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Colonisation of EHEC in ruminants and the development of super-shedding status – Knowledge and gaps

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Executive summary

[Enterohaemorrhagic *Escherichia coli* (EHEC) are foodborne zoonotic pathogens that were first recognised in 1983 and cause clinical disease such as diarrhoea and haemorrhagic colitis in humans. Cattle are a major reservoir of EHEC and longitudinal studies of cattle have shown that most, if not all, farms and feedlots have positive animals at some time with feedlot cattle showing temporal peaks of up to 80% for within pen prevalence of O157. Published prevalence rates from overseas studies vary considerably with reported rates ranging from <1% to 36%. These studies confirm that there are major variations in the carriage of O157 by cattle. However, until recently, few studies had considered the relative importance of individual animals and their overall effect on prevalence. Recent investigations have demonstrated that relatively few cattle can be responsible for excreting the majority (>95%) of *E. coli* O157 present in a herd. Such variations in shedding rates cannot be explained by a single distribution that represents one homogenous population and this has prompted the development of the term 'super-shedder'. Super-shedding cattle typically excrete *E. coli* O157 at levels greater than 10^3 CFU/g and it is thought that colonisation of the cattle gastrointestinal tract at the rectoanal junction is required to achieve the elevated shedding levels.

Research effort into factors affecting the colonisation of *E. coli* O157 has increased in recent years; however this is a complex area requiring detailed investigation of numerous components including the pathogen, the ruminant host, and the environment. Pathogen specific factors such as the role of LEE and non-Lee-encoded effector molecules are being rapidly deduced although there remains much speculation about the cascade of events or the function of various molecules leading to persistent colonisation of the cattle gastrointestinal tract. The lack of a small animal model for EHEC colonisation, the expense and difficulty of working with large animals, and the need to use artificially inoculated animals are impediments that have limited the understanding of host and environmental factors. Studies that have attempted to address host and environmental factors such as the effect of diet, the effect of seasonality, the role of chemical sensing and the location of colonisation often produce conflicting results. Whilst this is often regarded as a function of biological variability it is clear that the reliance on artificially inoculated cattle, the use of prevalence as opposed to quantitative data and the inability to identify either cattle or specific sub-types of *E. coli* O157 that are or are likely to become super-shedders adds to the variability of results. It is clear that outcomes of future research in the area of colonisation must be aimed at reducing the uncertainty and variability currently present in these studies. Fundamental to this is an ability to rapidly identify super-shedding cattle and associated *E. coli* O157 that give rise to the super-shedder status.

Gaps exist in the understanding of how the pathogen, host and the environment each effect colonisation, persistence and super-shedding status. Specific knowledge gaps for each of the three key areas include:

- importance of pathogen factors – triggers for increased numbers,
- impact of animal factors on super shedding – age, sex, breed, stress and diet
- impact of environmental factors – season, climate, geographic location

The evidence available to date suggests that the pathogen has the greatest influence on super-shedding and when combined with the difficulty and costs associated with measuring the impact of environmental factors and performing large animal experiments, the most effective means for investigating super-shedding will be to focus on the pathogen and its interaction with bovine colonic mucosa and understand the factors associated with this. Subsequent research efforts could then utilise the findings from this research to adequately examine and interpret studies

investigating specific host and/or environmental factors. Recommendations for future research are outlined below:

Overall aim: Increase understanding of the factors influencing the colonisation and persistence of *E. coli* O157 in cattle and determine the factors that enhance the super-shedding status by:

- Examining the ability of *E. coli* O157 from human and animal sources to attach to bovine intestinal cell lines *in vitro*.
- Determining the presence of key colonisation factors in isolates with increased attachment capability
- Developing a method for the rapid identification of cattle or cattle herds that contain super-shedders.

Identifying physiological changes in naturally colonised cattle that correlate with the super-shedding of *E. coli* O157

Contents

1 Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) are foodborne zoonotic pathogens that were first recognised in 1983 and cause clinical disease such as diarrhoea and haemorrhagic colitis in humans. On occasion, treatment of disease caused by EHEC is complicated by life-threatening sequalae, including haemolytic uremic syndrome (HUS) and thrombocytopaenic purpura (Karmali et al. 1983; Paton and Paton 1998). During the past 27 years EHEC, in particular *E. coli* O157, has become a well recognised cause of human intestinal disease. Routine screening for *E. coli* O157 from stool samples indicates that there is a negligible carriage rate of *E. coli* O157 in the human population and its presence in the stool is usually associated with disease (Smith et al. 2002). By contrast, cattle which are the principal reservoir of *E. coli* O157, do not exhibit clinical disease when colonised or transiently shedding *E. coli* O157 (Russell et al. 2000). The asymptomatic status of cattle remains even when numbers being shed reach 10^5 and 10^8 CFU/g in naturally and artificially inoculated animals respectively (Besser et al. 1997; Dean-Nystrom et al. 1999; Sanderson et al. 1999). Longitudinal studies of cattle have shown that most, if not all, farms and feedlots have positive animals at some time (Gannon et al. 2002; Sargeant et al. 2003; Fegan et al. 2004b) with feedlot cattle showing temporal peaks of up to 80% for within pen prevalence of O157 (Khaitsa et al. 2003). Published prevalence rates from overseas studies vary considerably with reported rates ranging from <1% to 36% (Faith et al. 1996; Naylor et al. 2005a; Gunn et al. 2007). In Australia, the prevalence of *E. coli* O157 in cattle also varies and has been shown to range from 1.9% to 13% (Cobbold and Desmarchelier 2001; Fegan et al. 2004b).

These studies confirm that there are major variations in the carriage of O157 by cattle. However, until recently, few studies had considered the relative importance of individual animals and their overall effect on prevalence. Recent investigations have demonstrated that relatively few cattle can be responsible for excreting the majority (>95%) of *E. coli* O157 present in a herd (Omisakin et al. 2003; Chase-Topping et al. 2007). Such variations in shedding rates cannot be explained by a single distribution that represents one homogenous population and this has prompted the development of the term ‘super-shedder’. Currently there is no formal definition for a ‘super-shedder’ although a few studies have arbitrarily designated carriage of *E. coli* O157 at levels greater than 10^3 CFU/g as being indicative of super-shedding status (Omisakin et al. 2003; Fegan et al. 2004a; Low et al. 2005). In 2003, Naylor and colleagues (Naylor et al. 2003) demonstrated that some *E. coli* O157 could colonise the mucosal epithelium of cattle at the rectoanal junction (RAJ). Further investigation of this phenomenon via field studies has indicated that cattle colonised at the terminal rectum are more likely to exhibit greater levels and duration of *E. coli* O157 shedding (Low et al. 2005; Cobbold et al. 2007; Lim et al. 2007). The association

of increased shedding and duration with colonisation of the RAJ led to the proposal of a working definition for super-shedding. Chase-Topping and colleagues (Chase-Topping et al. 2008) proposed that an *E. coli* O157 super-shedder is an animal that excretes >10^4 CFU/g of faeces. The working definition does not include a parameter related to the duration of shedding but it is generally accepted that such high levels of excretion are unlikely to occur without colonisation. Formalising the super-shedding definition is dependent on gaining an understanding of the molecular mechanisms underlying the process of colonisation and identifying factors that differentiate colonising *E. coli* O157 from those that are transiently shed. Research into the factors effecting colonisation of cattle by EHEC and the effect of super-shedders is continuing, however this is a complex area requiring detailed investigation of numerous components (i.e host, pathogen, environment). The lack of a small animal model for EHEC colonisation, the expense and difficulty of working with large animals, and the need to use artificially inoculated animals are impediments limiting scientific progression. Despite these impediments, progress has been made over the last decade. This review will summarise current knowledge on the bacterial, host and environmental factors involved in the colonisation of cattle by EHEC and will identify future areas of investigation.

2 Bacterial factors

2.1 Role of EHEC virulence factors in cattle colonisation

EHEC are a subgroup of Shiga toxin-producing *E. coli* (STEC) that possess unique virulence determinants that mediate disease development. From a diagnostic viewpoint, EHEC are defined by the presence of Shiga toxin(s) (Stx), the locus of enterocyte effacement (LEE) pathogenicity island, and the pO157 virulence plasmid which encodes for, amongst other things, an enterohaemolysin (EhxA) (Wick et al. 2005). EhxA is a cell-associated, pore-forming toxin that belongs to the repeats-in-toxin (RTX) family (Saitoh et al. 2008). It is upregulated in the clinical genotype and presumably causes injury to the microvascular endothelium (Aldick et al. 2007), however it's exact role in EHEC pathogenesis is not clear. There is no evidence that EhxA is associated with colonisation of EHEC in cattle. Similarly, whilst the importance of Stxs in the causation of clinical syndromes in humans is without question, the role of Stxs in intestinal colonisation and disease in cattle is poorly understood. Nevertheless Stxs can be detected at biologically active levels in cattle thereby suggesting that they play an important role during infection of cattle and have presumably contributed to the widespread dissemination of STEC in this host (Smith et al. 2002). That said, there are clear differences between the effects of Stx in the human and cattle host with the absence of overt pathogenicity in the latter. It was originally

thought that cattle were refractory to the effects of Stx through an absence of globotriaosyl ceramide (Gb3) receptor (Pruimboom-Rees et al. 2000). It has since been shown that Stx (particularly Stx1) localises to proliferating crypt cells of the small and large intestine of cattle where receptors are present (Hoey et al. 2002). Despite receptor expression, Stx does not exhibit cytotoxic activity at this site presumably as a result of intracellular trafficking which excludes the toxin from the endoplasmic reticulum and localises it to the lysosomes where it is inactivated (Hoey et al. 2003). Support for the role of Stx in ability of EHEC to colonise cattle stems from the demonstration that Stx1 has a direct and significant effect on bovine lymphocytes (Menge et al. 2004). However, many studies have established persistent colonisation in cattle using Stx-negative EHEC strains thereby suggesting that the role of Stx in natural infection and colonisation is limited (Naylor et al. 2005a). In addition, a recent comparison of human-origin O157 strains and bovine O157 strains determined that Stx1 was more important to the infection process in humans and was unlikely to be critical for the colonisation of cattle (Lowe et al. 2009). Contrasting this suggestion is the demonstration that Stx2 increases colonisation in the intestine of cattle but it is not cytotoxic to epithelial cells from the jejuna and descending colons of cattle (Baines et al. 2008a). The cellular mechanisms behind this finding remain unknown but an increase in the expression of nucleolin is known to play a role (Robinson et al. 2006).

Of most interest to researchers is the role of the LEE pathogenicity island in the colonisation of EHEC in cattle. The LEE pathogenicity island is a 36 kb DNA region that comprises 41 open reading frames organised in five major operons, LEE1, LEE2, LEE3, LEE4 and LEE5 (Smith et al. 2002; Moxley 2004). Included in the LEE are *sep* and *esc* genes, encoding a type III secretion system (T3SS); the *eae* gene, encoding intimin; *tir*, encoding the translocated intimin receptor; the *espABD* genes, which encode proteins secreted by the T3SS; and *ler* (LEE-encoded regulator), which encodes an H-NS-like protein that activates the expression of the LEE genes (Nataro and Kaper 1998). One of the first LEE products identified was the outer membrane protein intimin (Jerse et al. 1990). Intimin is a fundamental requirement for the intimate attachment of bacterial cells to intestinal epithelial cells and is produced by all bacteria that induce the attaching and effacing (A/E) lesion. Although A/E lesions are readily observed in human clinical cases and the intestines of neonatal calves with clinical *E. coli* O157 infections (Dean-Nystrom et al. 1997), they either do not occur during the carrier state in adult cattle or affect such small areas of the intestine that they are below the limit of detection (Cray and Moon 1995; Brown et al. 1997). In support of this, a recent study identified that the LEE of clinical *E. coli* O157 (genotype 1) was upregulated in comparison to the LEE of bovine biased *E. coli* O157 (genotype 5) when placed in a model stomach system. They concluded that because strains of

the clinical genotype expressed key LEE genes at higher levels it was more likely that these strains would adhere to the intestinal epithelium and cause the A/E lesions that initiate the disease process (Vanaja et al. 2010). In addition, an increase in *gadE* expression was observed in the bovine biased genotype. GadE is the central activator of the GAD system (acid resistance system of *E. coli*) and negatively regulates LEE in *E. coli* O157 strains. It is possible that increased expression of negative regulators of LEE could suppress the expression of LEE genes and consequently reduce adherence to bovine intestinal tissue (Vanaja et al. 2010). In 2005, Naylor and colleagues (Naylor et al. 2005c) documented for the first time the presence of A/E lesions in a naturally colonised animal. In the same study they demonstrated that genes present in the LEE4 operon were critical for the colonisation process (Naylor et al. 2005c). The LEE4 operon encodes factors essential for the translocation of Tir and the T3SS and therefore it's involvement in attachment and colonisation is not surprising. It does however, suggest that vaccination strategies using preparations including proteins encoded by the LEE4 operon are likely to prevent cattle-to-cattle transmission and protect human health.

2.2 Proteins involved in colonisation

In addition to the virulence factors mentioned above, EHEC secrete many other proteins that promote the colonisation of cattle. Research effort has primarily focused on the effect of LEE-encoded proteins, however the realisation that intimin-negative strains can still cause haemorrhagic colitis or HUS in human patients stimulated the search for additional adherence factors encoded for outside of the LEE. The contribution of LEE-encoded and non-LEE-encoded factors to the colonisation of cattle by EHEC is discussed below.

2.2.1 LEE-encoded colonisation factors

The LEE pathogenicity island contains a number of genes that encode for proteins called *E. coli*-secreted proteins (Esp) that play a role in the pathogenesis of A/E lesion development. In addition, components of the T3SS (*E. coli* secretion apparatus; Esc), the outer membrane protein intimin, and its receptor Tir are also encoded on the LEE pathogenicity island. Intimin and Tir, and several effector proteins such as EspA and EspB are secreted by the T3SS and are required for efficient colonisation of bovine terminal rectum by *E. coli* O157 (Sheng et al. 2008). When *E. coli* O157 adhere to epithelial cells, secreted Tir (a LEE5 gene product) is translocated into the host cell membranes, where it serves as the receptor for intimin, an extracellular bacterial adhesin. *E. coli* O157 contact host cells via the interaction of intimin and Tir receptor. EspA and EspB (LEE4 gene products) are translocators required for the targeting of Tir. Work with *tir* and

eae (intimin) deletion mutants confirmed that their absence decreased the adherence of *E. coli* O157 at the terminal rectum and affected the percentage of colonised steers both short term (2 weeks) and long term (1 month) (Sheng et al. 2006).

Additional investigations observed that the T3SS has a greater effect on the colonisation of animals than either intimin or Tir. The study demonstrated that in the absence of T3SS, but with intimin expressed, the bacteria are unable to colonise cattle thereby implying a role for other type III translocated effector proteins in cattle colonisation (Naylor et al. 2005b). The exact functions and roles of effector proteins are still being deduced, however, they mostly appear to be involved in modifying host cell functions to favour bacterial persistence in the host by inhibiting apoptosis and disrupting tight junctions (Spears et al. 2006). Research has shown that not all *E. coli* O157 strains regulate Esp expression in the same way and this may account for differences observed in colonisation in human and animal hosts. A study of EspD secretion levels in *E. coli* O157 isolates from cattle and humans determined that human isolates had 90-fold greater EspD secretion levels than cattle isolates. All strains had the capacity to cause cytoskeletal rearrangements but the efficiency of lesion formation was greater with the high secretors (Roe et al. 2004). This observation is characteristic of *E. coli* O157 isolates belonging to human lineages and it is likely that the decreased secretion of Esp's in cattle lineage isolates is responsible for the lack of A/E lesions observed in cattle. Similarly, the increased secretion of some Esp's may confer enhanced persistence. Shames and colleagues (Shames et al. 2010) proposed that EspZ enhances host cell survival and therefore it is plausible to suggest that EspZ is a key effector molecule involved in the long-term persistence of cattle by EHEC.

2.2.2 Non-Lee-encoded colonisation factors

The identification of LEE-negative EHEC (atypical EHEC) that are able to cause clinical symptoms in human hosts identical to that of EHEC isolates indicated that some strains may carry additional colonisation factors. Initial investigations identified several proteins that were implicated as novel adhesion factors, including Iha (IrgA homologue adhesin), Saa (STEC autoagglutinating adhesin) and Efa-1 (EHEC factor for adherence) (Tarr et al. 2000; Paton et al. 2001; Stevens et al. 2002b). Whilst Saa has been shown to be specific to LEE-negative disease causing strains, the availability of genome sequences for EHEC strains has enabled the identification of numerous putative colonisation factors (Ogura et al. 2009) and consequently focus has shifted to the role of these factors in *E. coli* O157. A collection of non-LEE-encoded effector (*nle*) genes that encode translocated substrates of the T3SS have been identified and their relative prevalence in EHEC and STEC strains has been explored. *Nle* genes are typically

associated with pathogenicity islands in *E. coli* O157 and the presence of *nleB, nleE,* and *nleH1-2* are strong signatures of human-pathogenic EHEC (Bugarel et al. 2010). As is the case with many of the LEE-encoded proteins, the role of most *nle* genes is not well understood. NleA has been shown to be involved in the disruption of intestinal tight junctions in EPEC pathogenesis (Thanabalasuriar et al. 2010) and presumably performs a similar role in EHEC pathogenesis. Investigations into the functions of other *nle* genes have yielded limited data and it has been suggested that specific, as yet unidentified, environmental stimuli may be required to facilitate expression (Roe et al. 2007).

The *efa1* gene is found in almost all bacteria capable of producing A/E lesions but it is not required for A/E lesion formation (Badea et al. 2003). Efa1 is involved in host cell adherence and is required for efficient colonisation of the bovine intestinal tract for non-O157 EHEC (Stevens et al. 2002b). *E. coli* O157 carries a truncated form of *efa1* which does not confer the same properties as the full-length version found in other EHEC. Instead, *E. coli* O157 harbour a homologue of Efa1 called ToxB that appears to mediate similar functions as Efa1 (Tatsuno et al. 2001). Vaccination studies using antibodies to Efa1 have indicated that Efa1 acts as an adhesin, however debate continues as to whether Efa-1 acts as an adhesin or whether it functions by effecting the expression and secretion of LEE-encoded proteins (van Diemen et al. 2007). One of the most recent effector molecules to be identified is $EspF_u$. $EspF_u$ is a non-LEE-encoded protein that is also known as the Tir cytoskeleton coupling protein. It's dual name is a result of it being simultaneously discovered by two research groups a few years ago (Campellone et al. 2004; Garmendia et al. 2004). $EspF_u$ has a dual function in EHEC-mediated pedestal formation. First, it directly binds to and activates N-WASP during pedestal formation, and second, it contributes to N-WASP recruitment to the bacterial attachment site. Interestingly $EspF_u$ only has a role in EHEC pedestal formation with EPEC-Tir relying on phosphorylation by host family kinases instead of $EspF_u$ (Weiss et al. 2009). It is therefore plausible to suggest that other LEE- and non-LEE-encoded proteins specific to EHEC are yet to be identified or at least the specific function of effectors may be pathotype specific.

2.3 Adhesins involved in colonisation

The first step in EHEC colonisation / infection is the initial adherence of bacteria to intestinal cells. It is proposed that this adherence step may be the basis of any host specificity via the production of colonisation factors, such as the bundle-forming pilus adhesin of typical EPEC strains (Bardiau et al. 2009). Reports of fimbrial adhesins contributing to the initial attachment and persistence of EHEC date back to 1987 (Karch et al. 1987) however, early findings were

conflicted by sequencing data that failed to demonstrate the presence of adhesin gene clusters on the large plasmid of *E. coli* O157 (Burland et al. 1998). Recent work contrasts with the early sequencing work, and interrogation of the EHEC O157 Sakai sequence has demonstrated the presence of at least 14 fimbrial gene clusters, including long polar fimbriae (LpfA1 and LpfA2), F9, type 1 fimbriae, and curli fimbriae (Low et al. 2006). A Japanese study that investigated the presence of adhesins in cattle and human isolates determined that a novel autotransporter protein called EhaA was present in greater than 95% of all isolates (Wu et al. 2010). EhaA has been shown to be involved with adhesion and biofilm formation (Wells et al. 2008) and it's presence in a substantial number of EHEC strains makes it a suitable vaccine candidate. Similar studies are investigating the relative prevalence of adhesins in isolates from cattle and humans (Bardiau et al. 2009) with a view that some adhesins may influence tissue tropism thereby explaining why some isolates colonise more efficiently than others. However, these relationships are yet to be established in animal models (Ho et al. 2008).

2.4 Other bacterial factors involved in colonisation

The role of pO157 in *E. coli* O157:H7 colonisation and persistence in cattle remains poorly understood. Sequencing of the plasmid identified 100 ORFs, however the vast majority of these remain uncharacterised. Nevertheless, it has been demonstrated that pO157 is required for efficient colonisation (>30-fold increase) at the bovine terminal rectal mucosa (Sheng et al. 2006). Furthermore, a myristoyl transferase gene in the plasmid *ecf* operon and a similar chromosomal gene *lpxM* are thought to be involved with persistence as deletion of the genes results in reduced survival in the bovine gastrointestinal tract and reduced persistence in water troughs (Yoon et al. 2005). Ho and colleagues (Ho et al. 2008) detailed the involvement of a pO157-associated type 2 secretion system with the colonisation of EHEC to intestinal epithelia. They identified three EHEC genes *adfO*, *yodA*, and *etpC* that contributed to the adherence of EHEC to HeLa cell monolayers. An isolate deficient in EtpC was shown to have a ~6-fold reduction in colonisation of rabbit intestine (Ho et al. 2008) but the exact mechanism involved and the potential effect of EtpC in the cattle intestine were not deduced. Further characterisation of the ORF's of pO157 and pathogenicity islands will continue to yield additional putative colonisation factors.

3 Host factors

3.1 Tissue tropism

Initial reports detailing the location of EHEC colonisation and persistence in cattle were conflicting. Brown and colleagues (Brown et al. 1997) reported that the forestomachs were the primary sites of *E. coli* O157 localisation and proliferation, while others suggested that the likely area of colonisation was the rectum and caecum (Dean-Nystrom et al. 1999; Buchko et al. 2000). In 2003, Naylor and colleagues (Naylor et al. 2003) provided evidence that lymphoid follicle-dense mucosa at the terminal rectum was the principal site of *E. coli* O157 colonisation in cattle. This finding was correlated by a study of naturally-colonised cattle that identified significant numbers of *E. coli* O157 at the terminal rectum (Low et al. 2005). Subsequent studies have utilised this phenomenon as a more sensitive means of detecting *E. coli* O157 (Cobbold et al. 2007; Fox et al. 2008). Furthermore, a potential consequence of EHEC colonising the terminal rectum is that it results in heavier contamination of the faecal surface during defecation, a finding that has been observed in natural stool samples (Pearce et al. 2004) and has practical implications for researchers examining faecal material for the presence of *E. coli* O157.

Despite the identification of the rectoanal junction as the principal site of colonisation of *E. coli* O157 (Naylor et al. 2003), evidence has recently emerged that the heterogeneity of *E. coli* O157 shedding in cattle may be related to *E. coli* O157 colonisation of the small and large intestines. Baines and colleagues (Baines et al. 2008b) identified mild to severe forms of pathology associated with *E. coli* O157 infections in experimentally challenged cattle that had shed for >4 months and a residual pathology present in the intestinal tracts of cattle that ceased shedding at 5-12 weeks. The hierarchy for the amount of intestinal pathology in cattle was shown to be jejunum >> ileum > caecum, ascending colon >> duodenum, transverse colon, descending colon, sigmoid colon, rectum, and rectonanal junction and it was concluded that the rectoanal junction (terminal rectum) is not an important site for maintaining *E. coli* O157 infections in cattle (Baines et al. 2008b). Debate will continue about the ramifications of using experimentally challenged cattle as opposed to naturally-colonised cattle. Whilst there is clear preference for examining pathological changes in naturally-colonised cattle, concern exists about the ability to adequately target super-shedders instead of low-colonised cattle.

Although the abovementioned study casts some doubt on the rectoanal junction as the preferential site of colonisation for *E. coli* O157, the majority of studies that investigated the presence of *E. coli* O157 throughout the cattle GI tract support the rectoanal junction as a

preferred colonisation site in cattle (Naylor et al. 2003; Low et al. 2005; Naylor et al. 2005b; Cobbold et al. 2007; Walker et al. 2010) . However, the reason(s) for persistence of *E. coli* O157 at the rectoanal junction and to a lesser extent in the hindgut of cattle is not known (Walker et al. 2010). Possibly, conditions in the hindgut of cattle such as higher pH, lower volatile fatty acids concentrations, absence of ciliated protozoa, slower rate of passage of digesta, mucous production compared with the rumen are more favourable to survival and growth of *E. coli* O157 (Fox et al. 2007).

3.2 Super-shedding cattle

Whilst the biological basis of *E. coli* O157 colonisation of cattle is still to be fully elucidated there is one practical observation that is consistent in all cattle herds that have been exposed to *E. coli* O157. Quantitative studies investigating the concentrations of *E. coli* O157 shed by cattle have identified that some cattle shed the organism in much higher numbers that the majority of cattle in that particular herd (Omisakin et al. 2003; Low et al. 2005; Chase-Topping et al. 2007; Fegan et al. 2009). These cattle were defined as super-shedders and typically excrete >3 x 10^3 CFU per gram of faeces. They generally represent less than 10% of cattle in a herd but can account for >96% of all *E. coli* O157 shed within a particular herd (Omisakin et al. 2003; Chase-Topping et al. 2007). Cobbold and colleagues (Cobbold et al. 2007) proposed an alternative definition for super-shedders that related specifically to the colonisation of the rectoanal junction as opposed to any faecal parameters. Application of the alternative definition was shown to have minimal effect on the overall outcomes of the study. A current working hypothesis for super-shedders that takes into account colonisation and faecal parameters states that super-shedders are the subset of animals that are colonised at the terminal rectum and that replication at this site leads to excretion in the faeces at levels greater than this threshold (Chase-Topping et al. 2008). By contrast, most animals that shed *E. coli* O157 at lower levels do so because the bacterium is amplified in the faeces during transient passage through the animal or colonises with a lower level of replication at sites other than the terminal rectum (Chase-Topping et al. 2008).

Aside from the probable need for super-shedders to be colonised by *E. coli* O157 at the terminal rectum, additional factors leading to the development of super-shedding status are poorly understood. Pathogen related factors, specifically infections with *E. coli* O157 of particular phage types or lineage specific polymorphisms have been documented (Chase-Topping et al. 2007; Ziebell et al. 2008). The association between phage type and super-shedders is likely to represent differences in the O island phage repertoire and consequently differences in the types of effector molecules and adhesins present. These associations are yet to be observed in

isolates from geographically distinct regions and therefore research is required to correlate the presence of particular O islands with epidemiological data. Genotypic and phenotypic host factors and environmental factors such as the route of transmission and exposure dose are undoubtedly of significance to the development of super-shedder cattle but have not yet been investigated. The transmission dynamics of *E. coli* O157 have been examined using mathematical modelling. Matthews and colleagues (Matthews et al. 2006) concluded that a model that incorporates a proportion of animals that excrete at much higher levels than the rest (i.e super-shedders) best represented cross-sectional epidemiological data. The model provides estimates for parameters such as shedding duration, cattle to cattle transmission rates and immigration rates of infection from external sources and will be a useful tool in objectively assessing the effect of factors (host, pathogen or environmental) involved in the development of super-shedding status. Practical and effective methods capable of identifying super-shedders are therefore required. Such methods would provide obvious benefits to the understanding of super-shedders but perhaps more importantly, could guide pre-slaughter intervention strategies towards high risk herds so that the food safety risk associated with *E. coli* O157 can be further minimised.

3.3 Effect of diet on shedding

The effect of diet on the prevalence and shedding rates of *E. coli* O157 continues to be a subject of great debate. Results of studies that attempt to investigate relationships between diets or diet additives and the prevalence of O157 are often conflicting and not repeatable. The controversy surrounding work in this area highlights the complexity of hindgut ecology and mechanisms responsible for increased or decreased colonisation and faecal shedding (Jacob et al. 2009). Nevertheless diet continues to be an area of focus for the pre-harvest control of *E. coli* O157.

Feedlot cattle are typically fed high-energy grain diets to maximise weight gain and efficiency of feed conversion. The effect of different grain types on *E. coli* O157 prevalence has been studied extensively (Dargatz et al. 1997; Diez-Gonzalez et al. 1998; Buchko et al. 2000; Berg et al. 2004). Research has focused on the effect of starch digestion and the effect on pH and volatile fatty acid concentrations in the hindgut. Grains with lower starch content (e.g barley) are digested rapidly in the rumen and little to no starch enters the hindgut for secondary fermentation. The use of low starch grains such as barley has been positively associated with *E. coli* O157 shedding in both observational and experimental studies (Dargatz et al. 1997; Buchko et al. 2000; Berg et al. 2004). More specifically, cattle fed a barley-based grain diet were shown to shed *E. coli* O157 at a higher concentration than cattle fed a higher starch corn-based diet (Berg et al. 2004).

Interestingly, survival of *E. coli* O157 in manure from corn- and barley-fed cattle is similar; therefore survival in the faeces is not responsible for the increased *E. coli* O157 prevalence in barley-fed cattle (Bach et al. 2005).

The processing method used to prepare the grains for cattle diets has an effect on the availability of starch in the hindgut. Processing grains with heat, moisture or mechanical treatment will increase starch breakdown in the rumen and decrease the availability of starch for secondary fermentation in the hindgut (Huntington 1997). Steam-flaked grains were shown to increase *E. coli* O157 shedding compared to diets composed of dry-rolled grains (Fox et al. 2007). This finding was initially corroborated in a similar study where cattle fed a steam-flaked grain diet showed a higher prevalence of *E. coli* O157 than cattle on a dry-rolled grains diet for 30 days (Depenbusch et al. 2008). An Australian survey made similar observations when comparing steam-flaked sorghum or rolled barley diets with whole sorghum or barley (Gilbert et al. 2005).

Studies that have attempted to directly compare the effects of forage and grain diets on the faecal shedding of *E. coli* O157 are numerous and conflicting. The data available still indicates that more *E. coli* (including O157) are present in the faeces of cattle fed grain diets (Callaway et al. 2009). However, experimental inoculation studies have shown the forage based diets result in a longer period of *E. coli* O157 shedding than animals on a grain based diet (Hovde et al. 1999; Van Baale et al. 2004). In addition, there are studies that found no significant differences between forage- and grain-based diets (Tkalcic et al. 2000; Fegan et al. 2004b). The switching of diets has also been investigated. Research has demonstrated that switching feedlot cattle from grain diets to hay prior to slaughter can reduce *E. coli* O157 (Diez-Gonzalez et al. 1998). However, there are significant practical issues involved in doing this at a commercial level and the practice also results in an undesirable decrease in carcase weight (Callaway et al. 2009). Fasting of animals before and during transport to slaughtering facilities is a relatively common practice that can impact on the shedding of *E. coli* O157. Feed withdrawal results in a decrease of volatile fatty acids which research indicates results in increased shedding of *E. coli* O157 or makes cattle more susceptible to colonisation (Callaway et al. 2009).

The use of feed additives and direct fed antimicrobials as pre-harvest control strategies continue to be investigated. Ionophores, such as monensin are routinely included in feedlot rations and are designed to target gram positive organisms. It was postulated that the exclusion of gram-positive organisms may yield an environment that would favour an increase in *E. coli* O157 but initial studies failed to routinely demonstrate increases in shedding (Dargatz et al. 1997; Herriott

et al. 1998). More recent studies have demonstrated that monensin in combination with particular diet types may actually reduce *E. coli* O157 shedding. Cattle fed a forage diet that included monensin shed *E. coli* O157 for a shorter period of time than those fed forage without monensin (Van Baale et al. 2004). Similarly, an *in vitro* study found that a combination of monensin and tylosin reduced *E. coli* O157 populations by up to 2 $\log_{10}$ CFU/ml in ruminal fermentations from cows fed forage (McAllister et al. 2006). The effect observed did appear to be diet specific as no effect was seen in ruminal fermentations from cows fed corn (McAllister et al. 2006). Beta-agonists are another type of feed additive that are commonly added to feedlot rations to improve performance traits and carcase leanness. Initial studies in sheep determined that increases in *E. coli* O157 shedding could be attributed to the use of the beta-agonist ractopamine (Edrington et al. 2006b). Follow up studies in cattle using ractopamine and zilpaterol contradicted the finding in sheep by demonstrating no effect on the shedding of *E. coli* O157 (Edrington et al. 2009a). A number of studies have found that direct fed microbials or probiotics can reduce faecal shedding of *E. coli* O157 in cattle (Stevens et al. 2002a; Brashears et al. 2003; Elam et al. 2003). Reductions in shedding rates of more than 50% were observed when a cattle rumen derived *Lactobacillus acidophilus* was fed to feedlot cattle (Brashears et al. 2003). The addition of *Propionibacterium freudenreichii* reduced the prevalence of *E. coli* O157 in the faeces from 27% to 16% and on hides from 14% to 4% (Elam et al. 2003; Younts-Dahl et al. 2004). Probiotics have the additional benefit of increasing growth efficiency which helps to offset the cost of the inclusion in cattle rations.

The difficulty in identifying super-shedding cattle prior to conducting diet-based studies has meant that the effect of diet on the super-shedding status of cattle has not been adequately addressed. Studies in this research area have primarily focussed on the effect of diet on the prevalence of *E. coli* O157 and therefore they could not quantify the effect of diet on super-shedding animals. Additionally, the transient and individual nature of super-shedding animals casts doubt on the relevance of herd-based studies that cannot account for or describe the specific individual factors that give rise to super-shedder status. Clearly there are substantial knowledge gaps in relation to the identification, monitoring and quantification of super-shedding cattle before appropriate diet-based studies can be conducted.

4 Environmental factors

4.1 Chemical sensing

E. coli O157 can act as a commensal or a pathogen depending on its host. A number of virulence related genes in *E. coli* O157 such as the LEE genes, Stx genes and the flagella regulon are activated through the bacterial cell-to-cell signalling mechanism known as quorum sensing (Sperandio et al. 2001). Quorum sensing uses hormone-like compounds known as auto-inducers to allow the organism to sense environmental changes and to subsequently alter gene expression. *E. coli* O157 employs a number of quorum sensing systems including the autoinducer-3 (AI-3)/epinephrine/norepinephrine system (Sperandio et al. 2003) and the LuxR homolog SdiA that senses acyl-homoserine lactones (AHLs) (Michael et al. 2001; Ahmer 2004; Smith et al. 2008). The AI-3/epinephrine/norepinephrine system has been shown to activate EHEC virulence in animal models by responding to signals produced by intestinal flora (AI-3) and to human stress hormones (epinephrine/norepinephrine) (Rasko et al. 2008).

Whilst the AI-3 system appears to be specifically aligned to increasing the expression of EHEC virulence, the SdiA-AHL system has been shown to be necessary for EHEC colonisation of cattle (Hughes et al. 2010). This is despite the fact that EHEC do not produce AHL's themselves and presumably rely upon as yet unidentified bacteria to produced these compounds. AHL's are present in the rumen of cattle where they act to repress the expression of the LEE and increase expression of the *gad* acid resistant system necessary for survival within the bovine rumen and subsequent acidic stomachs (Hughes et al. 2010). AHL levels decrease during passage through bovine digestive tract, presumably as a result of the lower pH or via an absence of AHL-producing bacteria (Edrington et al. 2009b). Ultimately this results in an increase in LEE expression and AE lesion formation. Enhanced colonisation of the RAJ was observed in cattle challenged with wild-type *E. coli* O157 compared with those challenged with SdiA mutants (Hughes et al. 2010). Interfering with the AHL signalling that occurs in the rumen could lead to reductions in the numbers of *E. coli* O157 able to successfully navigate the low pH environment of the bovine digestive tract thereby reducing colonisation and shedding. There is currently no data available that quantifies the level of reduction in colonisation and shedding in AHL deficient cattle.

The effect of hormonal changes in cattle on the shedding of *E. coli* O157 has been investigated. To date this has focused on the role of hormones produced by the thyroid or pineal gland (discussed below) with a view that they stimulate a cascade of events, involving numerous

hormones and target tissues that are yet to be elucidated (Edrington et al. 2007). There is some evidence to suggest that animals exhibiting a hyperthyroid state may play a role in increasing the shedding of *E. coli* O157 (Schultz et al. 2005) however contradictory results have been obtained in follow-up studies (Edrington et al. 2007). Aside from the studies mentioned above, understanding the role of hormone producing glands in cattle on the shedding of *E. coli* O157 appears to have received little direct attention because of the difficulty associated with linking 'cause and effect' in a complex biological system such as a bovine.

4.2 Seasonality of shedding

Seasonal shedding of *E. coli* O157 in ruminants has been documented (van Donkersgoed et al. 1999; Barkocy-Gallagher et al. 2003; Edrington et al. 2006a). Faecal shedding of *E. coli* O157 typically peaks in summer before decreasing in autumn to low winter levels which then rise during spring to the peaks of summer. Human outbreaks of *E. coli* O157 tend to reflect the shedding patterns observed in cattle with a predominance of cases in summer months (Besser et al. 1999; Rangel et al. 2005). Despite the mounting evidence in support of seasonal shedding of *E. coli* O157, the biological basis of seasonality remains poorly understood. In addition, there are a number of studies described where shedding has not followed a seasonal pattern (Smith et al; Ogden et al. 2004; Alam and Zurek 2006; Pearce et al. 2009).

Not surprisingly, initial investigations into seasonality of shedding pointed to climatic conditions, specifically ambient temperature as the most plausible cause of increased shedding in summer. However, epidemiological data did not support this hypothesis as northern latitudes of the United States and Canada contribute more greatly to human outbreaks of *E. coli* O157 than southern regions with higher ambient temperatures (Griffin and Tauxe 1991; Meyer-Broseta et al. 2001). A relationship between shedding and day length has been proposed with early results indicating that the hormone melatonin is secreted by the pineal gland in a pattern reflecting the seasonality of *E. coli* O157 (Edrington et al. 2006a). Understanding the effect of melatonin has been problematic to date as cattle also produce gastrointestinal melatonin at much higher levels than the pineal gland (Bubenik 2002; Edrington et al. 2008).

The effect of super-shedders has been discussed in relation to seasonal shedding studies that demonstrated higher prevalence of *E. coli* O157 in winter months. In a series of Scottish studies the prevalence of *E. coli* O157 was higher in winter and autumn than in the warmer months, however the concentration of *E. coli* O157 was shown to be 6-fold higher in samples collected in summer compared to those collected in winter (Ogden et al. 2004). Such a substantial change in

the concentration of *E. coli* O157 being shed could only be attributed to the presence of super-shedding animals (Ogden et al. 2004). The results suggest that the use of prevalence data in determining seasonal variation may be inadequate and the use of quantitative methodology is required if the seasonality of *E. coli* O157 shedding is to be understood.

5 Conclusion

Since the designation of *E. coli* O157 as a foodborne pathogen in the 1980's and the identification of cattle as the principal reservoir there has been a concerted effort to understand many aspects of this organism, its hosts and the diseases that it causes. The advent of more rapid and powerful molecular techniques has provided a basis for a greater understanding of these pathogens. The colonisation of cattle by *E. coli* O157 and other EHEC is now an intense international research area. Although there remains much speculation about the cascade of events or the function of various molecules leading to persistent colonisation of the cattle gastrointestinal tract, these are rapidly being deduced. It is clear that only some strains of EHEC are able to cause pathological changes in the human or bovine gastrointestinal tracts. Congruent with this is that *E. coli* O157 super-shedders cause similar (though less prevalent) pathological changes during colonisation of the terminal rectum or other proposed cattle gastrointestinal tract locations. Understanding the factors that facilitate these processes will provide avenues for control of the colonisation, growth and transmission of *E. coli* O157 which ultimately will reduce carriage of these pathogens in cattle as well as assisting in the prevention of human disease via the food chain or associated environmental contamination.

6 Identification of key research objectives

Studies that have attempted to address host and environmental factors such as the effect of diet, the effect of seasonality, the role of chemical sensing and the location of colonisation often produce conflicting results. Whilst this is often regarded as a function of biological variability it is clear that the reliance on artificially inoculated cattle, the use of prevalence as opposed to quantitative data and the inability to identify either cattle or specific sub-types of *E. coli* O157 that are or are likely to become super-shedders adds to the variability of results. It is clear that outcomes of future research in the area of colonisation must be aimed at reducing the uncertainty and variability currently present in these studies. Fundamental to this is an ability to rapidly identify super-shedding cattle and associated *E. coli* O157 that give rise to the super-shedder status.

Gaps exist in the understanding of how the pathogen, host and the environment each effect colonisation, persistence and super-shedding status. Specific knowledge gaps for each of the three key areas include:

- importance of pathogen factors – triggers for increased numbers,
- impact of animal factors on super shedding – age, sex, breed, stress and diet
- impact of environmental factors – season, climate, geographic location

The evidence available to date suggests that the pathogen has the greatest influence on super-shedding and when combined with the difficulty and costs associated with measuring the impact of environmental factors and performing large animal experiments, the most effective means for investigating super-shedding will be to focus on the pathogen and its interaction with bovine colonic mucosa and understand the factors associated with this. Subsequent research efforts could then utilise the findings from this research to adequately examine and interpret studies investigating specific host and/or environmental factors. Recommendations for future research are outlined below:

Overall aim: Increase understanding of the factors influencing the colonisation and persistence of E. coli O157 in cattle and determine the factors that enhance the super-shedding status by:

- Examining the ability of E. coli O157 from human and animal sources to attach to bovine intestinal cell lines in vitro.

- Determining the presence of key colonisation factors in isolates with increased attachment capability
- Developing a method for the rapid identification of cattle or cattle herds that contain super-shedders.
- Identifying physiological changes in naturally colonised cattle that correlate with the super-shedding of E. coli O157

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