higher level and colonised cells more effectively than both PT 32 and strain W3. The ability of PT 21/28 to out-compete the other strains of *E. coli* O157:H7 at the terminal rectum, the point that is considered a specific tropism, provides a substantial advantage to its persistence in the cattle population and may explain why PT 21/28 is more prevalent and associated with higher bacterial counts in faecal shedding of colonised animals than PT 32. However, the strain differences cannot be attributed to possession of Stx alone as many other strains contain Stx2 and strain W3, which contains no Stx, behaved more like PT 21/28 than PT 32.

In conclusion, this Chapter has confirmed that rumen protozoa, can affect survival of *E. coli* O157:H7 in cattle and although Stx may provide some advantage to survival in the rumen other factors have a greater effect on survival and replication. There are also significant strain differences in rates of replication in terminal rectum mucus, attachment to and colonisation of BTRE cells between strains with and without Stx and of different phage types. While the strain differences cannot be attributed to possession of Stx alone they do confirm that PT 21/28, the most prevalent phage type in cattle and humans, has significant advantages in colonisation over the less common PT 32 which could explain the epidemiological patterns seen in livestock and human populations.

Chapter 5

General Discussion

5.1 Summary of thesis aims

The overall aim of this thesis was to improve current understanding of *Escherichia coli* O157:H7 shedding and colonisation in cattle and the impact that these processes have on transmission. To address this, firstly the contributions to observed shedding patterns of replication in the gut leading to passive shedding and rates of colonisation were investigated. Secondly, rates of transmission of different *E. coli* O157:H7 strains were measured and the role of dose and environmental exposure explored. Thirdly, rates of replication and colonisation in the bovine gut of different strains were compared to determine whether differences in these parameters could explain the observed patterns of shedding and transmission of the strains.

5.2 Role of passive and active shedding in understanding *E. coli* O157:H7 epidemiology

Shedding levels drive transmission dynamics determining both environmental contamination and exposure to naïve animals however shedding patterns of *E. coli* O157:H7 in cattle are not well understood. This thesis set out to answer a number of key questions on the potential for passive shedding and rates of colonisation in cattle, as well as the effect of dose, transmission routes and pathogen factors on shedding patterns in cattle. Firstly, the replication seen in gut contents in Chapter 2 demonstrated the potential for *E. coli* O157:H7 to cause passive shedding in cattle. In particular, *E. coli* O157:H7 was exceptionally good at growing in terminal rectal mucus which has consequences both for passive shedding rates and rates of colonisation. The results showed that passive shedding could not only be responsible for low level shedding but also high level shedding when replication occurs in the mucus and could explain the patterns of shedding seen in epidemiological studies. This was despite the rumen being a point of die-off due to the action of microflora such as protozoa and other as yet unidentified biological factors which reduced the counts of both Stx⁻ and Stx⁺ strains. Along with its ability to remain viable for long periods in the environment, the potential for passive shedding has fundamental implications for understanding *E. coli* O157:H7 epidemiology as it suggests the potential for it to be maintained in the environment without the need for colonisation of cattle.

Another question this thesis aimed to address was what constitutes a colonised animal by determining rates of colonisation in animals both on BTRE cells and in

the live animal. In Chapter 2, coinciding with high replication rates in the mucus were high rates of attachment and colonisation on BTRE cells which meant that once the *E. coli* O157:H7 reaches the mucus, it would be hard to distinguish what is colonised and what is passive shedding. With attachment rates of 10^5 C.F.U. bacteria per 10^5 terminal rectum cells within 15 minutes and doubling times in both mucus and on cells of ~30 minutes it can be anticipated that high level shedding could be due to either mucus replication or mucosal colonisation or a combination of the two. Even if one cannot be distinguished from the other through faecal sampling, both replication in mucus and attachment to cells appear to be key processes in determining shedding levels. There is much evidence now for the importance of particular carbohydrates in terminal rectum mucus and that blocking the ability to utilise these reduces shedding of *E. coli* O157:H7 in cattle (Snider et al, 2009). This evidence, along with the results from this thesis suggests that the goal of colonisation actually may be to anchor the bacteria in the mucus rather being necessary in itself. Indeed the preferred site of colonisation of commensal *E. coli* is the mucous layer of the colon (Kaper et al, 2004) and *E. coli* O157:H7's tropism may be an extension of that with adherence acting mainly to prevent bacteria being shed in faeces and extend the duration of shedding. The importance of mucus replication is further highlighted by Chapter 4 where the highly successful PT 21/28 strain was able to replicate in mucus and colonise in greater numbers than the other strains. This high capacity for colonisation and reproduction may help it to out-compete other strains, and may help explain its high prevalence compared with other strains (Chase-Topping et al, 2007; Pearce et al, 2009).

Rates of shedding and colonisation in the live animal following a number of exposure routes were determined in Chapter 3 to identify important factors affecting shedding and transmission. Two Stx^+ strains were shed by in-contact calves within 3 days of challenge at levels and duration similar to those of their pen-mates indicating the potential for transmission and colonisation within 3 days in the presence of high-shedding animals. In contrast, Stx^- strains were not shed by calves either after exposure to shedding pen-mates and contaminated environments or after oral dosing of up to 10^7 C.F.U. in faeces from shedding animals. All three strains including Stx^- strains were able to colonise animals when challenged experimentally at high doses, confirming that *Stx* is not essential for colonisation at the terminal rectum if given at

a high challenge dose orally or rectally (Naylor et al, 2007b; Sheng et al, 2006b). *Stx*⁻ strains were at a disadvantage for transmission in cattle compared to *Stx*⁺ ones which could explain why, despite not being essential for colonisation, 99 % of cattle *E. coli* O157:H7 strains are *Stx*⁺ (Chase-Topping et al, 2007).

There were also differences between PT 21/28 and PT 32 both in terms of shedding level and their ability to replicate in and colonise the bovine gut (Chapters 3 and 4). PT 21/28 was shed at a higher level in both challenged and in-contact calves than PT 32 in Chapter 3. PT 21/28 also showed faster replication in mucus and higher levels of attachment and colonisation on BTRE cells than PT 32 in Chapter 4 which could go some way to explaining the differences in shedding between them in Chapter 3. These strains differ in their phage status with PT 21/28 having both *Stx* 2 and *Stx*2c and PT 32 having only *Stx*2c but are otherwise similar. Between the two strains there was a definite advantage to PT 21/28 having the extra phage but the results of *Stx*⁻ W3 were higher than PT 32 and lower than PT 21/28 suggesting that the strain differences are more complex than just phage combination.

The results from this study support current definitions that animals shedding at levels above 10³ C.F.U./g faeces persistently over a number of occasions can be considered colonised at the terminal rectum and/or have high levels of replication in terminal rectum mucus. The study has also highlighted the potential importance of passive shedding to maintenance of *E. coli* O157:H7 populations on farms.

5.3 Implications of this work for future research and possibilities for control

Due to their fast growth and the ease with which they can be cultured *E. coli* strains such as K-12 and B have been extensively used in laboratory work and are of particular importance for cloning in recombinant DNA technology because they can be easily grown in large quantities (Bachmann, 1972; Daegelen et al, 2009; Kaper et al, 2004; Lee, 1996). However, such strains are unlikely to be useful in examining disease transmission in live animal studies and this work was based upon *E. coli* O157:H7 strains recovered from cattle or humans, which are less well categorised than laboratory strains although a number of *E. coli* O157:H7 strains have now been fully sequenced (Lukjancenko et al, 2010). The ability to easily isolate and culture *E. coli* strains within 24 hours of sampling means that pathogenic *E. coli* provide the opportunity to study transmission dynamics of infectious diseases in experimental

situations more easily than some pathogens such as mycobacteria which cause bovine tuberculosis (*M. bovis*) and paratuberculosis (*M. avium*) and take up 16 weeks to culture from faeces (Beard et al, 2001). *E. coli* O157:H7 is highly pathogenic to humans however and in the UK, use of fully virulent strains requires a high-containment level facility that significantly increases the costs of animal work. Attenuated strains of *E. coli* O157:H7 without Stx are commonly used in laboratory work and have been used in animal models of colonisation which allows the work to be done under less stringent regulations and greatly reduces the cost. Both Stx⁻ and Stx⁺ strains were used in the transmission experiments in Chapter 3 with varying results. While the Stx⁺ strains transmitted to naïve animals easily, Stx⁻ strains did not transmit at all indicating that, while appropriate for studying colonisation for example in vaccine trials, Stx⁻ strains are not appropriate for use in transmission studies.

The results of this study have implications for potential control measures for both passive shedding and colonisation. Reducing exposure of livestock to *E. coli* O157:H7 is one possible route of control. *E. coli* O157:H7 is transmitted via the faecal-oral route and rates of ingestion will depend on levels of exposure to the organism in faeces or other environmental sources and vary depending on the farming system and the time of year. Farm management practices can have a significant effect on the level of contact of grazing animals with faecal contamination and hence pathogens (Smith et al, 2009). Stocking density on pasture, for example in situations where animals are limited to one area in which to graze for a set period, the pressure to graze contaminated areas will increase once all the uncontaminated pasture has been grazed and livestock will be more likely to encounter fresh faeces containing micro-parasites such as *E. coli* O157:H7 (Smith et al, 2009). Housed animals are also in closer proximity to one another and may not be able to avoid lying down in faecal contamination or ingesting it in feed, water or through grooming (Mcgee et al, 2004; Stanford et al, 2011). Wet bedding and inadequate cleaning of housing have been associated with *E. coli* O157:H7 transmission (Ellis-Iversen et al, 2007; Lahti et al, 2003). The high rate of transmission of Stx⁺ strains within 3 days in rooms with minimal bedding to absorb waste in Chapter 3 highlights how quickly contact with a contaminated environment or shedding animals can lead to transmission. Management factors which minimise

contact with faeces both on pasture during grazing and in housing through shed-cleaning could reduce exposure of naïve cattle to *E. coli* O157:H7. For example, animals kept on pasture are more likely to stop shedding than housed animals and over-wintering fields without animals reduces the chance of transmission when new animals are introduced (Jonsson et al, 2009; Schouten et al, 2009). To determine the most effective control measures however, quantification of rates of contact with faeces in different management systems would be an appropriate first step.

This thesis has also identified the rumen as a key point of control due to a combination to microflora and other biological factors which acted to reduce counts of *E. coli* O157:H7 strains over time. These results would support efforts towards development of diets and treatments that could enhance this restricting effect and have impact in reducing both passive shedding and rates of colonisation. Next steps for this work could include further exploration of the components of rumen fluid including protozoa that reduce *E. coli* O157:H7 replication in the rumen.

Future work on colonisation could focus on the mechanisms which allow PT 21/28 to replicate in mucus and attach at the terminal rectum more readily to shed further light on why it is able to out-compete other strains *in vitro* and *in vivo*. Control measures for colonisation could include either measured to physically remove the organism from the terminal rectum or protecting the animals from colonisation in the first place. For controlling colonisation when it is already there, lavaging the terminal rectum with chlorhexidine is one method that has been shown to reduce numbers of O157:H7 in the faeces in the short term (Naylor et al, 2007b). However, as this only removes the bacteria mechanically, it is possible that passive or even active shedding could occur again if colonisation re-occurred in the same animals. Biocontrol is another possibility as oral or rectal dosing with bacteriophage that are virulent to *E. coli* O157:H7 have been shown to reduce shedding in colonised animals (Callaway et al, 2008; Kudva et al, 1999; Sheng et al, 2006a). The timing of such treatments could be specifically targeted to reduce exposure to naïve animals in a new setting or for clearance of bacteria from the mucosa and mucus to reduce overall shedding levels prior to slaughter for example. Oral phage treatment could also be employed to reduce counts of *E. coli* O157:H7 in the rumen and intestines of animals that are not yet colonised and application of phage to farm environments to

kill *E. coli* O157:H7 could also potentially help reduce exposure and passive shedding of the organism.

In terms of protecting cattle from colonisation, vaccines have been the focus of much study over the years. Attempts to develop vaccines using single or multiple bacterial components like H7 and intimin have so far been only partially successful in reducing shedding level or number of animals shedding *E. coli* O157:H7 (McNeilly et al, 2008; van Diemen et al, 2007). Cattle develop both mucosal and serum antibodies following inoculation with *E. coli* O157:H7 and while shedding may be reduced following re-challenge it is not prevented and animals can re-infected (Johnson et al, 1996; Naylor et al, 2007a). Chapters 2 and 4 have highlighted the importance of replication in mucus and suggest that mucosal colonisation could be a means of anchoring the bacteria near the mucus. If mucus composition is conserved between animals there is the potential that any animal in which *E. coli* O157:H7 reaches the mucus could shed high levels but if there are differences between animals and between locations in the gut then these differences could be investigated for potential points of control and future vaccine targets. Whether replication in mucus should be considered an active process requiring interaction with the host and triggering of the immune response is a matter for debate which could determine the success of vaccines. The second peak observed in some animals near the end of their shedding curve has been attributed to immune-mediated changes in rates of mucus replication or attachment or detachment to terminal rectum cells (Tildesley et al, 2012). If vaccines can cause detachment of attached bacteria this could lead to high level shedding in the short-term as bacteria are sloughed off into the mucus and then shed which could then be controlled by exposure management to prevent re-infection.

5.4 Conclusions

This study has contributed to current understanding of the epidemiology of *E. coli* O157:H7 in cattle. Prior to this work, the reasons behind low and high level faecal shedding and rates of passive shedding and colonisation in cattle were unclear. This study has shown that passive shedding alone could explain observed shedding patterns through a combination of replication in gut contents and exceptionally high replication in terminal rectum mucus. Terminal rectum mucus may in fact be the key factor in *E. coli* O157:H7's tropism and colonisation helps to keep the bacteria in its

ideal niche. The high rates of attachment and colonisation on BTRE cells also demonstrated the high potential terminal rectum colonisation rates in cattle although this could be secondary to replication in the mucus in terms of importance for replication.

Transmission studies have demonstrated how quickly high-level shedding can occur in in-contact calves exposed to wild-type cattle strains of different phage types and Stx status. The study has also shown that Stx⁻ strains are at a disadvantage in transmission in cattle and should not be used in transmission studies. The differences in shedding between the strains were then shown to be due to higher replication in mucus and attachment to BTRE cells of the PT 21/28 strain. These differences were more complex than just Stx status as Stx⁻ W3 was more similar to PT 21/28 than the Stx2c⁺ PT 32.

Replication in terminal rectal mucus has been identified as particularly important to *E. coli* O157:H7 growth, perhaps more so than colonisation itself and could be targeted for control. The potential for passive shedding and replication of *E. coli* O157:H7 in the environment opens up the possibility that *E. coli* O157:H7 populations could be maintained without the need for active colonisation of the host and has implications when considering control strategies. This study has also identified the rumen and rumen microflora as having a role in reducing *E. coli* O157:H7 numbers in the gut which could be targeted through manipulation of diet or rumen conditions to minimise gut transit of the organism.

Abbreviations

°C	Degrees Celsius
A/E	Attaching and effacing
AUC	Area under curve
BTRE	Bovine terminal rectum epithelium/epithelial
C.F.U./g	Colony Forming Units per Gram
DAEC	Diffuse-adherent <i>Escherichia coli</i>
DMEM	Dulbecco's minimum essential medium
DNA	Deoxyribose nucleic acid
EAEC	Entero-aggregative <i>Escherichia coli</i>
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
EIEC	Entero-invasive <i>Escherichia coli</i>
EPEC	Enteropathogenic <i>Escherichia coli</i>
Esp	<i>Escherichia coli</i> secreted protein
ETEC	Entero-toxigenic <i>Escherichia coli</i>
ExPEC	Extra-intestinal <i>Escherichia coli</i>
FBS	Foetal bovine serum
g	G-force
Gb3	Globotriaosylceramide
h	Hour(s)
HBSS	Hank's balanced salt solution
HEPES	Hydroxyethyl-Piperazine Ethanesulafonic Acid
HUS	Haemolytic uraemic syndrome
IPTG	Isopropyl- β -D-thiogalactoside
LB	Luria-Bertani
LEE	Locus of enterocyte effacement
MCMs	Multiple Comparisons of Means
MEM	Minimal essential medium
min	Minute(s)
Nal	Nalidixic Acid
OD	Optical density
PBS	Phosphate buffered saline
PBST	Dulbecco A PBS with Tween-20
PCR	Polymerase chain reaction
PFGE	Pulse Field Gel Electrophoresis

PT	Phage type
R ₀	Basic Reproduction Ratio
rpm	Revolutions per minute
RT	Room temperature
SNPs	Single-Nucleotide Polymorphisms
SOS	Bacterial DNA-damage stress response
STEC	Shiga toxin-producing <i>Escherichia coli</i>
Stx	Shiga toxin
Stx1	Shiga toxin 1
Stx2	Shiga toxin 2
T3S	Type-3 secretion/secreted
T3SS	Type-3 secretion system
Tir	Translocated intimin receptor
UPEC	Uropathogenic <i>Escherichia coli</i>
V	Volts
v/v	Volume per volume (%)
VTEC	Vero-toxigenic <i>Escherichia coli</i>

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