# AN ECOLOGICAL STUDY OF *STREPTOCOCCUS UBERIS* IN THE DAIRY COW ENVIRONMENT

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## Abstract

Mastitis remains one of the most important endemic diseases of dairy cows worldwide, impacting on both the welfare of cows and profitability and sustainability of the dairy industry. *Streptococcus uberis* (*S. uberis*) is one of the most prevalent mastitis pathogens worldwide. Despite concerted efforts to increase the understanding of the pathogenesis of the bacterium its control is centred on treatment of infected animals and general environmental hygiene. Prevention of intramammary infection by *S. uberis* is particularly problematic because the organism has been shown to be present in various different sites in and around the cow. *S. uberis* has been isolated from cows, where it appears to be carried asymptomatically outside the mammary gland. This has led to the hypothesis that *S. uberis* primarily resides within the gastrointestinal tract of cows survives well in the environment and infects the mammary gland in a purely opportunistic fashion.

The aim of this project was to investigate different aspects of the environmental ecology of *S. uberis* that relate to the transmission of *S. uberis* to the mammary gland, in order to improve understanding of factors underpinning transmission of this recalcitrant mammary pathogen.

A robust PCR-based diagnostic test was developed using primers to the *S. uberis* specific gene *sub0888* and was shown to have a high analytical sensitivity of 12 cfu/g within bovine faeces. The sampling methodology was shown to have a significant impact on the ability to detect the presence of *S. uberis* within a freshly voided faecal sample,

an increased sample size of 1 gram, in comparison to 0.2g of a faecal swab, resulted in an increased probability of detection ($P<0.01$). Attempts to use of culture based methodology for the isolation of single strains of *S. uberis* was not successful and highlighted problems associated with isolation of single bacterial species present in low quantities from highly diverse and dense mixed bacterial populations.

The PCR diagnostic was used to investigate the presence of *S. uberis* within bovine faeces in two longitudinal studies. Study 1 investigated the detection rate of *S. uberis* in faeces of an approximately 220 cow herd over a prolonged time period, with samples taken every eight weeks and Study 2 involved the screening of faeces from 46 cows three times per week over a four week period for the presence of *S. uberis.* Both of these studies permitted further evaluation of factors that impact the gastrointestinal carriage of *S. uberis* and indicated that *S. uberis* was carried in a large proportion of cows, and that the maintenance of *S. uberis* in the environment was not due to a few 'super-shedding' cows within a herd. There was a significant effect of season on the detection rate of *S. uberis* in Study 1, with an increased odds of detection of *S. uberis* in faeces during the housed, colder winter months, in comparison to the grazing, warmer months (OR = 2.82). The mean detection rate was 11.2% during the grazing season and was 29.3% during the housed season. There were significant cow factors which impacted on the detection of *S. uberis* within the faeces at a herd level in Study 1 and included parity, stage of lactation and body condition score (BCS), which showed that there was a decreased odds

of detection in multiparous cows (OR = 0.69), later stage of lactation (OR for cows ≥250DIM compared to 0-49 DIM = 0.33) and decreased BCS (OR for BCS 2.5 compared to BCS ≥3.5 = 0.54).

None of these cow factors were found to significantly impact on the detection rate of *S. uberis* within faeces when screening occurred at shorter time intervals in Study 2, however there was an increased odds of detecting *S. uberis* if cows were housed in loose straw yard, in comparison to a straw cubicle house (OR = 1.77). It was also identified that *S. uberis* was more likely to be identified in a faecal sample if any of three recent faecal samples from that cow were *S. uberis* positive (OR=2.28).

This research also investigated differences in the ability of *S. uberis* to survive and replicate on six different bedding substrates. The use of sawdust bedding, whether it was unused or had been used by cows, inhibited the replication of *S. uberis; S. uberis* only survived for up to seven days at levels greater than or equal to 100 cfu. *S. uberis* was able to survive on both used and unused straw and sand for at least 35 days. These results suggested that in certain housing situations, *S. uberis* can remain at high levels for long periods of time and therefore the management of bedding substrates could have a large impact on the amount of *S. uberis* present within the environment.

In summary, this research has found that gastrointestinal carriage of *S. uberis* is common within dairy cows and that there are different

management and cow factors that impact carriage and the resulting contamination of the environment.

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## Table of Abbreviations

| | |
|---|---|
| AMR | Antimicrobial resistance |
| BHI | Brain heart infusion |
| bp | Base pairs |
| cfu | Colony forming units |
| cfu/ml | Colony forming units per millilitre |
| CI | Confidence interval |
| CM | Clinical mastitis |
| DIM | Days in milk |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| FVF | Freshly voided faeces |
| hasA | Hyaluronic acid capsule A |
| IMI | Intramammary infection |
| Kb | Kilobases |
| Log | Logarithm |
| MALDI-TOF | Matrix-assisted laser desoption/ionisation-Time of Flight |
| MLST | Multilocus sequence typing |
| MRAM | Modified Rambach's agar medium |
| mtuA | Metal transporter uberis A |
| PCR | Polymerase chain reaction |
| PFGE | Pulse-field gel electrophoresis |
| PFU/ml | Plaque forming unit per millilitre |
| REP-PCR | Repetitive element PCR |
| RFLP | Restriction fragment length polymorphism |
| RSUSM | Reduced salt uberis specific medium |
| SCC | Somatic cell count |
| srtA | Sortase A |
| ST | Sequence type |
| THB | Todd's Hewitt broth |
| URAM | Uberis Rambach's agar medium |

| | |
|---|---|
| USM | Uberis specific medium |
| vru | Virulence regulator uberis |

# Chapter 1. Introduction

## 1.1 An Overview of Bovine Mastitis

Bovine mastitis is one of the most prevalent diseases of dairy cows and represents a large economic loss to the industry, as well as a considerable welfare issue for affected cows (Bradley, 2002; Halasa *et al.*, 2007). Intramammary infections (IMI) are categorised as either clinical or subclinical mastitis. Clinical mastitis is defined as an IMI which results in visible clinical signs and vary from swollen, hard quarters and changes in milk composition to systemic illness (Royster and Wagner, 2015). Whilst there are no visible changes to the milk or clinical signs of disease in cows with subclinical mastitis, it can impact on the welfare of the cow, including changes in rumination times and pain responses (Peters, Silveira and Fischer, 2015), as well as impacting in the performance of the cow, including reproductive (Hudson *et al.*, 2012) and milk production (Halasa *et al.*, 2009).

### 1.1.1 Economics of Mastitis

Research has estimated the cost of a case of clinical mastitis to vary between $168 to $518 per cow (equivalent of approximately £110 to £340) (Kossaibati and Esslemont, 2000; Bar *et al.*, 2008; Hagnestam-Nielsen and Ostergaard, 2009; Huijps *et al.*, 2010; Down *et al.*, 2013). The rate of clinical mastitis in the United Kingdom was reported as 47 cases per 100 cows per year (A J Bradley *et al.*, 2007); therefore the

estimated number of cases of clinical mastitis for the 2.73 million dairy cows in the UK (AHDB, 2019) would be approximately 1.28 million cases per year, costing the dairy industry between £141 million and £436 million per annum. The cost of mastitis to the UK dairy industry is similar than that reported in New Zealand, based on a national herd size of 4.86 million dairy cows and an incidence rate of clinical mastitis of 0.19 cases per 305 days risk. The cost of mastitis can be categorised as direct costs including discarded milk ,diagnostic sampling, treatment costs, labour and risk of death and indirect costs including future production loss, increased risk of culling and increased risk of transmission (Bradley, 2002; Huijps *et al.*, 2010). Research has reported that the greatest influence on cost in mastitis cases is the risk of transmission of intramammary infection (IMI) to other cows (Down *et al.*, 2013), as even a relatively small increase in the rate of transmission could be associated with a large increase in cost. The calculated costs to the dairy industry do not include the cost of subclinical mastitis, which has been reported to result in decreased milk production, increased risk of financial penalties due to decreased milk quality, increased risk of spread of intramammary pathogens and increased risk of culling (Bradley, 2002). In the USA, the total production loss due to mastitis has been reported at $108.00 per cow (approximately £80) for herds with an average bulk milk tank somatic cell count (BTSCC) of 200,000–399,999 cells/ ml and $295.24 per cow (approximately £220) for herds with average BTSCC of 400,000 and above, which equated to losses of approximately $1 billion to the USA dairy industry due to subclinical

mastitis alone (Ott, 1999). The somatic cell count (SCC) generally provides a measure of the prevalence of subclinical mastitis, as it is the number of cells per ml of milk. An elevation in SCC is related to an influx of immune cells into the mammary gland in response to a pathogen (see section 1.1.3).

### 1.1.2 Aetiology and Incidence of Mastitis

There are more than 135 reported bacterial species, which have been identified as potential mastitis pathogens (Watts 1988). Mastitis pathogens are classified as either contagious or environmental pathogens, based on their primary method of transmission. The main mastitic pathogens are considered to be *Staphylococcus aureus* (*S. aureus*), *Streptococcus dysgalactiae* (*S. dysgalactiae*) and (*S. agalactiae*), which have traditionally been categorised as contagious pathogens, and *Streptococcus uberis* (*S. uberis*) and *Escherichia coli* (*E. coli*), which have been categorised as environmental pathogens (Hogan and Smith, 2012). In the early 1970s the incidence rate of clinical mastitis (IRCM) in the UK was reported to be greater than 150 cases per 100 cows per year, which decreased to 40 cases per 100 cows per year by the mid 1980s (Wilesmith, Francis and Wilson, 1986; Bradley, 2002). This reduction was thought to directly relate to the nationwide implementation of the five point control plan, which advocated the culling of persistently infected cows, therapeutic and prophylactic use of antibiotics, the use of post-milking teat disinfection and correct maintenance of the milking parlour (Leigh, 1999; Bradley, 2002). The five point plan resulted in a dramatic reduction in clinical

mastitis caused by contagious pathogens (Table 1.1). The prevalence of subclinical mastitis in the UK followed a similar pattern to the IRCM and has changed dramatically over the past 50 years, in particular in response to changes that have been measured through BMSCC based analysis, with the BMSCC decreasing from approximately 500,000 cells/ml in 1971 to 180,000 cells/ml by 1996 (Booth, 1997).

There are varying levels of prevalence of clinical mastitis reported across the world, possibly reflecting the difference of management systems between countries, as well as different aetiologies of pathogens and differing host genetic factors. Despite the differences between countries, wide variation of ICRM between herds within the studies is common and therefore there appears to be a number of farm-related factors which influence the incidence rates. Additionally it is thought that the incidence rates may be an underestimation of the true prevalence of infection, as it relies heavily on farmer detection, recording and/or sampling, which may provide additional bias (Wolff *et al.*, 2012). The incidence rate of clinical mastitis (IRCM) from different countries over different time points is summarised in Table 1.2. A comparison between different studies is difficult due to the use of various parameters being used to evaluate incidence rates, including both median and mean. There is the potential for the mean result to be skewed by a right sided distribution and therefore the median IRCM should be the preferred reported parameter. Another difficulty in comparing the results between studies is the use of different units to measure the IRCM.

**Table 1.1: A summary of the pathogen incidence rate in the UK over time (quarter cases per 100 cows per year)***

| Pathogen | 1967[a] | 1982[b] | 1998[c] |
|---|---|---|---|
| *Staphylococcus aureus* | 67 | 7 | 2.2 |
| *Streptococcus agalactiae* | 6 | 1 | - |
| *Streptococcus dysgalactiae* | 16 | 4 | 2.0 |
| *Streptococcus. uberis* | 7 | 9 | 5.3 |
| *Escherichia coli* | 7 | 10 | 14.4 |
| Other | 50 | 9 | 17.7 |
| Total | 153 | 40 | 41.6 |

***adapted from Bradley 2002.**

The five main mastitis pathogens, *S. aureus, S. agalactiae, S. dysgalactiae*, *S. uberis* and *E. coli* account for almost 80% of mastitis cases (Bradley, 2002), with different prevalence rates reported across the world and changing over time (Table 1.3). In some countries, including the UK, there has been a shift in the aetiology of clinical mastitis isolates with environmental pathogens such as *E. coli* and *S. uberis* increasing in prevalence (Bradley *et al.*, 2007; Shum *et al.*, 2009; Oliveira, Hulland and Ruegg, 2013; Joren Verbeke *et al.*, 2014). The shift in dominance from contagious to environmental pathogens has occurred in line with the introduction of five point mastitis control plan in the 1960s (Bramley and Dodd, 1984), with *S .uberis* now the most prevalent pathogen in the UK (A J Bradley *et al.*, 2007).

### 1.1.3 Immune Response to Intramammary Infections

The introduction of a mastitic pathogen into the mammary gland induces an inflammatory immune response in one or more quarters, which can result in alterations of the milk quality and yield, as well as other possible detrimental effects to the health of the cow (Burton and Erskine, 2003). The host response involves a variety of mechanisms, which differ dependent upon the bacterial pathogen causing an infection and due to differences in bacterial growth patterns, host genetics and the host associated innate immune response. One immediate host response is a large influx of polymorphonuclear neutrophils (PMN) into the mammary gland, and are directly quantified, in milk samples as the

**Table 1.2: A summary of some of the literature regarding the incidence rate of clinical mastitis.**

| Reference | Year | Country | Number of herds | Method | Incidence rate of clinical mastitis | | | Comments |
|---|---|---|---|---|---|---|---|---|
| | | | | | Mean | Median | Range | |
| Wilesmith *et al.* 1986 | 1980 | United Kingdom | 273 | Survey based | 55 | NR | NR | Units: cases per 100 cows per year |
| | 1981 | | 209 | | 50 | | | |
| | 1982 | | 159 | | 41 | | | |
| Kossaibati *et al.* 1998 | 1994 to 1996 | United Kingdom | 144 | Farmer reported | 43 | NR | 8-113 | Units: cases per 100 cows per year |
| Peeler *et al.* 2000 | 1997 | United Kingdom | 1,835 | Questionnaire based | 22.8 | NR | 0.6-147.1 | Units: cases per 100 cows per year<br>Herds in the study had a BMSCC<100,000 cells/ml |
| A J Bradley *et al.* 2007 | Feb 2004 to Jan2005 | England and Wales | 84 | Farmer reported | 47 | 39 | 9- 162 | Units: cases per 100 cows per year |
| | | | 97 | Samples collected | 71 | 41 | 3 -849 | For sample collection, cases per 100 cows per year were calculated as the time between first and fifth clinical sample |
| Barkema *et al.* 1998 | Dec1992 to Jun 1994 | Netherlands | 274 | Samples collected and estimation of missed cases | 0.262 | 0.233 | 0- 0.99 | Units for incidence rate was the number of quarter cases per 365 cow-days at risk |
| Lam *et al.* 2013 | 2004 | Netherlands | 396 | Survey based | 33.5 | NR | NR | Units for incidence rate was the number of quarter cases per 100 cow-years at risk |
| | 2009 | | 266 | | 28.1 | | | |
| Santman-Berends *et al.* 2015 | 2013 | Netherlands | 227 | Farmer recorded data | 32.1 | 27.8 | NR | Units: cases per 100 cows per year<br>25$^{th}$ percentile: 16.7; 75$^{th}$ percentile: 44.0 |
| J Verbeke *et al.* 2014 | Sep 2012 to Sep 2013 | Netherlands | 50 | Survey based | 7.4 | 5.3 | 0- 21.3 | Units: quarter cases per 10,000 cow-days at risk |
| Barnouin *et al.* 2005 | Feb1999 to Aug 2001 | France | 297 | Farmer recorded | 20.1 | NR | 0- 116 | Units: cases per 100 cows per year |

| Reference | Year | Country | Number of herds | Method | Incidence rate of clinical mastitis | | | Comments |
|---|---|---|---|---|---|---|---|---|
| | | | | | Mean | Median | Range | |
| Sargeant *et al.*, 1998 | May 1993 to Mar 1995 | Ontario, USA | 65 | Farmer recorded | NR | NR | NR | 19.8% of cows had at least 1 case of clinical mastitis |
| McDougall, 1998 | 8th July to 21st Aug 1997 | New Zealand | 38 | Farmer recorded | 9.9% | NR | 0.9 - 21.4% | Herds from Waikato region, during calving season. |
| Petrovski *et al.*, 2009 | Jul 2005 to May 2006 | New Zealand | 14 | Trained farm personnel recorded and samples collected | 0.19 | NR | NR | Herds from Northland region. Units: cases per 305 days at risk. |

NR = not reported

**Table 1.3: A summary of literature reporting the prevalence of clinical mastitis pathogens from across the world.**

| Reference | Country | No of herds sampled | No of samples | Sample type[b] | Prevalence of the pathogens (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Environmental Streptococci | *S. uberis* | *E.coli* | *S. aureus* | *S. agalactiae* | *S. dysgalactiae* | No growth |
| Bradley *et al.* 2007 | England and Wales | 97 | 480 | CM | - | 23.5 | 19.8 | 3.3 | - | 1.5 | 26.5 |
| | | | | SCM | - | 13.8 | 3.0 | 5.2 | - | 0.4 | 38.6 |
| Verbeke *et al.* 2014 | Flanders | 48 | 845 | CM | - | 18.2 | 15.5 | 7.3 | - | 7.2 | 19.9 |
| Petrovski *et al.* 2009 | New Zealand | 14 | 559 | CM | - | 23.3 | | 23.7 | | | 19.9 |
| Shum *et al.* 2009 | Australia | 5 | 820 | CM | 41.6% | 13.4 | 33.6 | 2.4% (combined) | | 8.7 | 24.4 |
| Oliveira *et al.* 2013 | USA | 50 | 741 | CM | 12.8 | - | 22.5 | 2.8 | - | - | 27.3 |
| Olde Riekerink *et al.* 2008 | Canada | 106 | 3,033 | CM | | 6.3 | 8.4 | 10.3 | 0.1 | 4.0 | 43.9 |
| Sumathi *et al.* 2008 | India | - | 60 | CM | 16 | - | 20 | 24 | - | - | - |
| Pitkälä *et al.* 2004 | Finland | 270 | 4237[a] | CM and SCM | | 1.94 | 0.42 | 10.3 | 0.07 | 0.14 | 4.15 |

[a] Of the samples that were positive on culture - not recorded.

[b] CM refers to clinical mastitis and SCM refers to subclinical mastitis

SCC. The SCC can be measured at the level of the individual quarter, the individual cow or for the bulk milk tank.

A SCC over 200,000 cells/ml is considered to be the most practical value used to define an intramammary infection (IMI) (Bradley et al 2012). A high SCC can lead to reduced shelf life and lower processed yields (Troendle, Tauer and Gröhn, 2017) and therefore penalties are usually placed on farmers if the BMSCC increases above a certain threshold; with the legal threshold for human consumption in the European Union at <400,000 cells/ml (European Parliament, 2016). The efficacy of PMN against different bacterial pathogens has been shown to differ. PMN have been shown to be efficacious against *E. coli*, with one hour delay in recruiting PMN shown to result in an eight fold increase of *E. coli* in the mammary gland (Hill, 1981). However PMN have been reported to be ineffective against the clearance of *S. uberis* (Pedersen *et al.*, 2003; Rambeaud *et al.*, 2003).

The acute phase response to IMI involves the up-regulation of chemokine and cytokine pathways, Toll-like receptor (TLR) signalling pathways and leukocyte transendothelial migration. TLRs are host cell sensors that recognise pathogen-associated molecular patterns (PAMPs), which tend be conserved microbial molecules, such as lipopolysaccharide (LPS) within Gram negative bacteria and lipoteichoic acid (LTA) within Gram positive bacteria cell surfaces (Schukken *et al.*, 2011). There are thirteen TLRs which are linked to the detection of bacteria and these drive the activation of NF-ĸB factors, which promote the gene expression of pro-inflammatory regulators. Pro-inflammatory

regulators include cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL) -1 and CXCL8. IL-1β mediates both local and systemic inflammatory responses and has been shown to increase within 18 hours of infection with experimental infections with different *E. coli* strains (Shuster, Lee and Kehrli, 1996; Shuster *et al.*, 1997; Riollet, Rainard and Poutrel, 2000; Bannerman *et al.*, 2004). The induction of IL-1β is delayed after IMI with Gram-positive or mycoplasma compared with infection with Gram-negative bacteria (Rambeaud *et al.*, 2003; Kauf *et al.*, 2007); however, the magnitude of the IL-1β response is comparable. CXCL8 is a cytokine which mediates the recruitment of granulocytes, such as PMN, and has been detected after experimental infection with Gram negative bacteria and *S. uberis*, but is not detected after experimental infection with *S. aureus* (Bannerman *et al.*, 2004; Alnakip *et al.*, 2014).

Another component of the cellular immune response are resident monocytes/macrophages, which are sentinel cells within the mammary gland and facilitate both innate and acquired immune responses to bacterial infection through antigen processing and presentation to lymphocytes via MHC class II (Alnakip *et al.*, 2014). Whilst macrophages have been shown to be efficacious against *S. aureus*; they have been shown to be less effective in killing engulfed *S. uberis* and Mycoplasma species. Additionally there is involvement of B lymphocytes during IMI, which have two main functions; the production of antigen specific antibodies and the presentation of antigen via MHC II to CD4+ cells, which activate B cells to produce and secrete antibodies,

activate phagocytosis by macrophages and activate CD8+ T cells. The level of B cells remains fairly consistent regardless of the stage of lactation or infection; however the levels of antibodies increase during an IMI (Alnakip *et al.*, 2014)

Finally the induced adaptive immune system has the capacity to establish antigen specific memory for rapid response when the host is subsequently exposed to the same antigen (Thompson-Crispi *et al.*, 2014). This antigen specific memory is created by a subset of lymphocytes, B memory cells and T memory cells and allows for a rapid and augmented response upon subsequent exposure to the same antigen, which has the potential to not be present in all strains of the bacterial species (Thompson-Crispi *et al.*, 2014).

Vaccination offers an alternative method of treating IMIs, by inducing an active acquired immune response to a biological agent prior to challenge by the pathogen. The development of mastitis vaccines has been hampered due to the perceived prerequisite that infection must be controlled without induction of a significant inflammatory response, since this in itself contributes to the disease condition (Leigh, 1999; Fontaine *et al.*, 2002). As many mastitis-causing pathogens tend to be extracellular in nature, a robust antibody response may be required (Thompson-Crispi *et al.*, 2014) and it is believed that concentrating on inducing an antibody response to a specific well conserved sub-unit of the bacteria important for virulence could be more effective, inferring that a sub-unit vaccine may be more efficacious (Roth and Henderson, 2001). However it has also been speculated that vaccination could

operate through the reinforcement of neutrophil recruitment by CD4+ T helper type 1 lymphocytes for pathogens such as *E. coli* (Herry *et al.*, 2017).

There are currently two mastitis vaccines licensed in the United Kingdom, Startvac (HIPRA UK and Ireland) which is licensed against *S. aureus*, coliforms and coagulase-negative staphylococci, and UBAC (HPIRA UK and Ireland), which is licensed against *S. uberis.* Vaccination resulted in lower SCC and PMN counts and less of a reduction in milk production compared to non-vaccinated animals for both Startvac and UBAC (Piepers *et al.*, 2017; Collado *et al.*, 2018), however Startvac was reported to have no significant impact on the incidence of mastitis and udder health parameters in two randomised control trials (Bradley *et al.*, 2015; Freick *et al.*, 2016). The results of a randomised control trial for the UBAC vaccine in the UK have yet to be reported.

### 1.1.4 Treatment of Clinical Mastitis

The treatment of clinical mastitis relies heavily on antibiotic therapy with treatment occurring at the time of infection during lactation and also across the dry period (Royster and Wagner, 2015). As such, antibiotic usage on dairy farms is often related to udder health as the majority of medicines are used in prevention and control of mastitis (Stevens *et al.*, 2016). There are a large number of different antibiotics and combinations of antibiotics licensed for the prevention and treatment of

IMI. The definition of the successful treatment relies on the presence of a cure, which can be either a clinical cure, based on resolution of clinical signs, or a microbiological cure, which is defined as the elimination of the mastitis causing pathogen. A microbiological cure is the most accurate indicator of a resolution of the infection, however laboratory based confirmation of pathogen clearance from milk is impractical for most cases (Royster and Wagner, 2015). Therefore other predictors have been used such as milk production levels, SCC and resolution of clinical signs in the cow and the milk produced. There is an increasing drive to reduce the amount of antibiotics used within the dairy sector due to concerns of antimicrobial resistance (AMR) and the potential impact on human health, particularly through the consumption of milk (O'Neill, 2015). There is also the potential for livestock to act as reservoirs for pathogens with antimicrobial resistance genes which could be transferred to related strains and impact upon human health, for example the genes encoding resistance to penicillin and methicillin (Fey *et al.*, 2000) and methicillin-resistant *S. aureus* has been isolated from mastitic milk samples from Belgian cows (Vanderhaeghen *et al.*, 2010). A study in Canada investigating the prevalence of AMR in bacteria isolated in milk, found no extended-spectrum beta lactamase producers were identified, however multi-antibiotic resistance was reported to be common in *E. coli* (62.8%) and Klebsiella (55%) isolates (Saini *et al.*, 2012). A ten year study in France (2006 to 2016) identified that most resistance levels were below 20%, with resistance to oxacillin and gentamicin reported at levels 2.2% and

2.4% respectively (n= 13,826 isolates) (Boireau *et al.*, 2018). In addition, 15.5% of isolates which demonstrated resistance (n=879 out of 5,867) were classified as multidrug-resistant, with *S. uberis* showing an increased trend in AMR over time (Boireau *et al.*, 2018). Overall, these findings differ from a more recent study in New Zealand, where 53% of 265 *S. uberis* isolates were reported to have oxacillin resistance, identified through 101 single nucleotide polymorphisms within 5 separate penicillin binding protein (pbp) genes (McDougall *et al.*, 2020).

Another Canadian study reported that the most frequent single AMR reported amongst MLST sequence types was for penicillin and the most common multiple antimicrobial resistance phenotype reported was for ampicillin, penicillin, and tetracycline, which was based on phenotypical analysis of 62 of *S. uberis* isolates (Reyes *et al.*, 2019). Subsequent genomic evaluation of 56 isolates identified the presence of genes associated with potential AMR traits (Vélez *et al.*, 2017). This included *ANT* which has been reported to inactivate kanamycin and neomycin and was identified in 59% of isolates, alongside the presence of β-lactamase enzymes TEM-157 and bl2b in approximately 90% of isolates. These and other related TEM variants have been reported as the most common mechanism of β-lactam resistance in clinically important *E. coli* and *Klebsiella pneumoniae* isolates, both of which are also associated with IMI. The presence of the specific AMR genes were reported to have a trend towards higher MIC values, however differences in the phenotypic and genotypic resistance profiles were

also displayed by the isolates. This study suggests that further work is required into both the genotypic and phenotypic AMR profiles of *S. uberis.*

The concern over AMR has led to strategies to decrease the use of antibiotics through measures to prevent infection, for example decreasing the use of antibiotics through strategies such as selective dry cow therapy and the targeted use of antibiotics for clinical mastitis cases, as well as methods of reducing the incidence of new cases of mastitis. A reduction in the number of new cases of mastitis involves the identification of where a new infection is likely to occur from and using specific prevention methods (Down *et al.*, 2016). These target prevention methods usually involve decreasing the exposure of the cows to environmental pathogens through improved housing management, for example frequent removal of soiled bedding and decreasing the stocking density of the housing area. Therefore the identification of particular sources of IMI pathogens has the potential to help reduce the exposure to a particular IMI pathogen through targeted management strategies.

## 1.2 *Streptococcus uberis*

*S. uberis* has traditionally been considered an environmental pathogen, as it is ubiquitous within the dairy cow's environment and the dairy cow, including the tonsils, skin, rumen and genitourinary tract (Douglas *et al.* 2000; Zadoks *et al.* 2005b; Lopez-Benavides *et al.* 2005; Cullen and Little, 1969). *S. uberis* is an opportunistic pathogen in ruminants, adapted to challenging and changing environmental conditions and

capable of a nutritional flexibility based on multiple metabolic options. It has traditionally been associated with clinical disease of the mammary gland, but may also have a pathogenic role in other diseases such as metritis in ruminants (Wagener *et al.*, 2014). *S. uberis* has not been reported to cause disease in any other species and has been used as a probiotic in a clinical trial in humans aimed at contrasting the growth of periodontal pathogens (Domenico *et al.*, 2015). Clinical disease is apparent at *S. uberis* concentrations of between $10^6$ to $10^8$ CFU/ml in the mammary gland, but can peak as high as $10^9$ CFU/ml (Leigh *et al.*, 2010). The pathogenicity of a bacterium is conferred by both the bacterium and the host, as it is a result of the interplay between the immune status of the host and the virulence factors encoded by the bacterium, with a number of known virulence factors found identified to be important for infection and persistence within the environment (Table 1.4) (Priest *et al.*, 2012).

The ability of *S. uberis* to survive in the environment may be enhanced by the presence of a hyaluronic acid capsule (Oliver *et al.*, 1998), as it serves as a means of protection from desiccation. Research has shown that there are three genes within the operon which are responsible for the production of a hyaluronic acid capsule, *hasA* (hyaluronate synthase), *hasB* and *hasC* (Pullinger *et al.*, 2006; Ward *et al.*, 2009). Analysis of *S. uberis* isolates from several countries showed that the presence of genes responsible for the production of a hyaluronic acid capsule could be detected in isolates associated with clinical disease (Field *et al.*, 2003; Coffey *et al.*, 2006; Pullinger *et al.*, 2006); however

during experimental challenge with a capsular strain, 0140J and its acapsular *hasA* isogenic mutant showed that both strains were capable of causing mastitis (Field *et al.*, 2003). Therefore it is unlikely that the presence of the capsule genes directly relates to virulence on it is own; instead it is more likely that capsule prevents desiccation and enhances survival in the environment.

Sortases are transpeptidases which act by anchoring bacterial surface proteins through their cell sorting signal to peptidoglycan in the bacterial cell wall, allowing them to be displayed on the microbial surface (Marraffini *et al.*, 2006). Each sortase enzyme has been reported to anchor a specific and discrete subset of proteins by recognition of a specific amino acid sequence motif contained within the substrate protein and for *S. uberis* there are two sequences (LPXXG and LPXXXD) which function as cell wall attachment motifs (Egan *et al.*, 2010). Within the *S. uberis* genome there is a single coding sequence (*sub0881*) identified as sortase (SrtA) and ten identified coding sequences with the LPXXG or LPXXD motifs, 9 of which have been confirmed to be anchored to the cell wall (sub0135, sub0145, sub0207, sub0826, sub0888, sub1095, sub1154 and sub1370, sub1730) (Ward *et al.*, 2009; Egan *et al.*, 2010).

Experimental challenge of an isogenic mutant with an insertion within the srtA gene (ISS1) showed IMI was transient, as it did not colonise in high numbers after the first 24 hours of the infection, when compared to

the wild type strain (Leigh *et al.*, 2010). The virulence of mutants that failed to express individual sortase substrate proteins was determined using a challenge study; those strains lacking sub0145, sub1095 and sub1154 all showed levels of bacterial attenuation, with the presence of sub1154 shown to be essential for virulence (Leigh *et al.*, 2010). Therefore it would appear that a number of sortase anchored proteins play a distinct and important role in the pathogenesis of *S. uberis* infection in the mammary gland.

**Table 1.4: A summary of identified virulence factors of *S. uberis***

| Virulence Factor | Mechanism of Action | Absence of Virulence Factor | References |
|---|---|---|---|
| Virulence regulator uberis (Vru) | Transcriptional regulatory gene which regulates a number of virulent genes, including the hyaluronic acid capsule, sub0145, sub1095 and PauA plasminogen activator | Decreased ability to colonise the mammary gland and a decrease severity of clinical mastitis due to decreased expression of a number of linked genes | Egan et al 2012 |
| Hyaluronic acid capsule | Protection from desiccation and maintenance of a humid environment in close proximity to the bacterial cell | Both capsular and acapsular strains can result in mastitis | Field et al 2013 |
| Sortase | Transpeptidases modify surface proteins by recognising and cleaving carboxyl- terminal sorting signal and anchoring proteins to the bacterial cell wall. | An inability to colonise the mammary gland in high numbers 24 hours post challenge and a decrease severity of clinical mastitis. Mutants lacking sub0145, sub1095 and sub1154 showed levels of bacterial attenuation, with sub1154 essential for virulence. | Leigh et al 2010 |
| Plasminogen activator uberis A (PauA) | PauA converts plasminogen into the active form plasmin, which hydrolyses casein and releasing essential amino acids. | The presence of PauA alone is not essential for virulence | Ward et al 2003, Rosey et al 1999 |
| Metal transporter uberis A (mtuA ) | Encodes a lipoprotein receptor-associated antigen, which is extracytoplasmic and binds metal ions, including manganese. | Mutants with a single insertion in the mtuA gene fail to grow in milk without exogenous manganese. | Smith et al 2013 |
| Surface adhesion molecule (SUAM) | SUAM has an affinity for lactoferrin and appears to have a central role in the adherence and internalisation of *S. uberis* within the mammary gland | Mutants with a single insertion in the SUAM gene failed to cause mastitis in challenge experiments. | Chen et al 2011, Patel et al 2009 |
| Fibronectin-binding protein (fbpS) | FbpS appears to promote the adhesion and internalisation of bacteria into host cells, and to act by preventing phagocytosis through binding the host fibronectin, which can act as a bridge between the bacterium and host cell. | High similarity to CshA/PavA of *S. gordonnii*/ *S. pneumoniae* at protein level and shown to modulate adhesion to human fibronectin and essential for virulence and postulated to be important for *S. uberis* virulence | Almeida and Oliver, 2001<br><br>War*d et al*., 2009)<br>Holmes *et al.*, 2001 |
| CAMP factor | CAMP factor is a secreted pore-forming toxin which causes haemolysis of erythrocytes. | CAMP factor (*cfb* gene) is not present in all strains of *S. uberis,* including the virulent wildtype strain 0140J and thus is non-essential for virulence | Jiang et al., 1996<br>Ward et al 2009 |
| C5a peptidase precursor | C5a peptidase is thought to cleave the C5a complement factor, which is involved in the | The mutants lacking C5a peptidase (sub1154) showed impaired colonisation compared to the | Ji *et al.*, 1996 |

| | | | |
|---|---|---|---|
| | recruitment and activation of phagocytic cells to the site of infection, based on protein similarity with *ScpA* of *S. pyogenes* and *ScpB* of *S. agalactiae*. | wild type strain in challenge studies, to the point where it was not detected in any challenged quarter by the sixth milking after challenge. Somatic cell influx was markedly reduced in comparison to the wild type strain. | Leigh *et al.*, 2010 |
| Lactoferrin binding protein (Lbp) | Lbp binds to host lactoferrin to decrease the chelation of iron by the host during inflammation | Mutants lacking Lbp (sub0145) showed an impaired ability to colonise the mammary gland and was re-isolated at levels of 100 to 1000-fold less than the wild type strain in a challenge study | Leigh *et al.*, 2010<br>Moshynskyy *et al.*, 2003 |
| Collagen like surface anchored protein (sub1095) | Sub1095 has been shown to have a 46% protein sequence identity to SclB of *Streptocooccus pyogenes,* which is involved in the adhesion of fibroblasts and can bind host Thromobin-activatable fibrinolysis inhibitor. | Mutants lacking sub1095 showed decreased clinical signs of mastitis in a challenge study, despite a similar ability to colonise the udder to the wildtype strain for 24 hours. However the recoverable numbers of the mutant declined 24 hours after inoculation. | Leigh *et al.,* 2010 |

### 1.2.1 Ecology of *Streptococcus uberis*

Bedding, water, feed, faeces and mud have been suggested to be the potential primary source for exposure to environmental mastitis pathogens, by acting as a transferral agent to the teats, which can result in IMI (Schreiner and Ruegg, 2003; Pullinger *et al.*, 2006). A number of studies have investigated the risk of exposure to *S. uberis*, in terms of different environmental sources and risk factors for infection (Leigh, 1999; Zadoks *et al.*, 2005a; Lopez-Benavides *et al.*, 2007). The correlation between *S. uberis* pasture detection with the high incidence of *S. uberis* mastitis in pasture based herds has led to suggestions that soil could act as a natural niche for *S. uberis* and allow for bacterial multiplication in the environment (McDougall, 1999). One study detected *S. uberis* at the level of grass, surface soil and subsoil at a level of >3 cfu/10g in a grazing herd in New Zealand for up to two weeks after the cows had left the pasture (Lopez-Benavides *et al.,* 2007). Additionally, in the same study, *S. uberis* was not able to be cultured if cows had not previously been on the pasture, highlighting that the cow is as an important reservoir for pasture contamination. This has led to the hypothesis that the contamination of the pasture by *S. uberis* occurs directly by faecal excretion or milk secreted from infected udders. High concentrations of *S. uberis* have been detected in areas where cows gather or lie down and on farm tracks with high cow traffic volume (i.e. to and from the milking parlour), where cows tend to spend an increased proportion of their daily time (Zadoks *et al.*, 2005a; Lopez-Benavides *et al.*, 2007; Wente *et al.*, 2019). This is also suggestive that

the presence of cows is required for the environmental contamination with *S. uberis* and the amount of contamination is correlated to an increased contact time with cows.

Bedding has been considered an important reservoir for the growth of most environmental mastitis pathogens (Hogan *et al.*, 1990; Zdanowicz *et al.*, 2004; Proietto *et al.*, 2013). The detection of *S. uberis* within straw, sand, sawdust and recycled manure has been reported (Ward *et al.*, 2002; Zadoks *et al.*, 2005a; Bradley *et al.*, 2018; Wente *et al.*, 2019), with the detection of *S. uberis* in bedding at a concentration of greater than $10^6$ cfu/g linked to an increased incidences of mastitis (Bramley, 1982a). Discrepancies in the bacteria identification methods, particularly the differentiation of *S. uberis* from Enterococcus species may have resulted in the over reporting of the prevalence of *S. uberis* within different bedding materials; however it is still considered a potential source of infection.

Potential cattle reservoirs of *S. uberis* have also been investigated, with *S. uberis* more frequently isolated from cattle body sites in herds that experienced high rates of IMI (Kruze and Bramley, 1982a). Multiple different strains of *S. uberis* have been isolated from teat skin, feet and tail sites, with a correlation between isolation of strains associated with clinical mastitis and those isolated from the same cow's hind feet has been reported (Lacy-Hulbert *et al.*, 2005). No correlation was observed for anatomical location and subclinically associated strains. The potential original source of bacteria is postulated to be either from

leakage of milk containing high numbers of bacteria directly onto the feet or contamination directly from faeces or bedding material.

A seasonality of the isolation of *S. uberis* from cow sites has been reported, with isolation occurring more frequently during the winter than in the spring and autumn (Buddle *et al.*, 1988) which may be related to environmental factors such as change from pasture to housing over winter, changes in cattle response to infection or changes in management practices. Although *S. uberis* has been detected on numerous sites on and within the cow, it is unclear as to whether the presence of these strains is due to colonisation, if the bacterium forms part of the commensal flora or it is transient contamination from the environment (Zadoks *et al.* 2005a).

The rate of detection of *S. uberis* in the faeces of dairy cows has been investigated with varying detection rates reported, ranging from 0% of cattle within the herd to 23% and 34.1% (Bramley, 1982b; Zadoks *et al.*, 2005a; Pryor, 2008) A summary of the main methods of detection and detection rates are outline in Table 1.5. Each of the studies conducted used different identification methods for *S. uberis*, as well as different population sizes and time periods of sampling; therefore, the variation in percentage of isolates recovered is not unexpected.

**Table 1.5: A summary of the literature regarding the detection of *S. uberis* in faecal samples of dairy cows**

| Country | Sampling | Detection Method | Average *S. uberis* detection level (detection range) | Reference |
|---|---|---|---|---|
| New Zealand | Monthly sampling of100 dairy cows for 14 months using rectal swabs (July 2003 to November 2004) | Culture on *S. uberis* selective media, repetitive DNA sequence PCR | 5.6% (Range: 1.2-9.7%) | Lacy-Hulbert *et al.* 2005 |
| United Kingdom | Monthly sampling of 11 cows (Apr-Nov) using rectal swabs | Inhibitor typing system | 0% | Buddle *et al.* 1988 |
| New York, United States of America | Monthly sampling from 100 dairy cows for 8 months, using faecal samples obtained during a rectal examination (April 2003 to December 2003). | Culture on selective *S. uberis* specific media, Gram, catalase and 16S rRNA based PCR testing | 23% (Range 0 to 41%) | Zadoks *et al.* 2005 |
| United Kingdom | Swabs of 24 cows throughout lactation. | Special selective methods used for isolation | Rarely detected | Cullen 1966 |
| United Kingdom | Rectal swabs were taken weekly from 14 dairy cows on 7 occasions | Inhibitor typing system | 34% | Kruze & Bramley 1982b |

Recent research has indicated that *S. uberis* could be capable of contagious transmission, demonstrated by the presence of the same *S. uberis* strains being found in different IMI in different cows, alongside the evidence that in some herds there can be a predominance of one strain of *S. uberis* in clinical mastitis cases (Davies *et al.*, 2015; Wieliczko *et al.*, 2002; Oliver *et al.*, 1998; Phuektes *et al.,* 2001). In one particular study investigating the strain types of *S. uberis* clinical mastitis from 52 British dairy herds, 63% of herds had at least one pair of clinical mastitis incidences caused by the same sequence type of *S. uberis*, as defined by multilocus sequence typing (MLST) (Davies *et al.*, 2015). This has led to the re-classification of *S. uberis* is an environmental pathogen, which can also act as a contagious pathogen in some instances and therefore introduces additional factors which may require investigation for control of *S. uberis* IMI.

## 1.3 Genotypic and phenotypic differentiation of *S. uberis* strains

Whilst *S. uberis* has been reported to be present in bovine faeces and the environment, it has yet to be definitively proven that the same strains of *S. uberis* isolated from the environment have also been isolated from the mammary gland of a cow. One study in Germany showed the potential for five strains of *S. uberis* to have been isolated from the environment and the mammary gland of cows on 4 out of 15 farms sampled, using pulse gel field electrophoresis (PGFE) for strain

typing (Wente *et al.*, 2019). However, without definitive genomic sequencing, it is not possible to reliably report these isolates as being the same strain. This is similar to the ribotype based analysis of *S. uberis* strains reported to have found seven common ribotypes of *S. uberis* within faeces, the environment and milk from cows on a dairy farm in New York (Zadoks *et al.*, 2005a)

### 1.3.1 Methods of Strain Typing

For epidemiological surveillance and research, there is a need to differentiate related bacterial isolates (Van Belkum *et al.*, 2007). Bacterial epidemiological typing generates isolate-specific genotypic or phenotypic characteristics that can be used to determine the sources and routes of spread of bacteria and therefore typing applies distinct labels to bacterial isolates (Van Belkum *et al.*, 2007). With a high rate of genetic material exchange between strains, any given phenotype may not reflect the genotype of the species and therefore this has largely been replaced by genotypic or molecular-based typing methods. Whole-genome sequencing followed by genome comparison has been referred to as the gold standard method for elucidating the genetic variability within a bacterial species; however it can be expensive and labour intensive and a number of subtyping methods have been developed for rapid analysis of strain differentiation (Hwang *et al.*, 2019). Subtyping methods require a high discriminatory power and must be reproducible, in order to develop a reliable database. The methods of strain typing bacteria can be categorised into three categories; DNA banding pattern-

based genotyping, DNA sequencing-based methods and gene sequencing.

#### 1.3.1.1 DNA Banding Pattern-Based Genotyping

The different DNA banding pattern-based genotyping methods have previously been used to investigate the epidemiology of *S. uberis* strains. Pulse field gel electrophoresis (PFGE) was historically considered to be the gold standard of strain typing, prior to genomic sequencing becoming more readily available (Tenover *et al.*, 1995; Goodwin, McPherson and McCombie, 2016 ). PFGE separates large DNA molecules of 10kb to 10Mb using an algorithmically controlled switching electrical field, as DNA molecules of >20kb show the same mobility in a conventional constant electric field. The DNA is fragmented using restriction enzymes with uncommon recognition motifs, in order to produce large DNA fragments and therefore any differences in banding patterns reflect DNA polymorphisms at the restriction enzyme recognition sites. It has been used as a technique to strain type *S. uberis* isolates (Baseggio *et al.*, 1997; Wang *et al.*, 1999; Douglas *et al.*, 2000 ), including in a study investigating the epidemiology of *S. uberis* mastitis in 38 dairy cows from 4 different herds, which identified a high discriminatory rates, with a total of 62 strains identified from 138 isolates (Phuektes *et al.*, 2001). A recent study in Germany identified similar strains of *S. uberis* from 237 mastitis and environmental *S. uberis* isolates from 15 dairy farms, suggesting the potential presence of the same strain within environment and mastitis. In comparison to PFGE, ribotyping of *S. uberis* isolates in mastitic milk

samples and has successfully been used to identify the heterogenity of *S. uberis* isolates from different on farm sources (Zadoks *et al.*, 2005a). Ribotyping is a variation of random fragment length polymorphisms (RFLP) analysis which analyses variations within the genomic sequence of the 16s or 23s rRNA genes. Bacterial rRNA operons contain a family of highly conserved genes, each flanked by more variable DNA regions, which are present in several copies in most bacterial species and can also be used for whole gene sequencing based differentiation of bacterial strains (Yap *et al.*, 1999). The product patterns of the fragments to the relevant rRNA are called ribotypes and are composed of several bands, which depend upon the restriction enzyme used. Ribotyping has been used to investigate the epidemiology of *S. uberis,* with one study reporting 48 different ribotypes among 266 isolates from milk, environmental and faecal samples (Zadoks *et al.*, 2005). However there may be potential issues with the discriminatory power of ribotyping for *S. uberis,* as it has been reported to have a lower discriminatory power in comparison to methods, such as PFGE (Gillespie and Oliver, 2004).

Rep-PCR is based upon a series of naturally occurring repetitive DNA sequences which are dispersed as multiple copies throughout the genome and the amplification of DNA fragments between these sequences, using primers complementary to those interspersed repetitive consensus sequences which results in the generation of different sized amplicons, dependent on repetitive sequence location (Li *et al.*, 2009). REP-PCR has been utilised to investigate the variations

between *S. uberis* isolates taken from milk, teats, faeces and the environment of the cow and reported clustering of different isolates from different sources, indicating the potential for these isolates to be the same strain (Pryor, 2008). However this study did acknowledge issues with surrounding standardisation of the REP-PCR typing methodology, which means it may not have sufficient discriminatory power for strain typing. DNA sequencing-based methods

DNA sequencing-based genotyping methods generate the original sequence of nucleotide and discriminate among bacterial strains directly from polymorphisms in their DNA (Li *et al.*, 2009). These methods have the advantage over band pattern analysis methods of being highly reproducible, due to relying upon unambiguous DNA sequences that are easily stored in a database for future data mining.

#### 1.3.1.2 Gene Sequencing

Gene sequencing can be based on less-conserved genes, commonly those that encode bacterial surface proteins or virulence factors or regulatory genes that with conserved sequence function. Some genes, particularly those that encode surface associated proteins that are under immune pressure, can evolve quickly and therefore gene selection criteria is dependent on the downstream application. Two of the methods which have been used to investigate *S. uberis* strain epidemiology are discussed below.

MLST is based on DNA sequence analysis of five to seven housekeeping genes, whose evolution are constrained by their

requirement to encode functional products and are not affected by the rapid evolution that may be detected within other genes encoding proteins that influence bacterial survival (Coffey *et al.*, 2006). MLST investigates allelic variants within these so called housekeeping genes, with each unique sequence attributed a number. In the case of *S. uberis*, each strain is assigned a seven number allelic profile, with each unique combination designated as a single sequence type (Li *et al.,* 2009) The sequence types are then collated and stored on a global database (www.pubMLST.org). The typing scheme for *S. uberis* and is focused on analysis of glucose kinase (gki), acetyl CoA acetyle-transferase (yqiL), D-ala-D-ala ligase (ddl), transketolase (recP), thymidine kinase (tdk), carbamate kinase (arcC) and triiosephosphate isomerase (tpi) genes (Coffey et al 2006). MLST has successfully been used in *S. uberis* epidemiological studies where 70% of chronic IMI were able to be identified as the same sequence type (ST) (Pullinger *et al.*, 2007). It was also used to determine the genetic diversity of 854 clinical isolates of *S. uberis* from 20 herds in the UK; of which 195 unique STs were identified. This allowed classifications to be assigned to the observed epidemiological patterns, which highlighted the potential for certain STs of *S. uberis* to act as a potential contagious pathogen (Davies *et al.*, 2016). MLST has been shown to have a higher resolution than PGFE due to the phenotype diversity being only a proxy for DNA sequence diversity (Pullinger, *et al*, 2006) however MLST alone is not sufficient to discriminate between closely related strains,

due to the sequence conservation of the housekeeping genes, which can limit the usefulness of MLST in epidemiological studies

#### 1.3.1.3 Matrix-Assisted Laser Desorption Time of Flight Mass Spectrometry

An alternative method for strain differentiation has also been utilised through Matrix Assisted Laser Desorption Time of Flight mass spectrometry (MALDI-ToF or MALDI), which analyses bacterial peptides and proteins, rather than DNA and therefore will incorporate the phenotype of the strain into the identification (Sandrin *et al.*, 2013). It is based on the ionisation of co-crystallised sample material by short laser pulses, with the ions being accelerated and the time of flight measured (Wieser *et al.*, 2012). This process is repeated 40 times per sample to create a characteristic fingerprint for the isolate, with the peak intensity correlated with the amount of the corresponding protein. It has been used to determine the mass of proteins and peptides within the whole bacteria, in order to identify bacteria and bacterial species (Arnold and Reilly, 1998; DeMarco and Ford, 2013).

For strain identification, the peak patterns are compared to with spectral libraries of defined bacteria (Fuh *et al.*, no date). Strain typing using MALDI has been performed for a variety of bacteria, including *E. coli* (Christner *et al.*, 2014) and *Leptospira* strains (Rettinger *et al.*, 2012), as well as other *Streptococci* species (Kumar *et al.*, 2004; Moura *et al.*, 2008; Williamson *et al.*, 2008), including *S. uberis*. A recent study

analysed *S. uberis* isolates using MLST and MALDI-TOF and reported that the clonal complex of isolates which were genetically similar to ST6 (CC6) could be differentiated moderately well from the more genetically diverse and distantly related MLST sequence types by the MALDI phenotype. A moderate discriminatory power of the MALDI protein spectra profile for MLST sequence type indicated that the MLST sequence type of an isolate is associated with a reasonable proportion of the phenotypic variation between isolates (Davies, 2016). The use of MALDI provides an alternative method for strain typing that allows a combination of genotype information and epigenetic regulation of that genotype to be used; however further research is required to assess its suitability for large scale discriminatory strain analysis.

MALDI-TOF has been used to investigate the potential for contagious transmission of *S. uberis* within 29 herds in the UK, using principal component analysis (Esene*r et al*., 2018). This study showed that MALDI-TOF has the ability to be used for an intra-farm analysis tool to determine whether *S. uberis* strains are likely to be transferring through a contagious or environmental route of infection based on the ability to cluster isolates on phenotypic expression of key proteins, such as bacteriocins and ATP synthases.

#### 1.3.1.4 Whole Genome Sequencing

Whole genome sequencing is used to determine the complete DNA sequence of an organism's genome at a single time. There are two

main strategies for genome sequencing; the Sanger method, where the DNA sequence of a single-stranded template DNA is determined using DNA polymerase to synthesize a set of polynucleotide fragments of different lengths through the use of dideoxynucleotides that interrupt the elongation step of DNA amplification (Sanger et al., 1977), and pyrosequencing. Pyrosequencing is a nonelectrophoretic sequencing method, which is based on real-time quantitative detection of pyrophosphate released following nucleotide incorporation into a growing DNA chain during DNA synthesis (Li, et al., 2009) and includes technology such as the Illumina methodology and 16S rRNA gene sequencing. However pyrosequencing is more commonly being replaced by third generation sequencing methods, such as nanopore sequencing and minion based sequencing, which is based on the concept of the ability to induce different ionic current bursts during DNA traversing through a tiny channel, which a constant electric field is applied (Wang *et al.,* 2015).

The Sanger methodology was used to sequence and annotate the entire genome of *S. uberis* strain 0140J, providing the reference genome for the species (War*d et al*., 2009). This was functionally annotated using a transposon sequencing based strategy to identify those genes essential for *S. uberis* replication, termed the essential genome (Blanchard *et al.*, 2016). The concept of an “essential genome” assumes there is a minimum number of genes that bacteria require for survival and that the remainder can be disrupted or removed without obvious deleterious effects (Mushegian and Koonin, 1996). In general,

these genes encode proteins that maintain central metabolism and cellular structure, replication, translation and the uptake of necessary nutrients and export of any potential toxic metabolites. The analysis of the genome of 0140J identified a relative paucity of classical virulence determinants, as well as providing nutritional flexibility through a large number of differing carbohydrate metabolism pathways (Ward *et al.*, 2009). Additional sequencing of a  strain originating from New Zealand (NZ01) an extra 146 novel coding DNA sequences and had 163 absent coding DNA sequences, in comparison to strain 0140J  (Taiaroa *et al.*, 2018), however both strains were associated with cases of clinical mastitis, indicating a likely core set of genes likely to be related to bacterial virulence.

Pyrosequencing methodology has been utilised for a comparative analysis of 12 UK field isolates from *S. uberis* IMI cases, which allowed the characterisation of the pangenome and core genomes of all strains, as well as a comparison of the strains to each other and the reference strain 0140J (Hossain *et al.*, 2015). Differences between virulence factors were assessed, however no obvious individual genes which impacted the virulence of *S. uberis,* could be identified, with additional comparisons from an experimental challenge study with a known avirulent strain, EF20 also unable to provide evidence of specific gene sequences to differentiate virulent and avirulent strains. Potential differences were highlighted surrounding the gene promoter region *vru*, indicating that transcriptomic analysis may be required to further

understand the role of epigenetic regulation and its role in bacterial virulence (Hossain *et al.*, 2015).

### 1.3.2 The Epidemiology of Strains of *S. uberis*

For the purposes of this section, a ST refers to a group of bacterial strains which have the same unique allelic profile, based on analysis of 7 genes and are most likely derived from a recent common ancestor. A clonal complex refers to a group of bacterial strains derived from a recent common ancestor that share many alleles at various phylogenetically informative loci.

Many different strains of *S. uberis* have been isolated from milk samples from IMI (P. Phuektes *et al.*, 2001; Coffey *et al.*, 2006; Davies *et al.*, 2016) and previous research has established the presence of many different *S. uberis* STs from IMI, both within a farm unit but also between farms and different geographical regions. The presence of a predominant clonal complex within *S. uberis* clinical mastitis cases have been highlighted with clonal complex 5 (CC5) identified as predominant in the UK (Davies *et al.*, 2015) , which differs from those found in Australia (CC86) and New Zealand (CC143) (Douglas *et al.*, 2000; Pullinger, López-Benavides, *et al.*, 2006; Pullinger *et al.*, 2007), with

| Strain of *Streptococcus uberis* | Genome Size (base pairs) | Comment |
|---|---|---|
| 0140J | 1,852,352 | |
| EF20 | 1,933,244 | EF20 is reported to be avirulent strain of *S. uberis,* as evidenced in different challenge studies. EF20 is acapsular due to lacking the gene *SUB1027*. There is also a deletion of five bp in a region of vru. There are no identifiable prophage regions within the genome of EF20. |
| Ab71 | 1,849,250 | There is a four bp deletion in the region of vru. |
| 6780 | 1,960,858 | This strain lacks the gene *hasB* (*SUB1027).* |
| C6344 | 1,907,431 | |

CC143 isolates also been identified in China (Shome *et al.*, 2012). A recent study of 52 British herds showed that whilst there were multiple cases of the same strain attributed to causing IMI within a herd, 40% of the clinical mastitis cases were attributable to 9 sequence types (ST6, ST22, ST5, ST35, ST20, ST233, ST24, ST361 and ST512), with multiple “true singleton” strain types identified. Given the variation in strains identified between herds in terms of numbers of sequence types, (Davies *et al.*, 2015), this suggests a semi-diverse population of clinical mastitis *S. uberis* strains is present within UK farms.

**1.4 Epidemiological studies have also investigated the strain types present in the faeces of dairy cows. The same strain of *S. uberis* was identified in the same cow at different time points in one study, alongside a high diversity of strains identified from the surrounding environment (Pryor, 2008). As the same strain could be isolated from multiple cows in this study, cow to cow transmission of strains or cycling from an environmental source was considered a likely mode of strain transmission. The faecal isolates and strains of *S. uberis* within and between herds have been shown to vary considerably, using various different methods of assessment (Baseggio *et al.*, 1997; Douglas *et al.*, 2000; Lundberg *et al.*, 2014; Davies *et al.*, 2016). Ribotyping of *S. uberis* isolated from faeces, the environment and milk has reported multiple strains of *S. uberis* within faeces and that these strains were also be identified in the environment, as well as in milk (Zadoks *et al.*, 2005a). The same strains of *S. uberis* have also been isolated from faecal samples taken at different time points from the same cow**

**using REP-PCR, which lead to the speculation that the gastrointestinal tract of the cow may become colonised by a particular strain of *S. uberis* (Pryor, 2008). It has also been hypothesised that there is a subpopulation of "virulent" strains of *S. uberis* which existed within the environmental population of *S. uberis* strains, which can result in IMI (Pryor, 2008). Conclusion and Aims of the Study**

*S. uberis* is a major mastitis pathogen, which has welfare implications as well as economic implications for dairy farms worldwide. Whilst *S. uberis* is an opportunistic pathogen, it has been shown to be capable of causing both clinical and subclinical mastitis. From the literature, it is evident that there is an environmental reservoir for *S. uberis (*Zadoks *et al., 2005a;* Lopez-Benavides *et al.,* 2007; Davies *et al.,* 2015), with IMI most likely to involve the contamination of the udder of the cow with *S. uberis* from within the environment. With the presence of cows or effluent required for the detection of *S. uberis* in pasture, it is hypothesised that bovine faeces act as the source of environmental contamination (Lopez-Benavides *et al.*, 2007). Research studies have reported varying detection rates of *S. uberis* in the faeces of dairy cows worldwide; however a detection rate has not been reported for UK dairy herds for more than 35 years. It is unknown if the prevalence of faecal shedding has altered over this time period alongside the changing incidence of *S. uberis* clinical mastitis. Previous research has also

shown that there are the same ribotypes and STs of *S. uberis* in the environment and the faeces (Zadoks *et al.*, 2005a; Pryor, 2008) however the link between the same strains isolated from a cow with *S. uberis* IMI, the faeces or environment of the cow has yet to be determined and it is unknown whether the environmental or faecal strains isolated from the dairy are pathogenic, non-pathogenic or a mixture of both.

An infection model for this research has been hypothesised and suggests that there is the possibility of transmission of faecal *S. uberis* strains to the udder and subsequent IMI is through contaminated faeces and the propagation of this cycle continues through a faecal-oral based transmission (Figure 1.1). The faeco-oral transmission route is supported by evidence that *S. uberis* can be isolated from the lips and tonsils of cows as well as the gastrointestinal tract (Lacy-Hulbert *et al.*, 2005; Cullen 1969; Pryor 2008).

The first objective of this research was to develop a robust microbiological method of screening bovine faecal samples for the presence of *S. uberis*. After confirming the presence of *S. uberis* within a faecal samples, the aim was to isolate individual strains of *S. uberis* from bovine faeces for strain typing. A subsequent aim was to compare strain types of *S. uberis* isolated from faecal samples with mastitic milk samples from the same dairy herd, in order to explore the hypothesed infection model. Strain typing would also inform the epidemiology of *S.*

*uberis* present within faecal samples from the same cow over time, as well as between cows in the same dairy herd. This is addressed in Chapter 2.

The second objective this research was to report the prevalence of faecal shedding of *S. uberis* in a typical UK herd, as most recent reports of prevalence of faecal shedding have been based in other countries, where herds are predominantly at pasture during the sampling. This system is different to the vast majority of dairy systems in the UK, where housing occurs for some or all of the year. Alongside the aim of reporting the prevalence of faecal shedding of *S. uberis* in a herd within UK, another aim was to investigate the faecal shedding patterns of *S. uberis* of the cows within the herd over time and investigate potential explanatory variables that influence whether a cow sheds *S. uberis* or not. In order to perform this study, a robust screening test would be required which had a high analytical sensitivity and allow a high throughput of faecal samples. This is addressed in Chapter 3.

The third objective of this research was to investigate the potential for *S. uberis* to survive within the housed bedded environment of the dairy cow, because the ability to survive in different environments may have an important role in the epidemiology of *S. uberis* mastitis. It has been reported that *S. uberis* survives on pasture for up to 2 to 3 weeks (Lopez-Benavides *et al.*, 2007), however the survival of *S. uberis* on different bedding substrates is currently unknown. This research aimed

to investigate the survival and potential growth on different bedding substrates of *S. uberis* strains which had been isolated from mastitis cases, as well as the survival of *S. uberis* strain 0140J mutants, in order to investigate genes that may influence the survival of *S. uberis* on different bedding substrates. This is addressed in Chapter 4.

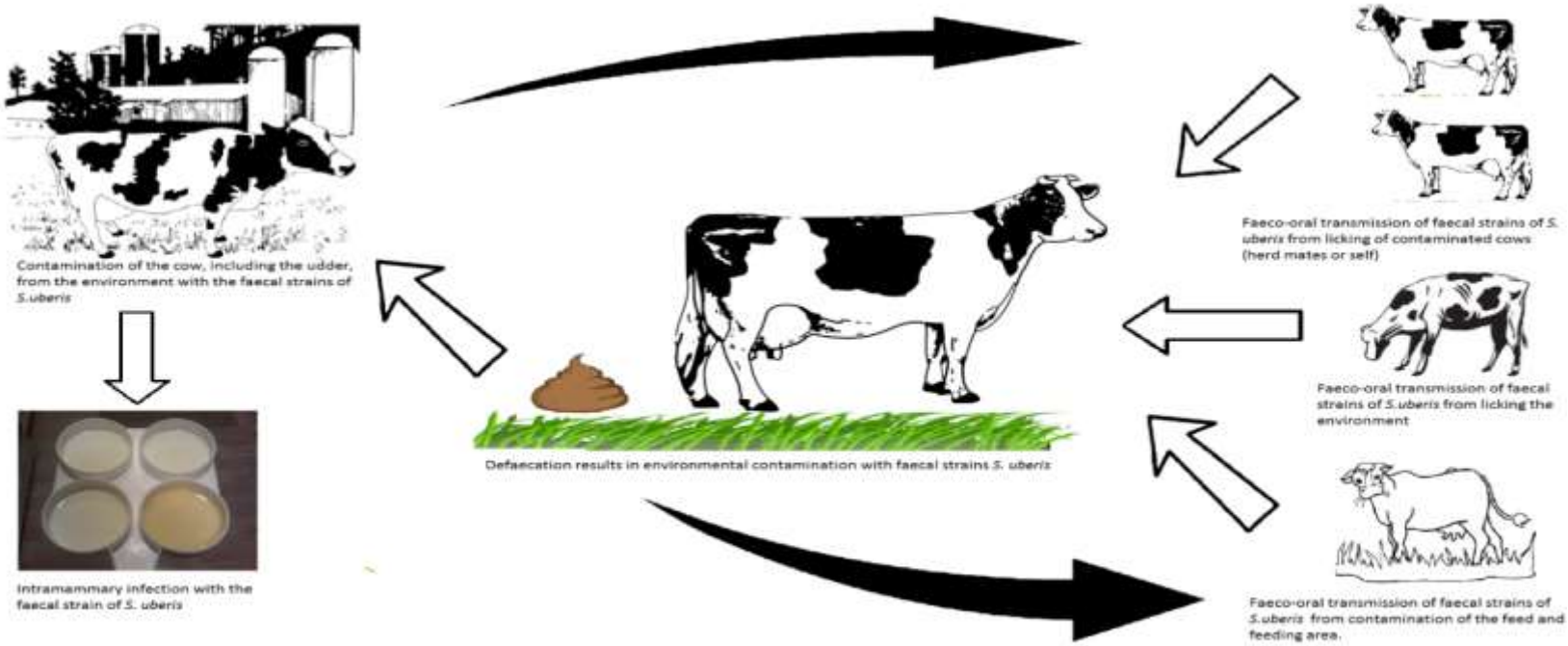


**Figure 1.1 The hypothesed model of infection for *S. uberis* faeco-oral transmission. .**

The cow acquires *S. uberis* bacteria by ingestion within the contaminated environment, either from potential commensal sites in or on the cow or other cows within the herd, or through ingestion of contaminated feed.

# Chapter 2. Detection and identification of *S. uberis* in bovine faeces

## 2.1 Introduction

The aim of this section of the study was to develop a diagnostic that could be used to detect the mastitis pathogen *S. uberis* in bovine faeces. It has been hypothesised that faeces are the main source of *S. uberis* contamination within the cattle environment (Lacy-Hulbert *et al.*, 2005; Zadoks *et al.,*2005a; Lopez-Benavides *et al.*, 2007) and such contamination may lead to an increased risk of *S. uberis* mastitis. The development of a diagnostic screening test would allow the investigation of the role of asymptomatic carriage play in the transmission intramammary infection by *S. uberis*. The second aim was to develop a methodology for isolation and identification of *S. uberis* from bovine faeces, in order to enable investigation of strain variation within and between animals over time and for comparison with isolates obtained from clinical mastitis within the herd.

Identification of causative agents responsible for mastitis is used to guide treatment decisions and to provide practical solutions for prevention through different management practices (Adkins and Middleton, 2018; Lago and Godden, 2018). The assessment of subclinical mastitis is often measured by SCC, where elevated SCC

numbers link to the influx of neutrophils into the gland as a response to infection and can be measured directly. Confirmation of the specific IMI pathogen by bacterial culture is still considered the gold standard practice, despite being labour intensive. However, a high proportion (up to 50%) of clinical mastitis samples do not yield bacteria following culture (Hogan et al 1999).

Traditionally, laboratory diagnosis of *S. uberis* as the causative agent of mastitis directly from milk has been based on the presence of a Gram-positive, catalase-negative, esculin hydrolysing, non-haemolytic colonies composed of coccoid cells that were non-typable by Lancefield antigen classification (Odierno *et al.*, 2006; Wald *et al.*, 2017; Hogan *et al.*, 1999; Koskinen *et al.*, 2010; Zadoks *et al.*, 2014). The most widely used bacterial media is esculin blood agar, where *S. uberis* bacterial colonies can be differentiated from other species through their ability to hydrolyse esculin, resulting in darkening of the surrounding medium. There are a number of other bacterial species including *Lactococcus*, *Enterococcus* and *Aerococcus* that can also hydrolyse esculin and have similar biochemical and colony morphology to *S. uberis*. Given these species are considerably less common as causative agents for mastitis than *S. uberis*, use of esculin blood agar can successfully differentiate *S. uberis* the other main species typically responsible for intramammary infection directly from milk.

Other selective media have been utilised to differentiate *S. uberis* from other species, including modified Rambach media (MRAM), which utilises the presence in *S. uberis* of β-D-galactosidase to cleave the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, resulting in distinguishable, blue *S. uberis* colonies (Watts, Salmon and Yancey Jr., 1993; Wald *et al.*, 2017). Additional studies have used *uberis* selective media (USM) to successfully detect *S. uberis* from cattle faecal samples utilising similar chromogenic substrates, suggesting the use of selective chromogenic media may be applicable for the isolation of single *S. uberis* colonies from bovine faeces.(Lacy-Hulbert *et al.*, 2005; Pullinger *et al.*, 2006; Pryor, 2008).

Molecular-based techniques, typically PCR, have been developed for the detection of different mastitis pathogens in clinical samples, including *S. uberis.* This involves amplification of a DNA target sequence that is diagnostic for a particular organism. Such assays can be scaled for use in high-throughput testing and / or enable detection of multiple pathogens simultaneously (Bursle and Robson, 2016). One of the drawbacks of PCR based detection is that a positive result only reflects the presence of the nucleic acid sequence and not the presence of a viable organism. For definitive confirmation of the organisms present, a combination of culture and molecular based detection would be considered optimal.

A PCR based systems are now routinely used as a diagnostic to identify mastitis pathogens from milk samples (Brikenmeyer and Mushahwar, 1991; Garibyan and Avashia, 2013). These include the “Pathoproof PCR assay” (Spittel and Hoedemaker, 2012; Hiitiö *et al.*, 2016; Taponen *et al.*, 2017), which is multiplex PCR able to detect twelve different bacterial species, including *S. uberis,* within one PCR assay. The sensitivities of detecting different bacterial species using this assay has been shown to vary, with *Staphylococcus aureus* detected at a greater sensitivity than *S. uberis* and differences in the ability to extract DNA from these different species thought to contribute to this discrepancy (Steele *et al.*, 2017).

Various methods have been used to detect the specific bacterial species within faecal samples, including culture and non-culture based methods. One difficulty encountered when using culture based techniques for identification of a specific bacterial population within faeces is the large number of other bacteria present, which is estimated to be approximately $10^{11}$ Cfu/g and includes the presence of potentially unknown bacterial species (Dowd *et al*., 2008a). This means that direct comparison of *S. uberis* isolation methods from mastitic milk and faecal/environmental sources can be problematic, given the differences in complexity and numbers of bacteria in the samples. Within a mastitic milk sample, *S. uberis* tends to be the dominate bacterial species present, typically at levels of between $10^3$ to $10^8$ cells/ml (Leigh, 1999) and even in the case of mixed infections, the

number of different species within the sample is low (Pryor *et al.*, 2009). This contrasts with faecal samples, where *Streptococcus* species have been found to represent less than 0.55% of the total bacterial DNA present and whilst the exact percentage of *S. uberis* that can be attributed is currently unknown, it is likely to be a low percentage (Dow*d et al*., 2008b; Mao *et al*., 2015). Numerous strains of *S. uberis* have been isolated and characterised worldwide, (Davies *et al.*, 2016, McDougall *et al.*, 2004), primarily from cases of bovine mastitis and do not include any strains specifically isolated from the faeces of cows. There are currently four complete *S. uberis* genomes that have been assembled and each were isolated from cases of mastitis (Altschul *et al*., 1990; Ward *et al*., 2009; Taiaroa *et al*., 2018)

Development of a protocol for identification and isolation of *S. uberis* from cattle faecal samples would allow for a direct epidemiological comparison of strains of different provenance and may highlight differential characteristics of these isolates based on genomic analysis. Given such genomic analysis of *S. uberis* strains has primarily been performed on strains isolated from cases of bovine mastitis, this may provide key information regarding environmental persistence and infection potential of faecally derived strains. This chapter focuses on the development of a method for the *S. uberis* identification and isolation from cattle faeces.

This chapter will describe the development of a molecular diagnostic test for the screening of bovine faeces for the presence of *S. uberis,* including determining optimal conditions for DNA extraction as well as the overall analytical sensitivity of the test. It will also describe the impact of the sampling methodology used to detect the presence of *S. uberis* within cattle faecal material. The last section of the chapter will discuss the potential use of culture based methodologies for the isolation of individual strains of *S. uberis* from bovine faeces.

## 2.2 Materials and Methods

Reagents used in this study were supplied from Sigma-Aldrich (Dorset, UK) or Thermo Fisher Oxoid (Basingstoke, UK) unless otherwise specified.

### 2.2.1 Growth of Bacterial Strains

Thirteen isolates obtained from clinical and subclinical mastitis, including the reference strain *S. uberis* strain 0140J were routinely cultured overnight in brain heart infusion (BHI) at 37°C (Table 2.1). As were other bacteria; *Escherichia coli* strain P4, *Enterococcus faecium, Enterococcus hirae, Streptococcus lutentis* and *Streptococcus equinus*. Overnight cultures (50µl) were plated on different selective solid media (blood esculin, Modified Rambach media (MRAM), *Uberis* Rambach media (URAM), *Uberis* specific media (USM), Reduced salt *Uberis* specific media (RSUSM)) and incubated overnight at 37°C.

Six different selective media were used throughout the growth experiment studies, each of which is summarised below.

Blood Esculin plates containing sheep blood agar and 0.1% esculin were purchased directly from Cherwell Laboratories (Bicester, UK). Mitis salivarius was prepared as per manufacturer's instructions.

Modified Rambach media (MRAM) was prepared as described previously (Watts, Salmon and Yancey Jr., 1993) and contained 5g peptone, 2g yeast extract, 0.03g neutral red, 10g propylene glycol and 0.1g 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside per litre.

**Table 2.1: Characteristics of the 13 strains of *S. uberis* used for methodology testing**

| ***S. uberis* Strains** | **Genome Sequence Size (base pairs)** | **Year of Isolation** | **Isolated from** | **MLST Sequence Type** |
|---|---|---|---|---|
| 0140J* | 1852352 | 1973 | Clinical mastitis | ST-5 |
| C6344 | 1907431 | 2002 | Clinical mastitis | ST-5 |
| C5072 | 1906212 | 2002 | Clinical mastitis | ST-5 |
| S6261 | 1868255 | 2002 | Clinical mastitis | ST-5 |
| EF20** | 1933244 | 1970 | Clinical mastitis | |
| 6736 | 1893525 | 2002 | Subclinical mastitis | ST-5 |
| C8329 | 1923969 | 2002 | Subclinical mastitis | ST-5 |
| Ab71 | 1849250 | 2002 | Clinical mastitis | ST-5 |
| C9359 | 1837707 | 2002 | Clinical mastitis | ST-5 |
| C5388 | 1837997 | 2002 | Subclinical mastitis | ST-5 |
| B190 | 1881868 | 2000 | Subclinical mastitis | ST-5 |
| 6780 | 1960858 | 2000 | Clinical mastitis | ST-86 |
| B362 | 1912672 | 2000 | Subclinical mastitis | ST-86 |

*Clinical reference virulent strain (Ward *et al.*, 2009)

**Avirulent strain (Hossain *et al.*, 2015)

*Uberis* Rambach media (URAM) was prepared as above, however 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside was omitted and 0.1g 5-bromo-4-chloro-3-inodyl β-D glucuronide sodium salt was included as the chromogenic substrate per litre.

*Uberis* specific media (USM) was prepared as previously described (Cursons and Wilkins, 2008) and contained 2.5g peptone, 8.5g yeast extract, 0.5g meat extract, 15g pancreatic digestion of casein, 7.5g NaCl, 10g inulin, 0.25g esculin or 0.1g 4-mehytlumbelliferyl-beta-D-glucuronide, 1g bromocresol purple, 1g gentamicin and 1g thallous acetate per litre with the pH adjusted to 6.8. Reduced salt *uberis* specific media (RSUSM) was made as above, however the concentration of NaCl was decreased to 5g per litre.

### 2.2.2 Cattle Faecal Sample Collection and Processing

Samples were collected directly from freshly voided faeces (FVF) from eleven dairy cows on two separate sampling dates (March and June 2016) from one herd. Samples were collected as either 1g aliquots or as swabs obtained directly from FVF and were cultured as above. Enriched faecal cultures were mixed with sterile glycerol (final concentration 25%, w/v) and stored at -80ºC. Bacteria from these stocks were used for DNA extraction.

A faecal sample was autoclaved twice at 121°C and confirmed to be culture negative after incubation in BHI as above. Samples (1g or 5g)

were spiked with *S. uberis* strain 0140J and cultured for 16-48 hours in BHI containing varying concentrations of Streptococcus selective supplement (SS) (10 mg of colistin sulfate and 5 mg of oxolonic acid per litre) or 4µg/ml gentamicin. Cultures from the various enrichments were stored as frozen stocks in 25% (w/v) glycerol, generated as described above and 10µl of each inoculated into 2ml of BHI containing 4µg/ml gentamicin, incubated overnight at 37°C prior to DNA extraction.

### 2.2.3 Comparison of DNA Extraction Methodologies

Three different DNA isolation methods were compared using the sterilised faecal sample that had been spiked with *S. uberis* 0140J. Extraction was performed on samples either after 30 minutes after the inoculation with *S. uberis,* or after culturing the samples for 16 hours after the inoculation, as above.

#### 2.2.3.1 Boilate DNA Extraction

Bacteria from 0.5ml of spiked faecal culture were harvested by centrifugation (13,000 x *g*, 5 minutes) and the supernatant discarded. The cell pellet was washed twice with 100µl TE buffer (1M Tris-HCl, 0.5M EDTA) and cells collected by centrifugation, as above. The washed cell pellet was re-suspended in 100µl of TE buffer and transferred incubated for 10 minutes at 99.9ºC, followed by rapid cooling and incubation at 12ºC for 2 minutes in a thermocycler (BioER). The samples were then stored at -20ºC until required.

#### 2.2.3.2 Phenol-based DNA Extraction

Bacterial cells were harvested by centrifugation (13000 x *g,* 10 minutes) from 1.5 ml overnight culture, the supernatant removed and cell pellet re-suspended in 0.5ml of TE buffer. Following centrifugation (as above), the bacterial pellet was re-suspended in 375µl of cell wall disruption buffer (10mg/ml lysozyme, 30U/ml mutanolysin in TE buffer) and incubated at 37ºC. After 30 minutes, 20µl of lysis buffer (20% SDS w/v in 50mM Tris, 20mM EDTA, pH 7.8) and 60µg of proteinase K were added, mixed by gentle inversion and incubated for a further 60 minutes at 37ºC. Saturated (~6M) NaCl 200µl was added to precipitate the cellular debris which was removed by centrifugation (12,500 x *g*, 10 minutes) and the supernatant transferred to a fresh tube and mixed with an equal amount of Tris-equilibrated phenol: chloroform: isoamyl alcohol (25:24:1). Samples were centrifuged (13000 x *g*, 3 minutes) in order to separate the phases and the upper phase was transferred to a fresh tube and DNA precipitated by the addition of 2 volumes of ice cold 100% ethanol followed by incubation at -20ºC for 30 minutes. Precipitated DNA was collected following centrifugation (13000 x g, 3 minutes), washed in 70% cold ethanol and the final DNA pellets left to air dry before re-suspension in 30µl of TE buffer containing RNAseA (20µg/ml) and incubation overnight at 4ºC. In order to completely remove RNA, the samples were incubated at 37ºC for 30 minutes and then stored at -20ºC until required.

#### 2.2.3.3 QIAamp DNA Stool Mini Kit DNA Extraction

A sample of between 180-220mg of faeces was suspended in 1.4ml of Buffer ASL; Qiagen, and mixed by vortexing (Denley Vibromix), until the sample was homogenized. The sample was heated at 95ºC for five minutes, followed by vortexing for 15 seconds and then clarified by centrifugation (20,000 x *g* for 1 minute). 1.2ml of the supernatant was removed and used to add to 1 InhibitEX tablet, this was mixed until the tablet was resuspended. The sample was incubated for a minimum of 1 minute, and debris removed by centrifugation (20,000 x *g* for 3 minutes). The supernatant was transferred to a fresh tube and mixed with 15µl of Proteinase K (>600mAU/ml), followed by the addition of 200µl of Buffer AL; Qiagen, and incubation at 70ºC for 10 minutes. After incubation, 200µl of 100% ethanol was added to the sample and the mixture transferred on to a QIAamp spin column and DNA isolated according to the manufactures protocol. All samples were stored at 4ºC prior to use.

### 2.2.4 Gel Electrophoresis

DNA was separated by gel electrophoresis using an 0.8% agarose/40 mM Tris-acetate and 1 mM EDTA, pH of 8.3 (TAE) gel in TAE buffer for approximately 45 minutes at 100V in the presence of DNA loading buffer (Bioline, Memphis, USA). Hyperladder 1 (Bioline) was routinely used as the DNA molecular weight marker. Gels were stained with 0.5µg/ml ethidium bromide for 30 minutes, before being visualised using a ultra-violet transilluminator (UVP).

### 2.2.5 Isolation of single strains of *S. uberis*

#### 2.2.5.1 Preparation of Faecal Samples

Faecal matter was inoculated into between 3-50 ml of BHI containing either 100%, 50% or 10% Streptococcal Supplement (SS) or 4µg/ml gentamicin and incubated for between16 to 48 hours at 37°C. Bacterial growth was determined after inoculation of either MRAM, URAM, USM or RSUSM agar plates with 10µl of liquid culture and further incubation at 37°C. *S. uberis* colonies were identified based on colony morphology and re-inoculated onto a secondary MRAM URAM, USM or RSUSM agar plates before inoculation of single colonies into BHI broth and growth of 16 hours at 37°C. Overnight cultures were streaked onto Enterococcus specific Mitis Salivarius agar plates and the remaining culture processed for DNA extraction or storage at -80°C in the presence of glycerol.

#### 2.2.5.2 The impact of *Enterococcus faecium* on the growth of *S. uberis*

Three URAM plates were seeded with 50µl of overnight culture of *S. uberis* 0140J and 10µl of *Enterococcus faecium* was inoculated as a single line halfway across the URAM plate and incubated at 37°C for 48 hours. Separate overnight cultures of *S. uberis* 0140J and *E. faecium* were grown in BHI media and then mixed to create the following ratios of *S. uberis*:*E. faecium*; 1:1, 1:10, 1:100 and 1:1000.A total of 40µl of each mixed culture was then plated on URAM agar and incubated for 48 hours.

### 2.2.6 Detection of *S. uberis* using PCR

PCR was performed on DNA extracted from 13 *S. uberis* strains and spiked faecal samples, using three separate primer pairs to amplify the genes *sub0888, sub1154* or *sub1730,* which had previously been identified as sequences present within the *S. uberis* 0140J genome (War*d et al*, 2009) (Table 2.2). Additionally 16S rDNA amplification was performed using primers previously described (Bentley and Leigh, 1995).

The PCR mastermix comprised of 1x standard Taq buffer reaction (NEB), 200µM of dNTPs (Thermo), 0.4µM of forward primer, 0.4µM of reverse primer and 1.25U Taq polymerase (NEB) and approximately 20ng of DNA. Initiation temperature was set to 95ºC for 30 seconds, followed by 35 cycles of denaturation, annealing and elongation at 95ºC for 20 seconds, 53.5ºC for 30 seconds and 68º for 60 seconds respectively. A final hold at 68ºC for 5 minutes constituted the final elongation. A negative control omitting DNA and a positive control using *S. uberis* 0140J DNA were included in all PCR assays.

Reactions using Phusion polymerase were generated as 25ul reactions to containing 1X concentration of Phusion HF reaction buffer (NEB), 200µM of dNTPs, 0.5µM of forward primer and 0.5µM of reverse primer, 1U of Phusion polymerase (NEB) and approximately 20ng bacterial DNA. Initial DNA denaturation was performed at 98ºC for 2 minutes, which was followed by 35 cycles of denaturation, annealing and

elongation at 98ºC for 15 seconds, 62ºC for 25 seconds and 72ºC for 45 seconds respectively. A final hold at 72ºC for 5 minutes constituted the final elongation stage.

### 2.2.7 Statistics and Bioinformatics

A dependent two-tailed t-test was performed to determine significance of starting material (swabs or 1g faeces aliquots) for detection of *S. uberis*. Primer sequences were analysed using the *S. uberis* BIGSdb genomics platform database (https://pubmlst.org/suberis/) (Coffe*y et al.*, 2006; Pullinger *et al*., 2006), as well as for potential cross-reactivity with other bacterial species within the NCBI non-redundant nucleotide basic local alignment data base (Altschul *et al.*, 1990).

**Table 2.2: Oligonucleotide primer sequences for identification of *S. uberis* by PCR**

| Gene | Sense Primer | Antisense Primer | Product Size (base pairs) |
|---|---|---|---|
| 16S | 5’-AGAGTTTGATCCTGGCTCAGGA-3’ | 5’-TTACCGCGGCTGCTGGCACGT-3’ | 529 |
| *Sub0888* | 5’-CTTTATGAAAATAGCCAAGCTGAAA-3’ | 5’-CTTAATTGTTGCTGGTCTTTCATTT-3’ | 1479 |
| Sub1730 | 5’-CAGGAGAAAGCTGCAAGCAC-3’ | 5’-GTATTTTGAGGTACCGGGGAAACTA-3’ | 1029 |
| Sub1154 | 5’-GAGCAATTGCAAAATGAAAAGC-3’ | 5’-ATGTCAAAAGCCCGGTACCTTTACAG-3’ | 3702 |

## 2.3 Results

### 2.3.1 Optimisation of PCR based detection of *S. uberis*

The ability of three different primer pairs to identify different strains of *S. uberis* were investigated. These were designed to amplify sequences unique to *S. uberis* within *sub0888*, *sub1154*or *sub1730*that encoded sortase anchored proteins (Egan *et al.*, 2010; Leigh *et al.*, 2010). The primer sequence conservation in *S. uberis* strains was confirmed using the *S. uberis* BIGSdb database (https://pubmlst.org/suberis/) containing 134 partial or completed *S. uberis* genomes (Coffey *et al*., 2006; Pullinger *et al.*, 2006). The presence of sequences complementary to the primers was investigated using the NCBI non-redundant nucleotide basic local alignment data base (Altschul *et al.*, 1990). Basis Local Alignment Search Tool (BLAST) analysis showed that these gene sequences had high identities, often at 100% for *S. uberis* bacteria strains and no other significant homology to other sequences in the non-redundant data base. The primer pairs were also screened using the primer BLAST program (Ye, Bioinformatics 2012, 13:134) and as a sequence pair were found to only amplify sequences from the 4 completed *S. uberis* genomes within the database (Accession number CP022435, AM946015, LS483397 and LS483408), including *S. uberis* 0140J.

Results indicated that the *sub0888* forward primer sequence had 100% homology with all *S. uberis* strains in the database and the 25

nucleotide reverse primer could be mapped to 79% of the strains, with a maximum of 2 base pair changes in the remaining 29% of strains (15% 1 mismatch, 14% 2 mismatches). Analysis of the *sub1154* forward primer showed alignment with all 22 nucleotides for all strains, with 1 nucleotide mismatch in 11% of strains and the reverse primer showed alignment of 23/26 nucleotides in 93% of strains, with the remaining 7% only showing alignment for 13 nucleotides of the primer sequence. Analysis of the *sub1730* forward primer found 59% aligned with all 20 nucleotides for the sequence, however the remaining mapped to only 16 nucleotides within the *S. uberis* sequences, and the reverse primer mapped to only 36.5% of sequences for the entire 25 nucleotides and the remaining only matched to between 12-13 nucleotides of the primer sequence (Table 10). Given this primer pair amplified a likely variable gene region, the use of this primer pair was discarded.

To assess the mismatch tolerances for the *sub0888* and *sub1154* PCR primer pairs, amplification was performed at an annealing temperature of 50ºC, using 12 sequenced strains of *S. uberis* (Hossain 2015), with the sequence alignment for these strains shown in Table 2.3 and Table 2.4. The *sub0888* and *sub1154* primer pairs were able to successfully amplify target from all 12 isolates, and strain 0140J (positive control), including the 3 strains (EF20, B362 and 6780) showing 1 or 2 nucleotide mismatches in the reverse primer. The *sub0888* primer pair

**Table 2.3: Alignment of *sub0888* primer sequences to *Streptococcus uberis* strains**

| *S. uberis* strain | Forward Primer | Reverse Primer |
|---|---|---|
| | C T T T A T G A A A A T A G C C A A G C T G A A A | C T T A A T T G T T G C T G G T C T T T C A T T T |
| C6344 | - - - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - - - - - - - - - - - |
| C5072 | - - - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - - - - - - - - - - - |
| S6162 | - - - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - - - - - - - - - - - |
| EF20 | - - - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - C - C - - - - - - - - - - - - - - - - |
| 6736 | - - - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - - - - - - - - - - - |
| AB71 | - - - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - - - - - - - - - - - |
| C9359 | - - - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - - - - - - - - - - - |
| C5388 | - - - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - - - - - - - - - - - |
| B190 | - - - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - - - - - - - - - - - |
| 6780 | - - - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - C - - - - - - - - - - - - - - - - |
| B362 | - - - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - C - - - - - - - - - - - - - - - - |
| C8329 | - - - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - - - - - - - - - - - |

*Missing base pair
- Indicates same base pair as the primer sequence

**Table 2.4: Alignment of *sub1154* primer sequences to *Streptococcus uberis* strains**

| *S. uberis* strain | Forward Primer | Reverse Primer |
|---|---|---|
| | G A G C A A T T G C A A A A T G A A A A G C | A T G T C A A A A G C C C G G T A C C T T T A C A G |
| C6344 | - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - T - - - T T - - - - - - - - - |
| C5072 | - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - T - - - T T - - - - - - - - - |
| S6162 | - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - T - - - T T - - - - - - - - - |
| EF20 | - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - T - - - T T - - - - - - - - - |
| 6736 | - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - T - - - T T - - - - - - - - - |
| AB71 | - - - - - - - - A - - - - - - - - - - - - - - | - - - - - - - - - - - - - T - - - T T - - - - - - - - - |
| C9359 | - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - T - - - T T - - - - - - - - - |
| C5388 | - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - T - - - T T - - - - - - - - - |
| B190 | - - - - - - - - A - - - - - - - - - - - - - - | - - - - - - - - - - - - - T - - - T T - - - - - - - - - |
| 6780 | - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - T - - - T T - - - - - - - - - |
| B362 | - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - T - - - T T - - - - - - - - - |
| C8329 | - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - T - - - T T - - - - - - - - - |

*Missing base pair
- Indicates same base pair as the primer sequence

**Table 2.5: Alignment of *sub1730* primer sequences to Streptococcus *uberis* strains**

| *S. uberis* strain | Forward Primer | Reverse Primer |
|---|---|---|
| | C A G G A G A A A G C T G C A A G C A C | C T T A A T T G T T G C T G G T C T T T C A T T T |
| C6344 | - - - - - - - - - - - - - - - - - - - - - | * - - - - - - - - - - - - - * * * * * * * * * * * * |
| C5072 | - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - A A A A - - - - - - - - - - |
| S6162 | - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - A A A A - - - - - - - - - - |
| EF20 | - - - - - - - - - - - - - - - - - - - - - | * - - - - - - - - - - - - - * * * * * * * * * * * * |
| 6736 | - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - A A A A - - - - - - - - - - |
| AB71 | - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - A A A A - - - - - - - - - - |
| C9359 | - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - A A A A - - - - - - - - - - |
| C5388 | - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - A A A A - - - - - - - - - - |
| B190 | - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - A A A A - - - - - - - - - - |
| 6780 | - - - - - - - - - - - - - A - - * * * * * | - - - - - - - - - - - - A A A A - - - - - - - - - - |
| B362 | * * * * * * - - - - - - - - - - - - - - * | - - - - - - - - - - - - A A A A - - - - - - - - - - |
| C8329 | - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - A A A A - - - - - - - - - - |

*Missing base pair
- Indicates same base pair as the primer sequence

were selected for detection of *S. uberis* in faecal samples, given the closer alignment of sequences when compared with known *S. uberis* strains on the BIGSdb database.

Two different DNA polymerases (Standard Taq and Phusion high fidelity polymerase) were compared for amplification of *16S rRNA* using the 13 *S. uberis* strains. 16S rRNA was successfully amplified for each strain using both types of polymerase, however despite repeated analysis, a product was repeatedly observed within negative control samples when using Phusion polymerase. Given all DNA samples extracted from faeces would need a positive control assay confirm isolation of bacterial DNA as well as *S. uberis* specific screening, Taq polymerase was chosen for the amplification of DNA in all remaining experiments.

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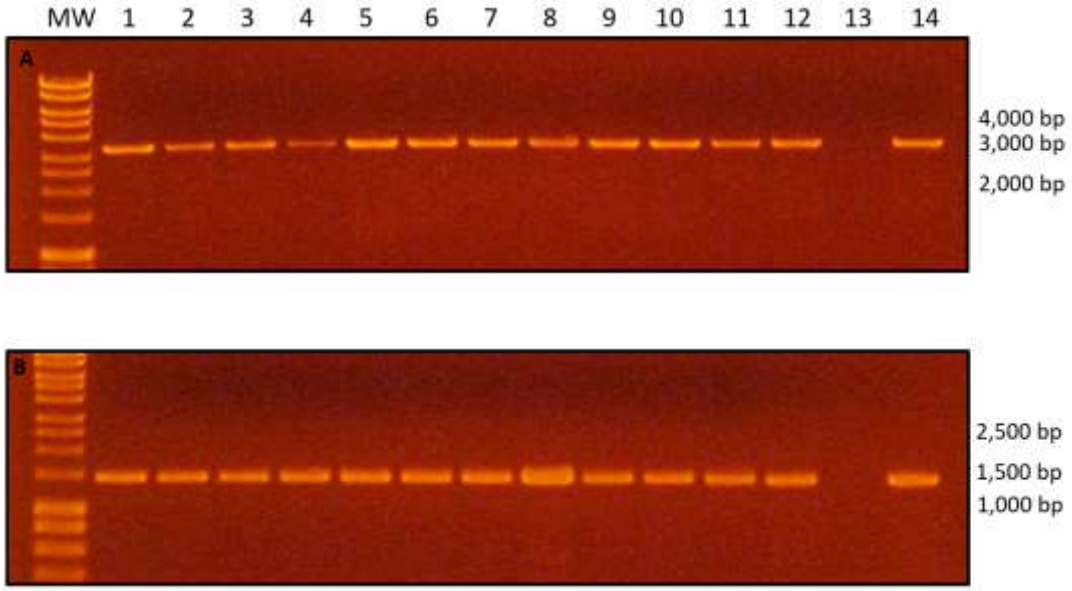



**Figure 2.1 Detection of *sub0888* and *sub1154* within *S. uberis* isolates by PCR.**

DNA was extracted from 12 *S. uberis* strains and used to amplify *sub0888* or *sub1154* by PCR using Taq polymerase. *S. uberis* strains were analysed as follows: C6344 (lane 1), C5072 (lane 2), S6162 (lane 3), EF20 (lane 4), 6736 (lane 5), Ab71 (lane 6), C9359 (lane 7), C5388 (lane 8), B190 Lane 9), B362 (lane 10), 6780 (lane 11) and C8329 (lane 12), with lane 13 omitting DNA, lane 14 amplified product using *S. uberis* 0140J DNA. Hyperladder 1Kb was used as the molecular weight marker.

A: Amplification of *sub1154* indicating a product size of 3,702bp present in each of the strains tested

B: Amplification of *sub0888* indicating a product size of 1,479bp present in each of the strains tested

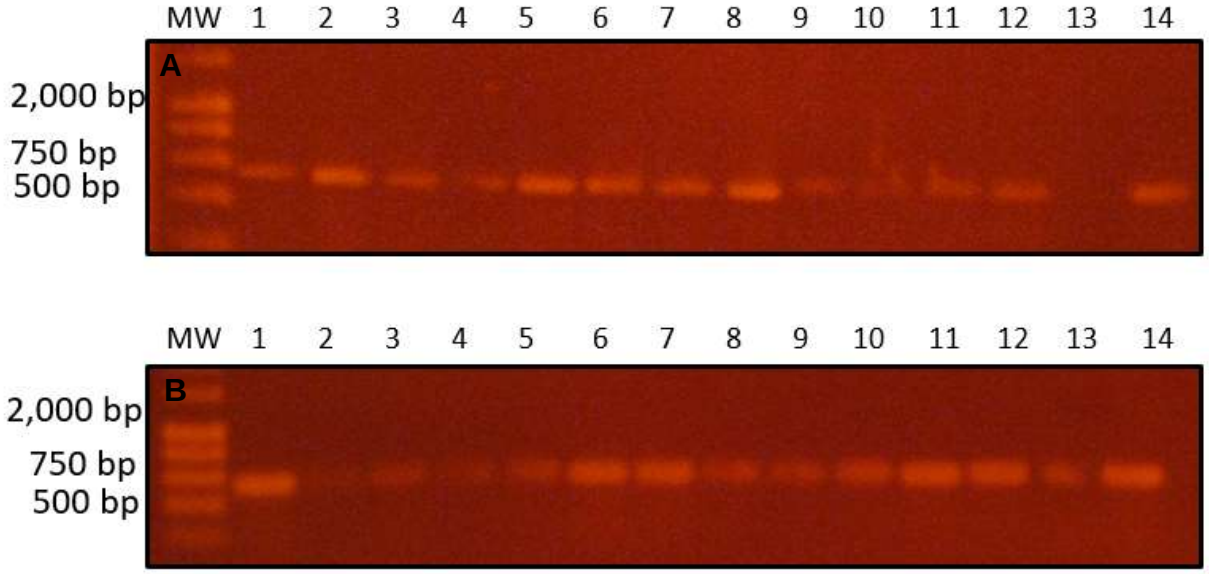



**Figure 2.2 Optimisation of 16S PCR, using either Phusion or Taq DNA polymerase**

DNA was extracted from 12 *S. uberis* strains and used to amplify 16S by PCR using either Phusion polymerase or Taq polymerase. *S. uberis* strains were analysed as follows: C6344 (lane 1), C5072 (lane 2), S6162 (lane 3), EF20 (lane 4), 6736 (lane 5), Ab71 (lane 6), C9359 (lane 7), C5388 (lane 8), B190 Lane 9), B362 (lane 10), 6780 (lane 11) and C8329 (lane 12), with lane 13 omitting DNA, lane 14 amplified product using *S. uberis* 0140J DNA. Hyperladder 1Kb was used as the molecular weight marker...

A: Amplification of 16S using Taq polymerase indicating the absence of an amplified product in Lane 13 (negative control)

B: Amplification of 16S using Phusion polymerase indicating the presence of an amplified product in Lane 13 (negative control)

### 2.3.2 DNA extraction from faeces

#### 2.3.2.1 Enrichment of *S. uberis* in faeces

In order to increase the chances of isolating *S. uberis* from bovine faeces, an enrichment based step was introduced to attempt to minimise the growth of Gram negative organisms, based on the use of the aminoglycoside antibiotic, gentamicin. To determine the minimum inhibitory concentration for *S. uberis*, $10^4$ cfu of bacteria were innoculated into BHI containing a range of gentamicin concentrations from 0-400ug/ml and assessed in triplicate. The bacteria were grown for 16 hours and growth recorded by measuring the absorbance at 550nm. No inhibition of growth was observed for the control samples where gentamicin was omitted, nor for samples containing gentamicin at a concentrations of 0.2, 0.4 or 2µg/ml. A slight decrease in growth in the presence of gentamicin at 4µg/ml (Figure 2.3), however this reduction was not statistically significant compared with the control value. No bacterial growth was observed at concentrations above 20µg/ml. Based on this result, a concentration of 4µg/ml of gentamicin was included in the liquid media for all culture based studies of cattle faecal material.

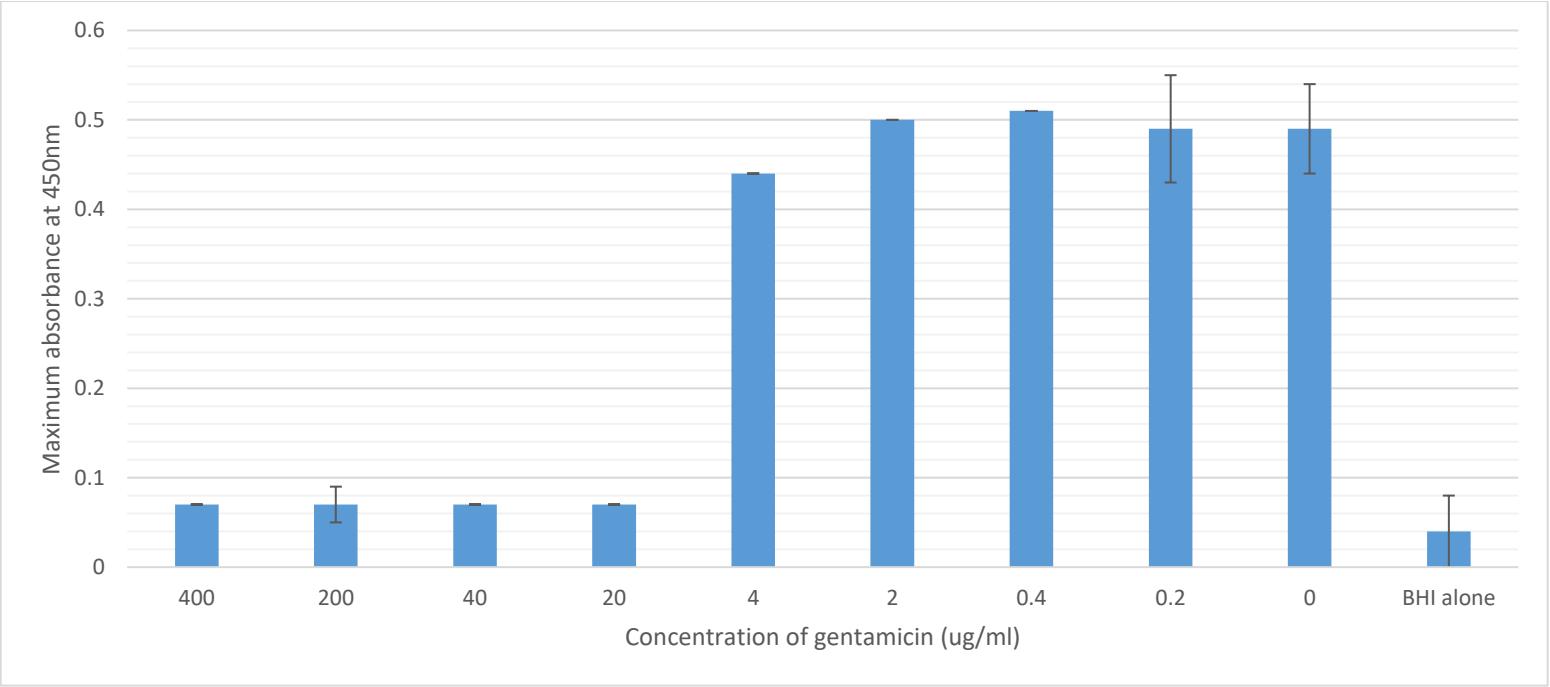



**Figure 2.3 Gentamicin minimum inhibitory concentration determination for *S. uberis*.**

The minimum inhibitory concentration for *S. uberis* 0140J growth in the presence of gentamicin was determined after culturing in concentrations ranging from 0-400µg/ml. Bacterial were cultured for 16 hours in triplicate and growth measured by optical density at $550_{nm}$. No growth was observed at concentrations above 20µg/ml of gentamicin. Growth at 4µg/ml showed a slight decrease in optical density compared with the positive control containing no gentamicin, indicating there was negligible effect on *S. uberis* growth at this concentration.

#### 2.3.2.2 Selection of DNA isolation method

Three different methods were compared for extraction of bacterial DNA from faecal samples (QIAamp DNA stool mini kit, boilate and phenol DNA extraction methods) before and after culture in BHI containing gentamicin. Cattle faeces, which were PCR negative for *sub0888,* were spiked with serial dilutions of an overnight culture of *S. uberis* to determine the limit of detection for and to determine the optimal method for DNA extraction.

DNA was obtained by each method from freshly spiked faecal samples following culture in BHI containing 4µg/ml of gentamicin. In addition, subcultures were made from each sample and DNA extracted from these to account for potential PCR inhibitors that may have been present in the faecal sample. The presence of bacterial DNA was confirmed by amplification of 16S rDNA and the presence of *S. uberis* determined by amplification of *sub0888*.

None of the extraction methods were able to amplify *sub0888* from any samples directly inoculated with *S. uberis* and processed however, the presence of bacterial DNA was confirmed using all methods by amplification of 16S rDNA from freshly spiked faeces and from the faecal samples which were cultured overnight (Figure 2.4).

DNA extracted using the QIAamp DNA stool mini kit did not permit amplification of *sub0888* from any of the faecal cultures, or subcultures.

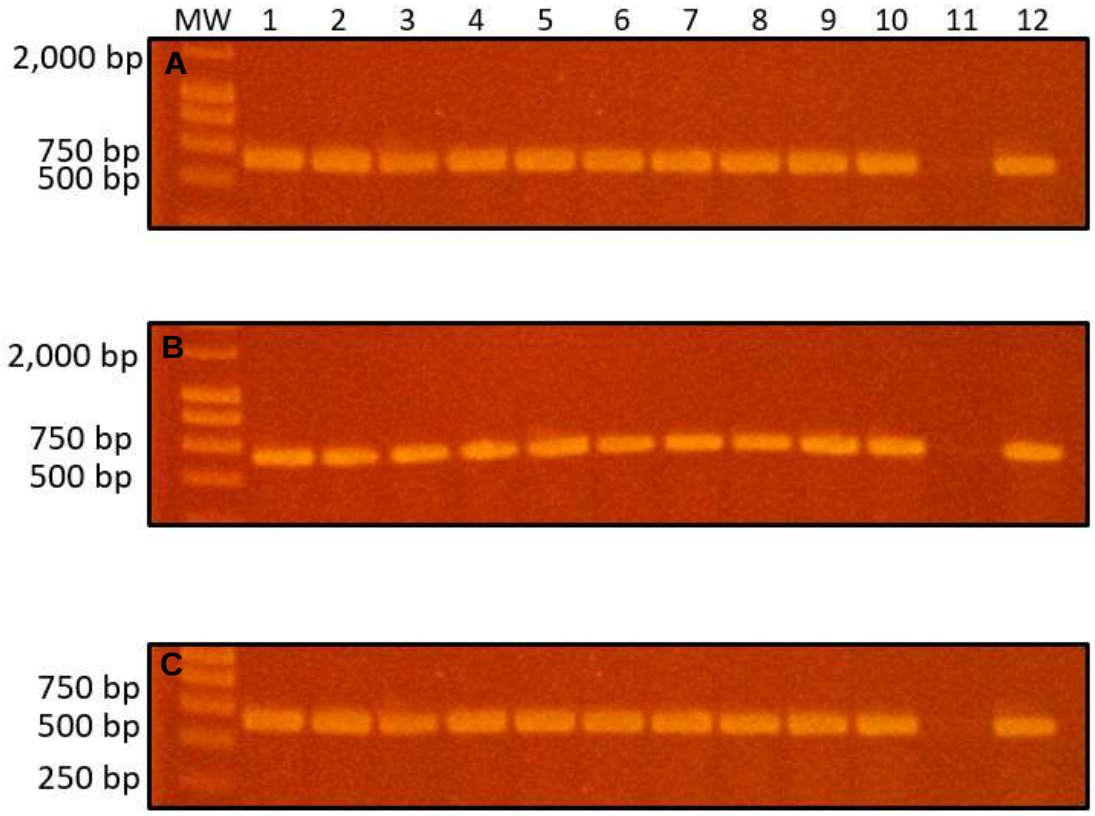



**Figure 2.4 Detection of bacterial DNA from faecal samples using three different extraction methods**

Autoclaved faecal samples inoculated into BHI containing 4µg/ml gentamicin were spiked with a serial dilutions of *S. uberis* 0140J beginning at 1.2 x $10^8$ cfu/sample. The samples were cultured overnight at 37°C. DNA was extracted using either QIAamp DNA stool mini kit, boilate, phenol method (lanes 1 to 10). The presence of bacterial DNA was determined using 16S PCR. Lane 11 contains a no DNA control. Lane 12 contains *S. uberis* 0140J control.

Figure A: DNA extracted using the QIAamp DNA stool mini kit

Figure B: DNA extracted using boilate method

Figure C: DNA extracted using phenol based method

DNA extracted using the boilate method did not permit amplification of *sub0888* from primary cultures (Figure 2.5A and B), but did permit successful amplification in the subcultured samples $10^8$ or $10^6$cfu (Figure 2.5C and D respectively).

DNA extracted from cultured faecal samples using the phenol based extraction successfully yielded DNA from which *sub0888* was amplified. Detection of *sub0888* was possible in samples in which as few as 12 cfu had been added to the original faecal sample. A similar result was apparent in sub-cultured samples (Figure 2.5 B, C and D).

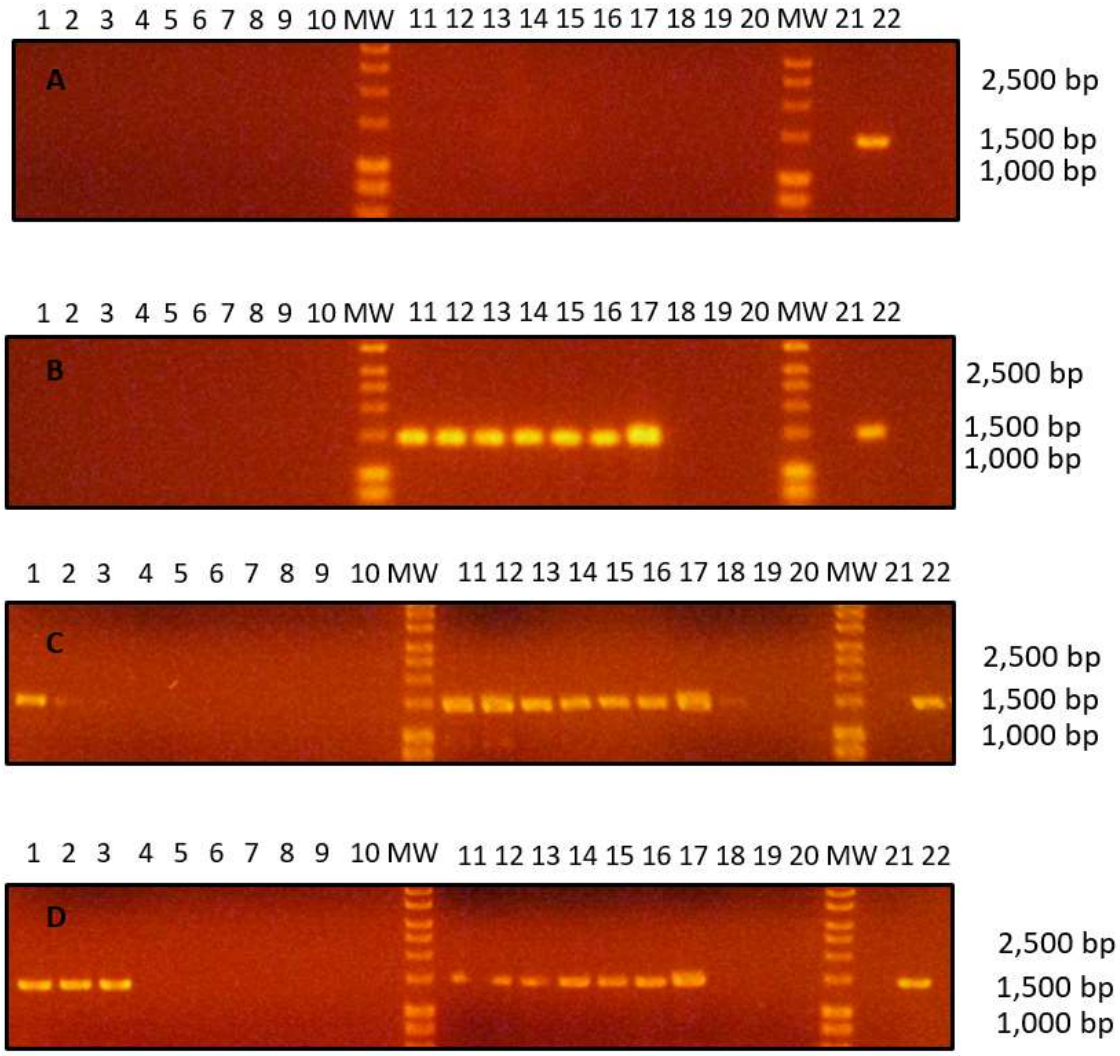



**Figure 2.5 Optimisation of faecal culture DNA extraction for detection of *S. uberis***

Faecal samples, which were PCR negative for *sub0888,* were inoculated into BHI containing 4µg/ml gentamicin were spiked with a serial dilutions of *S. uberis* 0140J beginning at 1.2 x $10^8$ cfu/sample. DNA was extracted using either boilate (lanes 1 to 10) or phenol-extraction methods (lanes 11 to 20). The presence of *S. uberis* DNA was confirmed by *sub0888* PCR Lane 21 contains a no DNA control, Lane 22 contains *S. uberis* 0140J control

Figure A: Faecal samples processed directly after spiking with *S. uberis* with no amplified product observed

Figure B: Faecal samples processed after spiking with *S. uberis* and overnight culture at 37°C, with amplified product at a level of 12 cfu/sample in phenol extracted DNA

Figure C: Bacterial glycerol stocks of directly spiked faecal samples subcultured overnight at 37°C with amplified product detected at 1.2 x $10^8$ cfu in boilate and 12 cfu/sample in phenol extracted DNA respectively

Figure D: Bacterial glycerol stocks of previously cultured, spiked faecal samples subcultured overnight at 37°C with amplified product detected at 1.2 x $10^6$ cfu in boilate and 12 cfu/sample in phenol extracted DNA respectively

#### 2.3.2.3 Direct detection of *S. uberis* from cattle faecal samples

The methodology developed above indicated that an example strain of *S. uberis* could be successfully detected in a spiked faecal sample, however it was important to confirm detection of true faecal *S. uberis*. Two different sampling methods were assessed to compare detection rates; either 1g aliquots or swabs containing between 0.11 to 0.31 g of faeces obtained directly from the same freshly voided faecal deposit (FVF) were cultured, DNA extracted and PCR *sub0888* conducted. The detection rate of *S. uberis* within one FVF was shown to be highly variable between different cows (Table 2.6, Figure 2.6).The results showed that in cow #44, *S. uberis* was not detected in either the 1g samples (n=20) or the faecal swabs (n=20). All other FVF from a further 10 animals tested yielded at least 2 *S. uberis* positive samples (of the 40 tested per animal). Overall detection per sample varied between 0-90% for 1g samples and 0-50% for swab faecal samples. The samples obtained from cows in March, when the animals were housed, had a mean detection rate of 64% for 1g aliquots and 23% for swabs. In contrast, the samples from June, when cows were at pasture, provided a mean detection rate of 24% for 1g aliquots and 8% for swabs.

There was a difference in the detection rate of the *S. uberis* within the faeces between the two different sampling methods, with all animals in which *S. uberis* identified, having a higher detection rate in 1g aliquot samples, compared to swabs samples. Of the ten cows' FVF in which *S. uberis* was detected, the mean prevalence of detection was 42.5%

for 1g aliquots and 14.5% for swabs and therefore there was a higher chance of detecting *S. uberis* from 1g of faeces, compared to swabs (Figure 2.6) which was confirmed using a dependent t-test, ($t=4.97$; $p = 0.000777$)

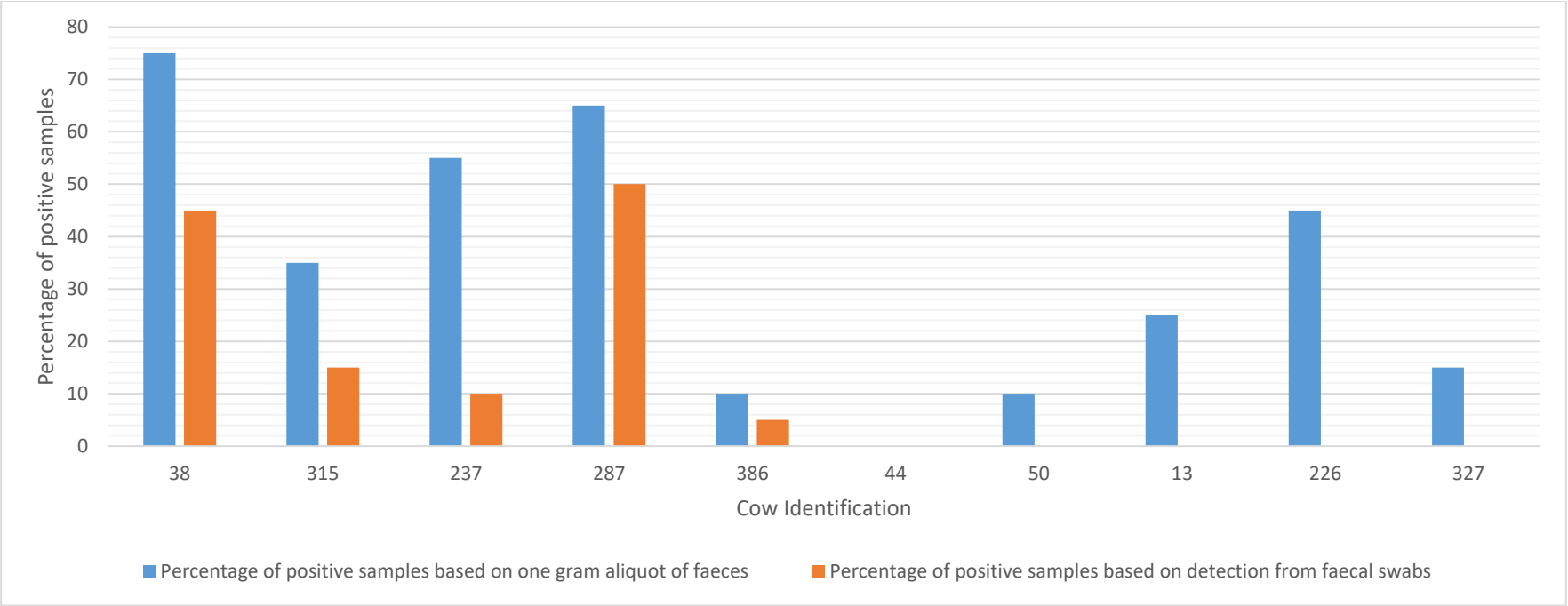



**Figure 2.6 The detection rate of *S. uberis* from one animal when assessing multiple faecal replicates**

Two different sample methods were used for collecting faeces from an individual cow's faecal pat; 20 faecal swabs or 20 one gram aliquots of faeces collected from each fresh faecal pat. DNA extraction was performed using the phenol-based method and PCR was performed using *sub0888* primer. The percentage of positive samples for each detection method are shown by the bars above (blue for one gram aliquot, orange for faecal swabs) for each of the faecal pats analysed, from ten different cows. The results showed that there was a higher detection rate using one gram aliquots compared to faecal swabs.

**Table 2.6: The detection rate of *S. uberis* from replicates analysed from a single faecal sample**

| Date of Samples | Cow ID | 1 gram faeces | | | Swabs | | |
|---|---|---|---|---|---|---|---|
| | | Number tested | Number *S. uberis* positive | *S. uberis* positive (%) | Number tested | Number *S. uberis* positive | *S. uberis* positive (%) |
| March 2016 | 38 | 20 | 18 | 90% | 20 | 9 | 45% |
| | 315 | 20 | 7 | 35% | 20 | 3 | 15% |
| | 237 | 20 | 11 | 55% | 20 | 2 | 10% |
| | 287 | 20 | 13 | 65% | 20 | 10 | 50% |
| June 2016 | 386 | 20 | 2 | 10% | 20 | 1 | 5% |
| | 44 | 20 | 0 | 0% | 20 | 0 | 0% |
| | 50 | 20 | 2 | 10% | 20 | 0 | 0% |
| | 13 | 20 | 5 | 25% | 20 | 0 | 0% |
| | 226 | 20 | 9 | 45% | 20 | 0 | 0% |
| | 372 | 20 | 3 | 15% | 20 | 0 | 0% |

### 2.3.3 The use of chromogenic selective media to isolate single colonies of *S. uberis*

#### 2.3.3.1 Colony morphologies on the different *S. uberis* selective media

The 13 clinical mastitis strains were cultured on five different selective media (esculin blood agar, MRAM, URAM, USM and RSUSM) and the colony morphology characteristics recorded (Table 2.7). Eleven of the strains showed consistent colony morphology on each medium, however two strains, C9359 and C5388, appeared as small pink circular colonies when grow on URAM, compared to the blue circular colonies produced by the other strains, indicating that C9359 and C5388 lacked β-glucuronidase, required for the metabolism of 5-bromo-4chloro-3-inodyl β-D glucuronide.

*S. uberis* colony morphology on MRAM appeared as small blue circular colonies with an entire margin. On URAM most strains showed a similar morphology, with the exception that colonies URAM were darker blue when compared to those on MRAM (Figure 2.7). Colonies of *S. uberis* on USM and RSUSM agar appeared as opaque circular colonies which induced a pH change in the surrounding medium, resulting in change from purple to yellow. On RSUSM, which contained less sodium chloride, colonies were of very similar appearance but larger (~ 1mm) in size. (Table 2.7).

Given the colony morphology of a number of bacterial species had the potential to be similar to *S. uberis*, or were reported to be found in high abundance in cattle faeces, a number of these strains were also cultured on blood esculin, MRAM, URAM, USM and RSUSM agar to allow direct comparison with *S. uberis* colony morphology (Table 2.8). Some bacterial species could be differentiated based on their colony morphology on MRAM, *E. hirae* appeared as circular, convex, 1mm dark pink colonies in comparison to *S. uberis* which appeared as circular, convex, 1mm blue colonies. Whereas, *S. lutentitis, S. equinus* and *E. faecalis* could not be differentiated from *S. uberis* on MRAM, with each producing circular colonies of a similar size, which hydrolysed 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside and therefore appeared blue (Table 2.8).

Comparison of growth on USM agar showed that, colony morphology for *E. coli, E. hirae* and *S. lutetiensis* appeared to be identical to that of *S. uberis,* whilst colony morphology for *S. lutetiensis* and *E. faecalis* appeared significantly larger in size than the punctiform *S. uberis* colonies. The use of RSUSM, the same bacterial agar but with reduced salt concentration resulted in the white colony morphology of *S. equinus* and *E. hirae* instead of translucent for *S. uberis;* with the morphology for *E. faecalis, E. coli* and *S. lutetiensis* very similar, as circular, raised, opaque, smooth and shiny 1mm colonies with an entire margin. It was not possible to differentiate between the *S. uberis* colonies and the colony morphology of *E. faecalis, E. coli* and *S. lutetiensis* using this media.

**Table 2.7: Description of colony morphology of S. uberis strains on selective media**

| Blood Esculin Plates | Modified Rambach Media | URAM* | USM | RSUSM |
|---|---|---|---|---|
| Circular, convex, white colonies, smooth with an entire margin and zone of black haemolysis surrounding the colony. The approximate size is 1mm. | Circular, convex, blue-grey, smooth colonies with an entire margin. The approximate colony size is 1mm. | Circular, convex*, dark blue, smooth colonies with an entire margin. The approximate colony size is 1mm. | Circular, flat, opaque, smooth and shiny colonies with an entire margin. The colonies were punctiform in size and surrounded by yellow discolouration of the media. | Circular, raised, opaque, smooth and shiny colonies with an entire margin. The approximate colony size is 1mm and is surrounded by yellow discolouration of the media |

* Strains *S. uberis* C9359 and C5388 appeared as circular, raised, pink, smooth colonies with an entire margin, approximate colony size of 0.5mm when cultured on this media

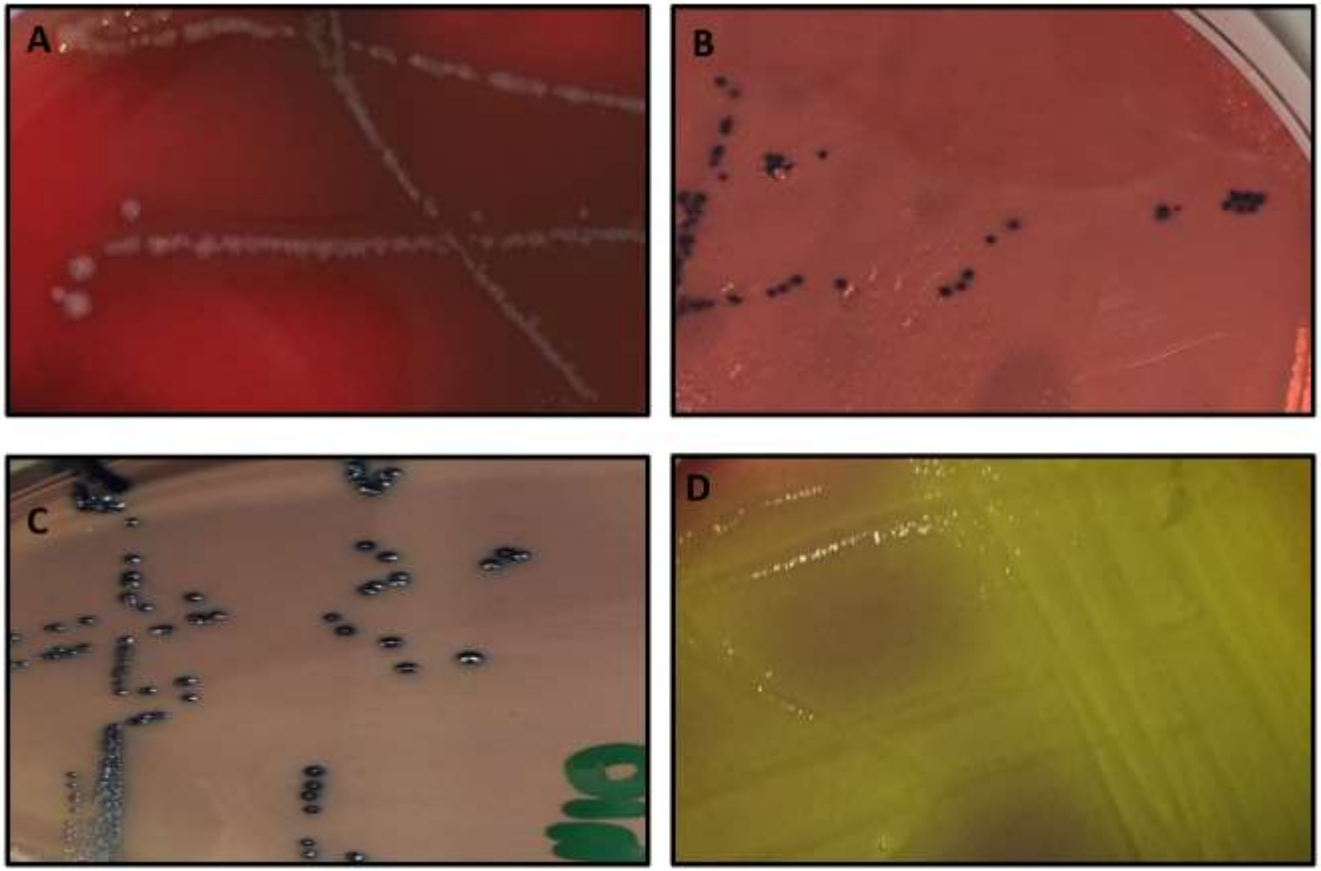



**Figure 2.7 Colony morphology of *Streptococcus uberis* strain 0140J on selective media**

A: *S. uberis* 0140J colonies on blood esculin plates.
B: *S. uberis* 0140J colonies on Modified Rambach media.
C: *S. uberis* 0140J on Uberis Rambach media.
D: *S. uberis* 0140J on reduced salt Uberis specific media.

**Table 2.8: Descriptions of the colony morphology of different faecal bacteria on different streptococcal selective media**

| Media | *S. uberis 0140J* | *S. lutetiensis* | *S. equinus* | *E. faecalis* | *E. hirae* | *E. coli* |
|---|---|---|---|---|---|---|
| **Esculin blood agar** | Circular, convex, white 1mm colonies, smooth with an entire margin and zone of black haemolysis. | Circular, convex, white 1mm colonies, smooth with an entire margin and zone of black haemolysis. | Circular, convex, white 1mm colonies, smooth with an entire margin and zone of black haemolysis. | Circular, convex, white 1mm colonies, smooth with an entire margin and zone of black haemolysis | Circular, convex, white 1mm colonies, smooth with an entire margin and zone of black haemolysis. | Circular, convex, white 2-3mm colonies, smooth with undulated margin and no zone of haemolysis. |
| **Modified Rambach media** | Circular, convex, blue-grey, smooth 1mm colonies with an entire margin. | Circular, convex, blue-grey, smooth 1mm colonies with an entire margin. | Circular, convex, blue-grey, smooth 1mm colonies with an entire margin. | Circular, convex, blue-grey, smooth 1mm colonies with an entire margin. | Circular, convex, dark pink, smooth 1mm colonies with an entire margin. | Circular, convex, blue-green, smooth 2mm colonies with an entire margin surrounded by a clear shiny zone. |
| **Uberis Rambach media** | Circular, convex, dark blue, smooth 1mm colonies with an entire margin. | Circular, convex, dark pink, smooth 2mm colonies with an entire margin. | Circular, convex, dark pink, smooth 1mm colonies with an entire margin. | Circular, convex, dark pink, smooth 2mm colonies with an entire margin. | Circular, convex, pink, smooth 2mm colonies with an entire margin. | Circular, convex, blue-green, smooth 2mm colonies with an entire margin surrounded by a clear shiny zone. |
| **USM** | Circular, flat, opaque, smooth and shiny punctiform colonies with an entire margin. | Circular, raised, white, smooth 1mm colonies with an entire margin. | Circular, flat, opaque, smooth and shiny punctiform colonies with an entire margin | Circular, flat, opaque, smooth and shiny 2mm colonies with an entire margin. | Circular, flat, opaque, smooth and shiny punctiform colonies with an entire margin | Circular, flat, opaque, smooth and shiny punctiform colonies with an entire margin |
| **RSUSM** | Circular, raised, opaque, smooth and shiny 1mm colonies with an entire margin. | Circular, raised, white, smooth 2mm colonies with an entire margin, discolouration of media | Circular, raised, opaque, smooth and shiny 1mm colonies with an entire margin | Circular, raised, opaque, smooth and shiny 1mm colonies with an entire margin | Circular, raised, opaque, smooth and shiny 1mm colonies with an entire margin | Circular, raised, opaque, smooth and shiny 1mm colonies with an entire margin |

#### 2.3.3.2 Detection of *S. uberis* on modified Rambach agar from mixed faecal cultures

A bovine faecal sample which had previously tested positive for the presence of *S. uberis* by *sub0888* PCR was selected for the isolation of *S. uberis* colonies utilising a previously published method which combined faecal culture in BHI media containing Streptococcus selective (SS) supplement and subsequent selection of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside hydrolysing colonies from MRAM agar (Zadoks *et al.,* 2005a)

A total of five FVFs were assessed, with 1g of faecal material cultured in a minimum of one sample from each animal. Faecal cultures were streaked onto multiple MRAM plates. A total 135 single colonies were isolated based on the colony morphology of blue circular colonies (as described in Table 5) from the MRAM plates; representing 12 to 56 isolates per faecal sample. However. none of the cultures from these 135 single colonies tested positive for *sub0888* by PCR and following analysis on Enterococcus selective mitis salivarius agar were determined to be most likely *Enterococcus* species.

### 2.3.4 Detection of *S. uberis* using URAM agar from mixed faecal culture

As none of the colonies isolated using the MRAM media were found to be *S. uberis,* it was decided to alter the chromogenic substrate from 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside to 5-bromo-4chloro-3-

inodyl β-D glucuronide, developing the newly defined URAM media. Unlike *S., uberis,* Enterococci lack the β-glucuronidase required to metabolise this substrate (Vancanneyt *et al.*, 2002). Six faecal samples were cultured overnight and plated onto URAM plates. A total of 180 colonies, (circular, convex, dark blue and smooth 1mm diameter) were isolated from 36 URAM plates. However, none of DNA extracted from subcultures derived from these permitted amplification *sub0888* (data not shown).

#### 2.3.4.1 Enhancement of detection of *S. uberis* on URAM

The impact of the pre-culture growth enhancement step was investigated, using *S. uberis* 0140J and different concentrations of SS in BHI (10%, 50% or 100%), using triplicate samples. There was a significant decrease in the recoverable amount of *S. uberis* when using 100% SS (10mg colistin sulphate, 5mg oxolinic acid), when compared to 50% SS, 10% and 0% SS, as shown in Figure 2.8. Based on this decision, it was decided to use a concentration of 50% SS in BHI for future growth based experiments.

The impact of different enrichment time periods on the recoverable amount of *S. uberis* was investigated, using BHI, BHI containing 4µg/ml gentamicin and BHI containing 50% SS. Triplicate samples of 5ml of each culture media, which had been inoculated with $10^4$ cfu *S. uberis* 0140J, was incubated at 37°C for a period of either 24 hours or 48 hours. After the incubation period, the samples were plated onto BHI for total bacterial counts.

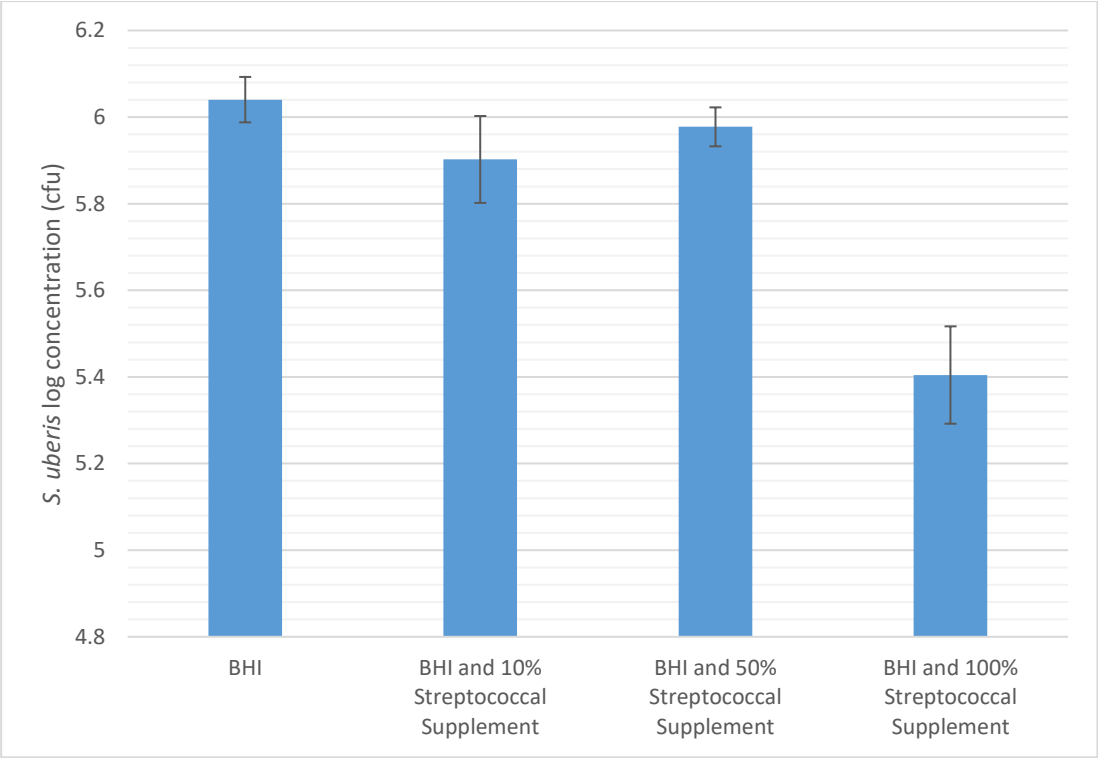



**Figure 2.8 Comparison of *S. uberis* 0140J growth in BHI containing different concentrations of Streptococcal supplement**

Triplicate samples of each culture media were incubated overnight at 37°C containing $10^4$ *S. uberis* 0140J. Each culture media contained brains heart infusion media and a different percentage of Streptococcal supplement (10mg colistin sulphate and 2.5mg oxolinic acid per litre for 100% concentration). The bars represent the mean concentration of *S. uberis* recovered from each media. The error bars represent the 95% confidence interval of the mean

There was a significant decrease in the recoverable amount of *S. uberis* between 24 hours and 48 hours incubation for the enrichment for all culture media, as shown in Figure 2.9. Based on these results, it was decided to use an enrichment time period of 24 hours for all subsequent experiments.

The impact of incubation time period on the URAM plates was investigated by incubating an overnight culture of *S. uberis* 0140J and overnight cultures of three faecal samples. 10µl of each sample was plated onto URAM and incubated at 37°C for a period of 24 hours and 48 hours. There were no significant differences in terms of the number of potential *S. uberis* blue colonies after 48 hours growth when compared to 24 hours, however the colonies did increase in both size and the intensity of blue pigmentation after 48 hours growth. A further 42 of these colonies were selected and tested for the presence of *sub0888* by PCR, however none were positive.

The impact of using different liquid culture media when using bovine faeces was investigated, using a faecal sample which was previously positive for the presence of *S. uberis* using *sub0888* PCR. The sample was cultured overnight in both 50% SS-BHI and BHI containing 4µg/ml gentamicin. The samples which had been cultured in the BHI containing 4µg/ml gentamicin had a large number of a lighter blue circular colonies, surrounded by a transparent mucoid substance, and similar to the *E. coli* P4 morphology. These differed from the circular, convex,

dark blue, smooth 1mm colonies of *S. uberis* strain 0140J, however there were two types of darker blue colonies; smaller discrete blue colonies and slightly larger colonies of the same colour blue which had blue colour dispersed into the agar. Single colony picks were performed on all types of blue colonies and tested for the presence of sub0888 (n=128 colonies). None of the colonies were positive on PCR for *sub 0888*; however 13 of the colonies produced a single amplified product which was approximately 200bp smaller than that obtained from the positive control strain 0140J. Amplification and sequencing of the *16s rDNA* product obtained from these samples indicated a variety of enterococci and streptococci, but not *S. uberis* (Table 2.9).

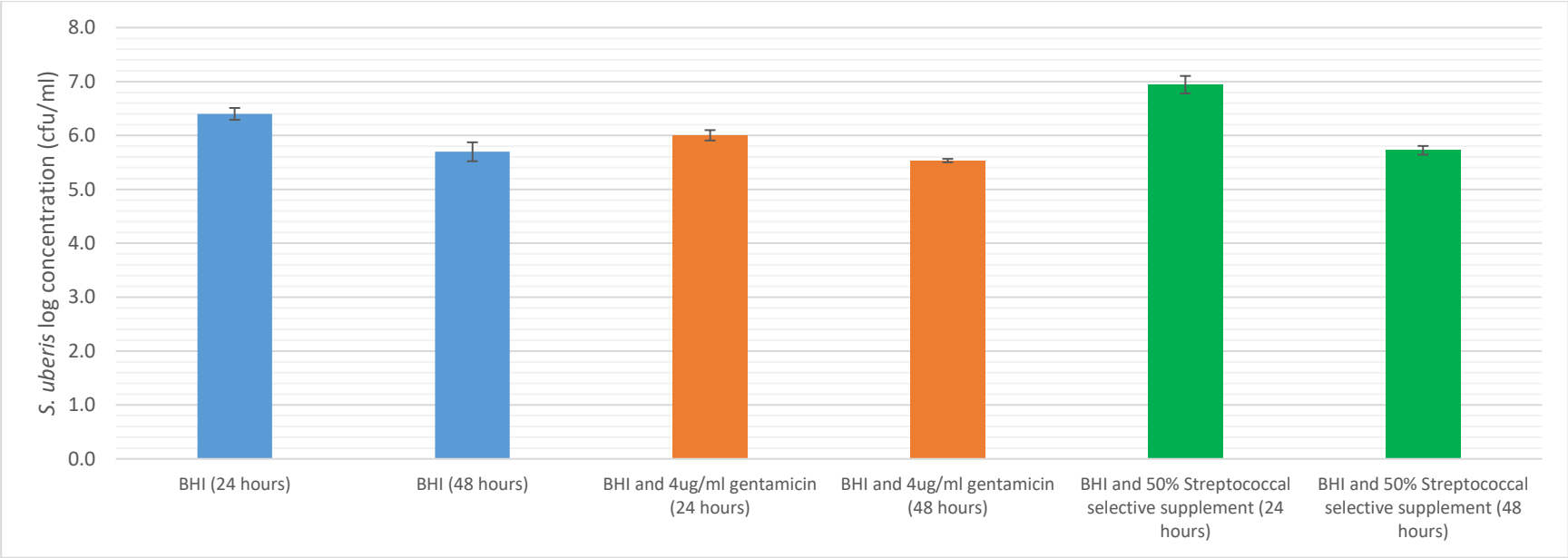



**Figure 2.9 Comparison of *S. uberis* 0140J growth in different liquid media at 24 and 48 hours.**

The green bars are *S. uberis* grown in brain-heart infusion media (BHI) containing 50% streptococcal selective supplement (5mg of colistin sulphate and 2.5mg oxolinic acid) with an incubation time of 24 hours and 48 hours. The blue bars are *S. uberis* grown in brain-heart infusion media (BHI) containing 4ug/ml gentamicin, with an incubation time of 24 hours or 48 hours The blue bars are *S. uberis* grown in brain-heart infusion media (BHI) with an incubation time of 24 hours or 48 hours. The error bars represent the 95% confidence interval of the mean.

**Table 2.9: The BLAST results for the sequence data from the 16S sequencing performed on 13 blue colonies picked from URAM plates**

| Sample Identity | Query length | Bacterial Identification* | Accession number | Identity (%) |
|---|---|---|---|---|
| 1 | 542 | *Streptococcus lutetiensis* PON175 | KF147890.1 | 95 |
| 2 | 536 | *Aneurinibacillus aneurinilyticus* WBC1 | KX954217.1 | 99 |
| 3 | 515 | *Streptococcus lutetiensis* PON175 | KF147890.1 | 94 |
| 4 | 518 | *Streptococcus* spp strain FMD1 | MK652875.1 | 95 |
| 5 | 501 | *Streptococcus lutetiensis* C22 | LC107931.1 | 92 |
| 6 | 551 | *Enterococcus hirae* BFT546 | MN420857.1 | 99 |
| 7 | 646 | *Enterococcus durans* XT1-1 | MH891655.1 | 99 |
| 8 | 437 | *Streptococcus equinus* EQ15 | KX073769.1 | 95 |
| 9 | 508 | *Streptococcus lutetiensis* L15 | LC107928.1 | 94 |
| 10 | 564 | *Enterococcus faecium* I19 | MN564965.1 | 100 |
| 11 | 516 | *Enterococcus hirae* ML06 | LC274614.1 | 100 |
| 12 | 524 | *Streptococcus equinus* EQ15 | KX073769.1 | 97 |
| 13 | 399 | *Streptococcus equinus* DMF7 | MF38037.1 | 96 |

*Bacterial identification was based on the best match on BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and may represent unknown species of bacteria

To maximise the chance of isolating *S. uberis* from cattle faecal samples, a number of different amounts of faecal material were cultured overnight in 50% SS-BHI at 37°C, either 5g, 1g or 0.2g in triplicate and were subsequently cultured on URAM agar. Overall the number of colonies which has a similar morphology to *S. uberis* 0140J appeared to increase if larger amounts of faeces were cultured; however this was not always the case, as some of the 0.2g cultures had a higher total bacterial count of *S. uberis* 0140J-like colonies (Table 2.7). A total of 70 single colonies were selected and tested for the presence of the *sub0888* gene, however each colony was negative by PCR and none could be confirmed as *S. uberis.*

#### 2.3.4.2 The use of different selective media to detect *S. uberis* from faecal samples

The growth conditions for the isolation of *S. uberis* from bovine faeces used a combination of overnight culture at 37°C in liquid BHI containing 4µg/ml gentamicin and incubation of streaking 10µl of the culture streaked on USM and RSUSM media for 48 hours at 37°C. A total 96 single colonies were isolated from the USM plates, based on the colony morphology of punctiform circular colonies. It was exceptionally difficult to isolate a single colony which was surrounded by a zone of yellow, or which fluoresced under ultraviolet light. Of these 96 single colonies isolated, none of the samples tested were identified as *sub0888* positive by PCR.

#### 2.3.4.3 The impact of *Enterococcus faecium* on the growth of *S. uberis*

Three URAM plates were seeded with 50µl of overnight culture of *S. uberis* 0140J with 10µl of overnight culture *Enterococcus faecium* inoculated as a single line halfway across the plate and incubated for 48 hours at 37°C. There was no growth observed of *S. uberis* along the *E. faecium* inoculum (Figure 2.10). Separate overnight cultures of *S. uberis* strain 0140J and *E. faecium* were grown in BHI media and then mixed to create the following ratios of *S. uberis*:*E. faecium*; 1:1, 1:10, 1:100 and 1:1000. A total of 40µl of each mixed culture was then inoculated onto URAM agar and incubated for 48 hours. Analysis of the growth of the mixed cultures found no presence of *S. uberis* in any of the ratios tested and was suggestive that *E. faecium* inhibited *S. uberis* growth.

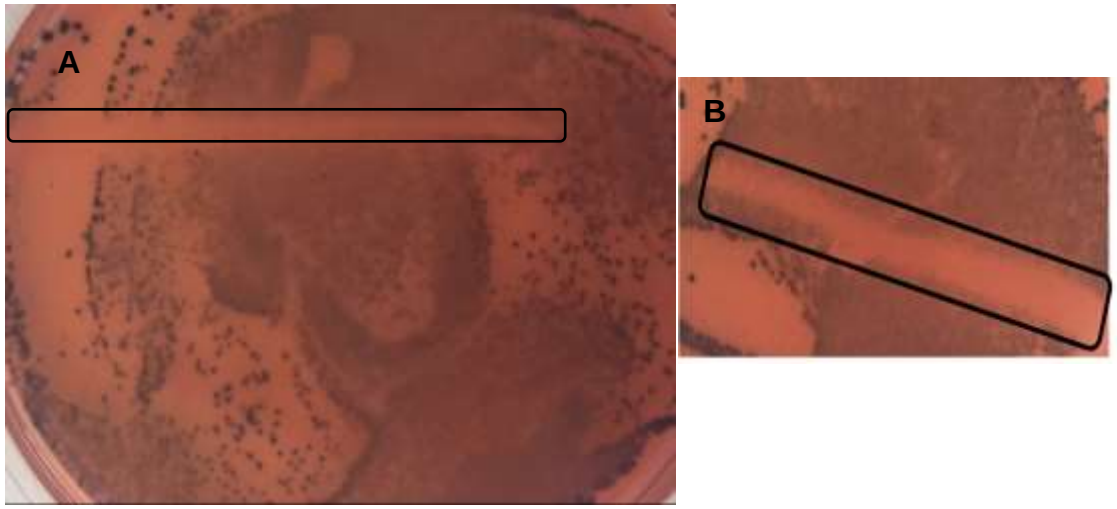



**Figure 2.10 Growth inhibition of *S. uberis* by *Enterococcus faecium* on URAM agar**

Selective URAM agar was inoculated with *S. uberis* 0140J, with *Enterococcus faecium* inoculated as indicated by the highlighted black box (A). No growth of *S. uberis* was observed in the area of *E. faecium* inoculum, with magnified image highlighted (B).

## 2.4 Discussion

Characterisation of *S. uberis* isolates has yet to identify specific strains to provide evidence for *S. uberis* faecal-oral transmission and a link between mastitis. This is despite a number of studies identifying similar bacterial strains by ribotyping, MLST, RFLP or REP-PCR based analysis and comparison of environmental or faecal strains with mastitis isolates (Zadoks *et al.,* 2005; Pullinger *et al.*, 2006; Pryor, 2008; Wente *et al.*, 2019). The development of a method which could identify the presence of *S. uberis* within faecal samples and then isolate specific strains for genomic based comparison would provide a potential to better understand the “life cycle” of *S. uberis* survival and persistence in the cattle environment.

### 2.4.1 Development of a molecular screening test for the presence of *S. uberis* in bovine faeces

Given cattle faeces contain a high concentration of a complex mixture of different bacterial species, of which *S. uberis* is considered to be a only a small proportion, the isolation of individual *S. uberis* from faeces is likely to be challenging. In contrast, the isolation of *S. uberis* directly from mastitic milk samples, where the main causative agent is often predominant within the sample, has made characterisation of *S. uberis* using selective media more likely to be successful. The adaption of differential media for isolation of *S. uberis* from faecal samples has been investigated in few studies, none of which have performed comprehensive genomic analysis of

acquired strains ( Zadoks *et al.,* 2005a; Pryor, 2008; Wente *et al.*, 2019). The methodology described in these studies was used as a basis for isolation of *S. uberis* isolates from UK cattle faecal samples, in which *S. uberis* was deemed to be present using a PCR based detection method.

#### 2.4.1.1 Design of *S. uberis* specific primers

A PCR assay was developed using primers based on sequences unique to *S. uberis* (*sub0888* and *sub1730*), or the very highly conserved sequence (*sub1154*) (Ward *et al.*, 2009). Each of these genes encoded for sortase anchored proteins, present on the cell surface of *S. uberis*, with *sub1154* identified as essential for colonisation and infection of the mammary gland in previous studies (Leigh *et al.* 2010; Egan *et al.*, 2010). The use of *sub0888* was found to be the most reliable for detecting *S. uberis* and was shown to be present in all *S. uberis* by *in silico* by analysis of known sequences on both the NCBI and *S. uberis* BIGSdb database (Jolley and Maiden, 2010) and by PCR amplification from 12 *S. uberis* isolates from cases of subclinical and clinical disease (Figure 2.1, Table 2.3). In addition, a number of mismatches within the primer sequences could be tolerated using this primer pair, increasing the chance of amplification of sequences that did not directly align perfectly within other *S. uberis* strains. In selecting *sub0888* as the target gene for PCR development, there was potential for bias, as sequence comparison could only be performed with *S. uberis* isolated from cases of mastitis as no sequences from faecal isolates of *S. uberis* have been reported. It is possible that the faecal

strains may have different genomic profiles and that the *sub0888* gene is not present in all *S. uberis* isolates. However the *sub0888* gene was present in all sequenced *S. uberis* and is, therefore, likely to be conserved between all strains.

One method of increasing the sensitivity of the PCR could be to perform parallel testing, using multiple targets, to account for discrepancies in a single gene. A multiplex PCR using both *sub0888* and *sub1154* targets has been shown to be effective at identifying *S. uberis* from milk samples taken from clinical mastitis cases (Sherwin *et al.,* in preparation), however this publication utilised alternative primer sequences than those used in this study, as it post-dated the sample collection and analysis performed in this study. The advantage of a singleplex PCR is that there is no primer-primer competition and therefore the relative abundance of target DNA is not an issue. The use of a multiplex PCR for the same bacterial species can act as an internal reference for each sample and could increase the sensitivity of detection of the PCR for detecting *S. uberis* and decrease the probability of false negatives.

Another factor which impacted on the sensitivity of the PCR was the sampling strategy used, with an increased probability of *S. uberis* detection within FVF if a larger quantity of faeces was sampled, or if multiple samples were tested (Figure 2.6). Quantities of faeces larger than 1g resulted in an increased risk of contamination of the DNA with faecal

organic matter (data not shown), which decreased the quality of DNA extracted. The impact of cow sampling strategy on the sensitivity of the PCR test would need to be considered when designing an epidemiology study, as processing multiple samples per cow may not be feasible for larger epidemiological studies, however this would reduce the sensitivity of the screening PCR test and potential result in false negative results.

#### 2.4.1.2 The impact of DNA polymerase on the reliability of the PCR

**2.4.1.3 One of the key components of the PCR assay is the DNA polymerase, which will amplify the DNA of the targeted gene. For the 16S PCR test, the Taq polymerase outperformed the Phusion polymerase in this study, with the Phusion polymerase producing a16S rDNA product, even in samples where DNA had been omitted (Figure 2.2). Phusion is manufactured as a recombinant protein in *E. coli* and BLAST analysis using the 16S rRNA primer sequence against an *E. coli* subset reported a 100% identity match with various known *E. coli* strains (data not shown), indicating that a small concentration of residual *E. coli* DNA could be amplified using this high fidelity enzyme. Given the negative control is important to validate the assay results, to indicate potential contamination or incorrect primer binding, the reproducibility provided by Taq polymerase was preferential in this case. Enrichment of the faecal sample**

One of the largest challenges for *S. uberis* detection from cattle faeces is the potentially small number of *S. uberis* present within the sample. Research has reported that of the number of bacteria present within the faeces of cattle is up to $10^{11}$ cfu/g of faeces (Hungate, 1966; Yokoyama *et al.,* 1988 ) and of the bacterial DNA present, approximately 0.55% belong to the *Streptococcus* genus, however this ranged between 0.17-1.16%

across 15 cattle with a further 5 animals negative for the presence of Streptococcus DNA (Figure 2.11) (Dowd *et al*., 2008b;).

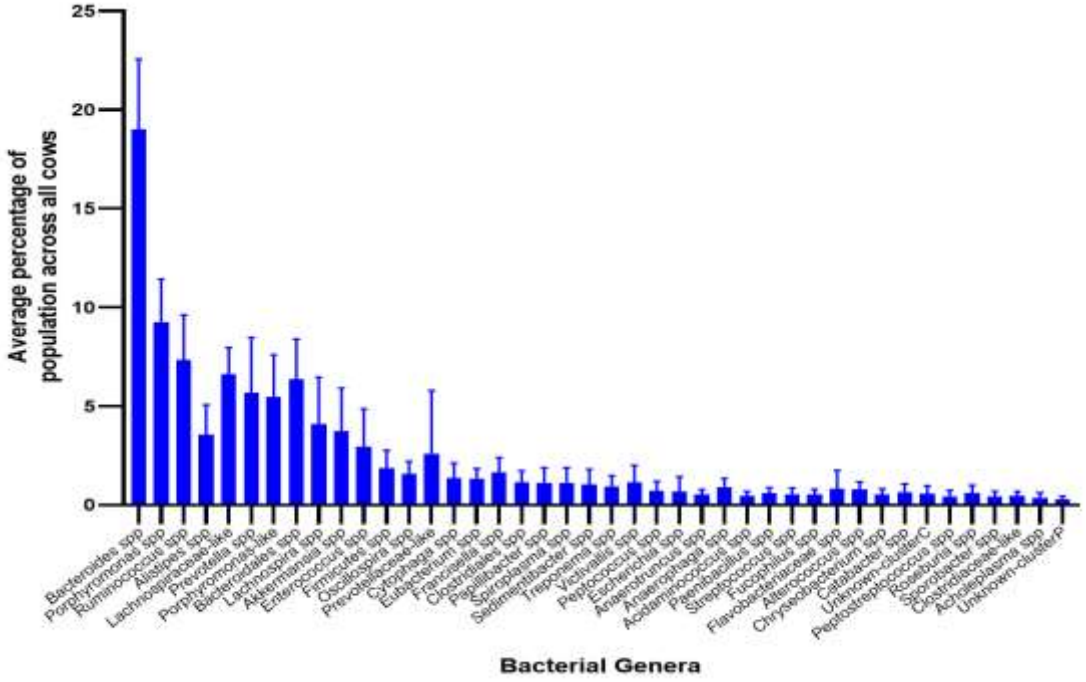



**Figure 2.11 Faecal bacterial population of dairy cows determined by genome sequencing**

The bacterial population of the faeces of 20 cows was analysed using bTEFAP PCR sequencing, based on the 16S gene. The results identified 274 bacterial species from 142 genera. There were 193 sequences of *Streptococcus* species, with only 15 out of the 20 cows testing positive for any *Streptococci,* with an average of 0.55% across the population of 20 cows (range 0.17-1.16%). (Dowd *et al.*, 2008a)

There are many different species of *Streptococcus* that have been detected within cattle faeces, including *Streptococcus bovis*, *Streptococcus suis* and *Streptococcus acidominimus* (Devriese *et al.*, 1992; Laukova, 1994; Golder *et al.*, 2014) alongside *S. uberis* and with the exact percentage that can be attributed is currently unknown. One method of increasing the detection rate for *S. uberis* is to culture faecal samples in conditions in which its proportion is enriched compared to other bacterial species.

In one sequence based study of bacterial diversity within cattle faeces (Dowd et al., 2008), over 57% of the bacteria were identified as Gram negative species and 40% identified as strict anaerobes (Figure 2.11). Given *S. uberis* is classified as facultatively anaerobic, the use of a non-anaerobic bacterial culture step could inhibit the growth of strictly anaerobic bacteria. In addition, the use of gentamicin, a bactericidal aminoglycoside antibiotic at a concentration of 4µg/ml, was also included to enrich for *S. uberis* growth (Figure 2.3). Gentamicin is a bactericidal aminoglycoside antibiotic, which passively diffuses through the outer membrane of Gram negative bacteria and binds to bacterial ribosomal 30S subunits, resulting in bacterial translational errors and cell death (Santucci and Krieger, 2000, Rasmussen *et al.*, 1993). As there are high proportion of Gram negative species identified within a number of cattle faecal sequencing studies including *Tenericutes*, *Bacteriodes* and *Proteobacteria* phyla (Dowd *et al.*, 2008a; Shanks *et al.*, 2011), gentamicin was selected

to reduce the potential number of Gram negative bacteria within the sample. The resistance of *Streptococcus* species to gentamicin has previously been utilised for the isolation of faecal streptococci in other selective media based studies (Thian and Hartman, 1981), further supporting its use within the culture enrichment step. Widespread resistance of *S. uberis* to gentamicin has been reported and has been attributed to the presence of *ant*, found in almost all sequenced isolates in some studies (Vélez *et al.*, 2017). This gene encodes for an aminoglycoside 6-adenylyltransferase, which has also been reported in other Gram positive bacteria, including Staphylococcus and Enterococcus species, and can contribute to resistance to streptomycin (Shaw *et al.*, 1993). Use of gentamicin for enrichment is also supported by a study where 138 *S. uberis* isolates were found to be resistant by gentamicin antibiotic disc diffusion based assays at a concentration of 10µg (Phuekt*es et al*., 2001). In this study, the growth of *S. uberis* decreased from the use of 2µg/ml to 4µg/ml concentration indicating that the concentration used was probably maximal. One pitfall of the current study is the fact that it was performed in bacterial media, without any faecal material present. The organic matter in the faecal sample could potentially interfere with the efficacy of the antibiotics. Gentamicin has been shown to have an enhanced effect in alkaline pH conditions (Xu *et al.*, 2017). The faecal culture conditions are predicted to be slightly alkaline, given the pH of THB is 7.8+/- 0.3 and bovine faeces has also been reported to be slightly alkaline in nature (Irshad *et al.*, 2013; Huang *et al.*, 2017), potentially

enhancing the effects of gentamicin may be enhanced in this environment. The use of a primary enrichment step also increased the sensitivity of the PCR assay, due to the increased amount of *S. uberis* present, resulting in a lower detection threshold for the initial sample, when compared to samples which were not enriched.

#### 2.4.1.4 Impact of DNA extraction on the sensitivity of the PCR

The PCR assay was able to detect *S. uberis* in samples taken directly from cattle faeces, however it was practically difficult to isolate high quality DNA samples from these samples due to the presence of contaminants, such as straw and other organic matter. The presence of contaminants has the potential to impact on the analytical sensitivity of the PCR, due to inhibition of the PCR (Wilde *et al.,* 1990; Jiang *et al.*, 1992; Clarridge *et al.*, 1993). The method of DNA extraction was shown to have a large impact on the sensitivity of the PCR, with the phenol-based method providing the best results and with a detection limit of 12cfu/g in enriched faecal samples. This may be related to the phenol-chloroform step increasing the removal of protein contaminants and polymerase inhibitors. The use of aqueous/non-aqueous separation systems has previously been shown to increase the sensitivity of PCR of faecal samples (Lantz *et al.*, 1997).

There are three basic steps required for DNA extraction; bacterial lysis, protein precipitation and nucleic acid purification. Given that *Streptococcus uberis* is a Gram positive bacteria, the lysis step of DNA extraction is critical to ensure DNA can be released from the cell and the efficacy of this

process will differ widely between bacterial species due to differences in cell wall structure and integrity. The three DNA extraction methods, boilate, phenol or commercial kit based extraction, showed that variable levels of *S. uberis* DNA could be detected by *sub0888* PCR, but were also tested alongside the ability to detect *16S rDNA*, to determine extraction of bacterial DNA had been successful.

The commercial QIAamp kit successfully isolated bacterial DNA as confirmed by the *16S rDNA* PCR, however failed to isolate *S. uberis* DNA, even when utilising the manufacturers' enhanced protocol recommended for the lysis of Gram positive bacteria. It is more likely that the bacterial DNA isolated using this method was from Gram negative bacteria, which are easier to lyse due to the differences in the cell wall structure (Wesolowska-Andersen *et al.*, 2014). It has been shown that DNA is more efficiently extracted from Gram positive bacteria present in faeces if a sample has been frozen and thawed, compared to fresh samples (Bahl, Bergström and Licht, 2012), which was also performed in this study; however this did not improve *S. uberis* DNA yields for the boilate or commercial QIAamp kit based extraction methods. Another study that compared different methods of DNA extraction for rumen fluid (Henderson *et al.*, 2013) indicated that none of the 15 extraction methods tested resulted in 100% comparable microbial community compositions, indicating the importance of consideration of the extraction procedure in data analysis. However, in that study, the most reliable methodology was that

incorporating a phenol-chloroform purification step. This yielded the highest concentration of total DNA, with other the kit based methodologies showing disproportionately selective DNA extraction from Gram negative genera. In addition the boilate DNA extraction method investigated in the present study was adapted from a method previously used for the extraction of Group B Streptococcal DNA (Mawn *et al.,* 1993),however, it was also poor at consistently releasing *S. uberis* DNA from the faecal samples.

#### 2.4.1.5 The analytical sensitivity of the PCR

The analytical sensitivity of the detection limit of the PCR test was important for determining the threshold level of detection for *S. uberis* within cultured faecal samples (Saah and Hoover, 1997). Previous research have reported analytical sensitivities for the detection of *S. uberis* using similar methodology, including a teat swab, culture and REP-PCR approach which could identify an analytical sensitivity of 10 cfu/ml (Pryor, 2008). This is the most similar diagnostic test, with a mixed sample reported within the literature for *S. uberis* detection. A high analytical sensitivity was thought to be required for the diagnostic screening test for *S. uberis* in cattle faeces due to potential low numbers of *S. uberis* present within samples. The reported levels of *S. uberis* detected within cattle faeces has been reported as low as 150 to 25,00 cfu/g in dairy effluent and 3.0 to 12,500 cfu/g within the dairy cattle environment (Lopez-Benavi*des et al*, 2007b). This indicates that *S. uberis* could be present at concentrations

less than 100 cfu/g within faecal samples; however the detection limit of 12 cfu/g of cultured faeces for the PCR assay should allow for the detection of the presence of *S. uberis* in the majority of cases, although separating and purifying this low number from the multiple other species present would likely be difficult at these low levels. One potential limitation of the study design was the use of only one *S. uberis* strain (0140J), throughout the study. This strain is the reference strain for *S. uberis*, isolated from a case of clinical mastitis and has been widely used in a number of mastitis studies as a typical clinical strain (Leigh and Lincoln, 1997; W*ard et al*., 2009; Günther *et al*., 2016).

### 2.4.2 Impact of sampling methodology on the detection rate of *S. uberis* in bovine faeces

One of the biggest challenges of detecting *S. uberis* within the faeces of cows is the volume of faecal matter an adult dairy cattle can produce, which is estimated to be around 40kg per day (Aland *et al.*, 2002; Whistance *et al.*, 2007). This would be the equivalent to 200,000 swabs based on average of 0.2g of faeces collected per swab and given the logistics for future longitudinal herd based studies, the processing of larger volumes of faeces for culture based enrichment would likely be unmanageable. Similar studies investigating *E. coli* and *S. uberis* have utilised faecal swabs (Chapman *et al.*, 1993, Hancock *et al*., 1997; Pryor, 2008) as an easy method for faecal sample collection from multiple

animals, which does limit the testing to a very small amount of faecal material and could reduce the likelihood of detecting *S. uberis*.

In studies investigating the presence of different bacteria within the faeces of an animal, it is common for only a single sample to be analysed (Chapman *et al*., 1993; Hancock *et al*., 1997; Zadoks *et al.*, 2005b; Pryor, 2008) and assumes a homogenous spread of the organism within the sample. The samples using swabs in this study were taken by randomly placing the swabs at multiple sites in freshly voided faeces and showed with a detection rate of between10% to 90% of cultured one gram aliquots collected. The variation in the detection rate of *S. uberis* within the samples taken from one FVF sample could reflect a heterogeneous distribution of *S. uberis* within the FVF. Heterogeneous distribution of *E. coli* in bovine faeces has been previously reported, with a difference of 76,800 cfu/g occurring between different areas of voided faeces (Pearce *et al.*, 2004), which was attributed to *E. coli* being located mainly on the GI mucosal surface, resulting in minimal opportunities for the bacteria to mix homogenously within the faeces. The location of *S. uberis* within the gastrointestinal tract is currently unknown and therefore the impact of differing sampling approaches, as well as the impact of using rectal swabs for the detection rate of *S. uberis* is still unknown.

### 2.4.3 The ability to use culture-based methodology for the isolation of *S. uberis* from bovine faeces

The second aim of this study was to develop a robust method for isolation of single colonies of *S. uberis* from a mixed faecal culture. Bovine faeces differs from mastitis samples, where selective media has been traditionally been used for *S. uberis* identification (Hogan *et al.*, 1999), as there tends to be only one bacterial species present in the majority of mastitis cases (Bradley *et al.*, 2007). It has also been shown that with mastitis caused by *S. uberis,* there tends to be only one strain of *S. uberis* dominating the intramammary infection, even if multiple strains are infused at the start of a challenge study (Pryor *et al.*, 2009). Numerous streptococcal and enterococcal species have been detected within bovine faeces using molecular based methods (Thian and Hartman, 1981; Herrera *et al.*, 2009; Golder *et al.*, 2014; Dowd *et al.*, 2008a; Jans *et al.*, 2015; Mao *et al.*, 2015), however sequence based studies do not necessarily confirm the presence of any live bacteria and therefore may misrepresent the viable bacterial numbers.

In the search for *S. uberis*, many studies have used enterococcal selective media, as there is currently no commercial bacterial media available to be able to accurately differentiate between streptococcal and enterococcal species. The difficulties differentiating between different bacterial species alongside the potential low numbers of *S. uberis* present within faecal samples decreased the chances of developing a robust method of isolation

of *S. uberis* from bovine faeces. Numerous different species with similarities to *S. uberis* have been reported in the faeces of cows including *E. faecium, E. faecalis, E. hirae, Streptococcus bovis/Streptococcus equinus* complex (SBSEC) and *Aerococcus viridians* (Devriese *et al.*, 1992). SBSEC is a group of non-β- haemolytic streptococci (Facklam, 2002), which have been categorised into biotypes depending upon their ability to produce acid from mannitol (biotype I) or not (biotype II) (Parker and Ball, 1976).

#### 2.4.3.1 The use of Uberis selective media

The identification of *S. uberis* with MRAM is based upon the cleavage of a chromogenic substrate galactopyranoside by the enzyme β-galactosidase, which results in the generation of the blue pigmentation of the bacterial colony. It has been reported that 95% of *S. uberis* isolates are positive for β-galactosidase production (Watts *et al*., 1993), however other bacteria found in bovine faeces which are able to produce β-galactosidase include *E. faecium, Lactococcus lactis, E. coli* and different biotypes of the *Streptococcus bovis/Streptococcus equinus* complex (SBSEC) (Citti *et al.*, 1965; Badarinath and Halami, 2011; Jans *et al.*, 2015). This was highlighted in the present investigation by the identical colony morphology for the species of enterococci, streptococci and *E. coli* strains when cultured using this media. To differentiate *S. uberis* from the large numbers of *Enterococcus* species and *E. coli* present in the faecal samples, an alternative selective media was developed (URAM) which substituted 5-

bromo-4-chloro-3-indoyl-β-D-galactopyranoside for a different chromogenic substrate, 5-bromo-4-chloro-3-inodyl β-D glucuronide, which requires the enzyme β-glucoronidase for cleavage and the generation of the blue pigment. β-glucoronidase has been reported to be produced by *S. uberis* but not *E. faecium* or *faecalis* (Devriese *et al.*, 1987, 1999). The use of β-glucoronide in URAM allowed differentiation between *Enterococci* and some strains of *S. uberis* however a number of *S. uberis* strains tested lacked the β-glucoronidase enzyme and appeared as pink colonies when grown on URAM media (Table 2.7). A comparison the β-glucuronidase sequence (identified as *sub1206* from strain 0140J) and 131 other *S. uberis* sequences (from BIGSDdb) (Jolley and Maiden, 2010) showed 75% of the isolates had a sequence with identity of greater than 98.0% over the entire1791bp. The remaining 33 isolates possessed a truncated (potentially inactive) version of the gene, with low homology. This indicates that URAM would not be able to detect all isolates *S. uberis* based purely on colony morphology and enzyme activity. Additionally, some of the bacterial species included in the SBSEC biotype II also exhibit β-glucuronidase and β-galactosidase activity, resulting in identical colony morphology to *S. uberis*. It was therefore unlikely to be a good choice as a *S. uberis* selective media.

The only major difference between the growth of SBSEC and *S. uberis* on the URAM media was the alteration of the media colour from red to yellow with SBSEC. SBSEC has been reported to produce lactate when proliferating at high growth rates (Jans *et al.*, 2015) which resulted in a

change of pH in the surrounding URAM. However, this colour change was only noticeable if numerous colonies were present and was not distinguishable at an individual colony level. Also given SBSEC have been reported at high numbers within the gastrointestinal tract, it is maybe not surprising that differentiation of *S. uberis* was problematic using both USM and RSUSM.

#### 2.4.3.2 The inhibitory interaction of *S. uberis* and *E. faecium*

Another problem encountered in attempting to isolate single colonies of *S. uberis* from faecal samples, was the possible interactions of other bacteria. Whilst it was not the purpose of this study to investigate the potential relationships between *S. uberis* and other faecal bacteria, it was highlighted that *E. faecium* had an inhibitory impact on the growth of *S. uberis* strain 0140J on URAM agar. This could have resulted in lower levels of *S. uberis* during the culture enrichment step which also appeared to enrich Enterococci species.

Analysis of bacterial DNA from bovine faeces indicated Enterococcus 2.95% of the bacterial population; ranging from 0.73 to 7.89% across 20 faecal samples (Figure 2.11) (Dow*d et al*., 2008a). This represents a 5:1 ratio of *Enterococcus* to *Streptococcus* species and as both have very similar growth requirements both are likely be enriched in the pre-culture step and thus there is likely to be a high concentration of enterococci with the potential to inhibit growth of *S. uberis.*

Growth retardation of other bacterial species has been reported due to the presence of enterococcal species within the gastrointestinal tract of humans. Commensal *Enterococci* species have been reported to have developed sophisticated defence mechanisms to eliminate exogenous competitors from the gut using bacteriocins (Dubin and Pamer, 2014) and can also secrete pheromones which act as chemical messengers between bacteria that can reduce growth and even mediate cell death. This area would benefit from further research as it indicates that there may be the potential to use the components secreted by *E. faecium,* as alternative antimicrobial agents, particularly for reduction of *S. uberis.*

The mechanism and impact of growth retardation on *S. uberis* by *E. faecium* is unknown (and was not part of this study), but this observation highlights the lack of understanding of the various relationships occurring within the faecal microbiota and then impact this might have on prevalence of *S. uberis*, and other pathogens. There have been reports of other bacteria having either an inhibitory relationship with *S. uberis* (Wuytack *et al.*, 2019), or a synergistic relationship (Wagener *et al.*, 2014), both of which have the potential to influence the survival and growth of *S. uberis* in bovine faeces. Further research into the potential interactions of different microbes within the bovine faecal microbiota is clearly justified.

#### 2.4.3.3 Optimisation of growth conditions for *S. uberis* within bovine faeces

All of the optimisation steps regarding the growth conditions surrounding the isolation of *S. uberis* strains from faeces using selective chromogenic media showed no real difference in terms of *S. uberis* identification as no isolates of *S. uberis* were isolated. One other problem with proposed method of identifying *S. uberis* may also be related to the use of using a PCR to categorise as the faecal sample as *S. uberis* positive or negative, prior to culture. The PCR diagnostic test had an analytical sensitivity of 12 cfu/g for *S. uberis* detection in faeces (Figure 2.5). This is a very low level of detection, which may be too low for isolation of *S. uberis,* especially in a mixed faecal culture. The other aspect of using PCR as the screening tool for deciding which samples to culture is the fact that PCR only requires the presence of DNA, not viable bacteria, therefore the samples may not contain viable *S. uberis* which is able to grow on the media during culture.

#### 2.4.3.4 Limitations of culture-based methodology for identification of *S. uberis* within bovine faeces

The use of MRAM in this study did not result in the isolation of any *S. uberis* isolates, which differs from a previous study reported by Zadoks et al in 2005. One of the main differences between the studies was the method of identification of the potential *S. uberis* isolates. Whilst both studies used PCR to confirm presence of *S. uberis,* the PCR used by Zadoks *et al* (2005) employed species-specific primers which targeted 16S

rDNA (Hassa*n et al*., 2001). Ribotyping was subsequently used for strain differentiation. However, ribotyping does not speciate bacteria and only highlights potential differences between bacterial isolates, based on the digestion of the DNA by a restriction enzyme. As this study also predates the release of the first annotated *S. uberis* genome, it cannot be certain whether *S. uberis* was isolated and correctly identified from cattle faeces based on the methodology of this study, particularly as the only species specific stage relied on amplification of 16SrDNA in a sample that would contain many, including as yet unidentified, bacterial species. Another study utilised MRAM for the detection and isolation of *S. uberis* from environmental samples (Went*e et al*., 2019). The samples were diluted in Ringer's solution prior to plating on Edwards modified medium supplemented with colistin sulphate and oxolinic acid. Single colonies were isolated and assessed to be catalase negative, Gram positive cocci which were subsequently cultured on MRAM to check for β-D-galactosidase activity. The isolates were also plated on D-sorbitol agar to investigate D-sorbitol reduction ability. The resulting presumptive *S. uberis* isolates were Gram-positive, esculin-positive, catalase-negative, β-D-galactosidase-positive, and D-sorbitol-positive. *Enterococcus* species have been reported to have differing abilities to reduce D-sorbitol, with species capable of reduction of D-sorbitol, the ability to hydrolyse esculin (Manero and Blanch, 1999), are catalase negative and have for β-D-galactosidase activity, as well as being Gram positive cocci (Lebreton *et al,* 2014). This is similar for some of the members of the SBSEC (Pompilio *et al.,*2019). Given the

relative ease of gaining annonated genome sequence data, it is surprising that these studies did not confirm the identity of *S. uberis* using 16SrDNA sequencing or did not undertake further genomic studies of these isolated strains. The results reported in this chapter cast questions regarding the reported prevalence of *S. uberis* in bovine faeces which have previously been reported within the literature, in particular as these studies utlisied culture based methodology, which could not be replicated in this study.

The other selective media utilised in this study was USM and a secondary version with reduced salt concentration (RSUSM), and based on the media previously described for the isolation of environmental and faecal isolates of *S. uberis* (Lacy-Hulber*t et al*., 2005; Pullinger *et al*., 2006) The USM theoretically worked as a selective media for *S. uberis* on a very similar base to the URAM, using 4-methylumbelliferyl-beta-D-glucuronide (MUG) as a substrate for the glucoronidase enzyme. MUG has traditionally used for the detection of *E. coli* in water which has been contaminated by faeces (Gauthier *et al.*, 1991; Farnleitner *et al.*, 2002). The USM resulted in *S. uberis* strain 0140J colonies appearing as very small translucent circular colonies, which fluoresced under UV light. These colonies were difficult to identify, especially if present in a mixed culture and the fluorescence was not around individual colonies, which did not help identify single *S. uberis* colonies. The poor growth of *S. uberis* strain 0140J was determined to be due to the high concentration of sodium chloride (7.5%), as *S. uberis* is

reported to not grow in concentrations higher than 6.5% (Odierno *et al.*, 2006). Once decreased to 5% in RSUSM agar, larger *S. uberis* colonies resulted; however, it was still not possible isolate single *S. uberis* colonies from mixed faecal cultures.

The results from this chapter indicate that using a culture based method alone maybe flawed for isolating single colonies of *S. uberis* from faeces. Another option which could provide a potential for isolation of single strains of *S .uberis* is the use of immunomagnetic separation (IMS). This technique has been reported to be successful for the isolation of both Gram negative bacteria such as *E. coli* and *Salmonella* species (Chapman *et al.*, 1994; Hsu *et al.*, 2014) and Gram positive bacteria such as *Clostridium perfringens* and *Listeria monocytogenes* from faecal samples (Cudjoe *et al.*, 1991; Bauwens *et al.*, 2003). IMS has also successfully been used to isolated different strains of *Streptococcus suis* from the tonsils of pigs (Gottschalk *et al.,* 1999). IMS relies upon the use of the Fc portion of species specific antibodies to covalently attaching to a magnetic bead, leaving the Fab region free to specifically bind the target bacteria. A magnetic field can then be used to recover the beads, with growth of the bacteria on a selective chromogenic media. This has been attempted in terms of a biosensor for mastitis pathogens, which use antibodies against the *S. uberis* adhesion molecule from strain 0140J complexed onto beads with a fluorescence marker to capture *S. uberis*. The study reported a high specificity of the anti *S. uberis*

antibody to antigen; however there was cross reactivity with nonspecific binding to *E. coli* and *S. aureu*s, suggesting a lack of antibody specificity (Mihklepp *et al.*, 2017). The *S. uberis* specific genes identified in Chapter 2 for the design of primers, *sub1154* and *sub0888,* alongside any of the other sortase anchored proteins previously identified (Leigh *et al.*; Egan *et al.*, 2010) would have the potential to make ideal target for antibodies as they are attached to the cell wall of the *S. uberis* bacterium. Further research is required into this area to determine whether the use of IMS, with or without the use of selective chromogenic media, such as URAM/RSUSM, could be used as a method of extracting single colonies of *S. uberis* from faecal samples.

#### 2.4.3.5 Conclusion

The results of this study indicate that selective media alone may not be suitable to differentiate *S. uberis* from other bacterial species which are present in bovine faeces, and that further research is required into alternative methodology, as well as the interactions between the bacteria in faecal microbiota. However the results of this study did show that the diagnostic test developed has a high analytical sensitivity and can detect the presence of low levels of *S. uberis* in the faeces of dairy cows. Whilst the biggest challenge to implementing the diagnostic test in epidemiological studies would be the sampling strategy used in order to get a representative detection rate of the population, the PCR based test

has shown to be a robust and reliable test for investigating the epidemiology of the presence of *S. uberis* in bovine faeces.

# Chapter 3. Epidemiological study of *S. uberis* in bovine faeces

## 3.1 Introduction

In a study investigating the main causes of clinical and subclinical mastitis in UK dairy herds, *S. uberis* and *E. coli* were the predominant pathogens isolated from the clinical mastitis cases and *S. uberis* the most predominant from subclinical cases; with both of these pathogens traditionally categorized as environmental pathogens (Bradley et al, 2007). Studies have investigated potential risk factors for environmental mastitis and most relate to specific cow factors which increase the risk of *S. uberis* IMI, for example stage of lactation or parity (Zadoks *et al*., 2001). There have been very few studies investigating the risk factors for *S. uberis* mastitis from the environment of both housed cows and cows at pasture. Previously reported risk factors for IMI relating to *S. uberis* including building ventilation, cow cleanliness (the amount and areas of dirt, faeces/mud on the cow) as well as pH, temperature and water content of the bedding substrates (Ward *et al.*, 2002; Schreiner and Ruegg, 2003), which suggests that the environmental conditions could substantially influence the survival and replication of environmental IMI pathogens.

Environmental pathogens have been hypothesized to survive and replicate in the environment surrounding cattle with the main mechanism for

infection of the mammary gland considered to be contamination of the tear from an environmental reservoir. It has been suggested that the key to reducing environmental mastitis within a herd is to reduce the exposure of cows to the pathogens (Hogan and Smith, 2012). Identification of reservoirs that seed the environment is key to controlling the level of environmental contamination by *S. uberis*.

*S. uberis* has been reported numerous times to be found in the environment of dairy cows, including pasture, used bedding, soil and plant material as well as in the faeces of dairy cows (Cullen, 1969; Lacy-Hulbert *et al.,* 2005; Zadoks *et al.,* 2005; Pryor, 2008). This had led to the hypothesis that contamination and persistence of *S. uberis* in the environment results from faecal-environment-oral transmission cycling of the pathogen within the population of cows. Increased environmental contamination results in the increased probability of teat end contamination by *S. uberis*, which increases the risk of transfer of bacteria into the mammary gland and subsequent infection. *S. uberis* has been detected at various rates in faecal detection studies, with a mean prevalence of 5.6% in a grazing herd in New Zealand (Lacy-Hulbert *et al.,* 2005) and 23% in a grazing herd in New York (Zadoks *et al.*, 2005); this shows wide variation between the two comprehensive studies that have been performed. These differences may be attributed to geographical differences, herd management and cow genetic differences or differences between the detection methods used.

This chapter focuses on investigating the faecal shedding patterns of *S. uberis* in a UK dairy herd that was housed over winter and at pasture other times of the year (a common husbandry system in dairy farming in the UK). The aim was to evaluate the impact of seasonality and housing on the bacterial shedding patterns of *S. uberis* in a longitudinal study.

This chapter describes a longitudinal study, in which one entire herd was sampled at bimonthly intervals throughout the year (Study 1) to investigate the prevalence of faecal *S. uberis* detection within a herd over time, and to investigate the impact of potential cow and management factors for *S. uberis* detection. A second study (Study 2) was designed to investigate the detection of faecal *S. uberis* over shorter time intervals in response to high detection rates observed during housed periods in Study 1. A cohort of 46 cows were sampled three times a week for 4 weeks during the housed period to determine any additional management or cow factors associated with *S. uberis* detection.

## 3.2 Materials and Methods

### 3.2.1 Cattle herd details and recording of mastitis

The dairy herd sampled as part of this study consisted of 228 adult lactating cows and was situated in Old Dalby, Leicestershire, United Kingdom. Sample collection began in October 2014 and concluded in June 2015 for study 1, with sampling for study 2 occurring in February and March 2016.

The average 305 day milk yield was 7,997 litres per cow, calculated based on cow yield and stage of lactation at the first sampling date in October. The median calving interval was 364 days and the mastitis incidence rate was 62 cases per 100 cows per year, based on the three months prior to the start of the study (target <30 cases per 100 cows per year). Previous bacteriology results (performed by QMMS laboratory, Somerset, UK) indicated that the main pathogen for IMI was *S. uberis*. The three month rolling mean bulk tank somatic cell count (SCC) was 80,000 cells/ml, with individual SCC milk recording occurring once a month for each animal.

Adult cows were housed from the beginning of November 2014 until the end of March 2015, spilt between two different housing systems. The majority of lactating cows were housed in cubicles, using wheat straw as the bedding substrate. The remaining cows, including freshly calved cows and dry cows, were housed in loose straw yards. The cows were fed a

partial mixed ration, as well as a flat rate of 6kg of concentrate in the parlour during the housing period.

From April to November, the lactating cows were turned out onto permanent pasture, which was rotated based on grass growth. Cows at pasture were also fed concentrates in the parlour, as well as twice daily access to a partial mixed ration after milking. Dry cows which were >3 weeks from calving were turned out in separate permanent grass paddocks during the grazing season, with no supplementary feed. These cows were housed on a separate unit during the winter and were unable to be sampled.

The transition cows, classified as <3 weeks before calving, were housed on a loose straw yard all year round. The transition cow diet remained consistent throughout the year, consisting of 4.3kg dry matter (DM) of haylage, 4.3kg DM chopped straw, 4.5kg maize silage and 0.9kg DM soya, resulting in 14kg DM/cow/day and an energy content of 130MJ/cow/day. DM is the proportion of the feed that is not water, which is measured when analysing ration ingredients, as there is a large variability in moisture content feed used in diets (Grummer, Mashek and Hayirli, 2004).

### 3.2.2 Sample Collection

Faecal samples were obtained by inserting a sterile cotton-tipped swab into the rectum of cows or obtained directly from a freshly voided faeces

(FVF) from each identified cow per sampling visit and stored at -20ºC until processing.

Clinical mastitis (CM) was diagnosed by trained farm staff at milking time, based on visible changes in the composition of the milk, including the presence of clots, discolouration or consistency and changes in the physiology of the udder, including swelling and hardness. Milk samples were collected in a sterile manner by the farm staff and stored at -18ºC prior to culture. The presence of *S. uberis* was confirmed after culture on esculin blood agar (QMMS Ltd, Somerset, UK).

### 3.2.3 DNA Extraction from bovine faeces and detection of *S. uberis* within the sample

The tip of the swab was removed and cultured in 3ml of Todd-Hewitt broth (THB) containing 4ug/ml of gentamicin at 37ºC overnight. . These cultures were preserved by the addition of an equal volume of glycerol (50%, v/v) stored at -80ºC.

DNA extraction was performed using the phenol based method as described in section 2.2.3.2.

The presence of bacterial DNA was tested by PCR, using primers for 16S rRNA and the presence of *S. uberis* was confirmed by PCR using the primer pair to amplify *sub0888* (Section 2.2.6, Table 2.2). The PCR master-mix compromised standard Taq buffer reaction (NEB), 200µM of

dNTPs (Thermo), 0.4µM of forward primer, 0.4µM of reverse primer, 1.25 units of Taq polymerase (NEB) in molecular biology grade water. Initiation temperature was set to 95ºC for 30 seconds, followed by 35 cycles of denaturation, annealing and elongation at 95ºC for 20 seconds, 53.5ºC for 30 seconds and 68ºC for 60 seconds, respectively. A final hold at 68ºC for 5 minutes constituted the final elongation. A negative control of molecular biology grade water and a positive control of genomic DNA from *S. uberis* strain 0140J strain were used.

### 3.2.4 Data Collection and Organisation

All of the data regarding the confirmation of *S. uberis* identity and the individual cow data were collated and analysed descriptively using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA). Cattle were identified as positive for shedding *S. uberis* if a faecal sample tested positive for *sub0888* in a PCR reaction. A binary indicator was used to represent the detection of *S. uberis* in the faecal sample, with detection being denoted as 1, and no detection denoted as 0. Any cows where samples were unable to be obtained, due to absence from the herd at the time of sampling, were denoted as 999. Confirmation of the presence of bacterial DNA was performed on all samples by 16S rRNA PCR and any samples that tested negative were retested or DNA was re-extracted from a duplicated sample from the corresponding faecal swab culture.

### 3.2.5 Herd Level Prevalence of Faecal Shedding of *S. uberis*

#### 3.2.5.1 Sampling

Investigating the herd level prevalence of *S. uberis* detection in faeces was undertaken as two separate studies. Study 1 was a longitudinal whole herd study, in which faecal samples were collected approximately every 8 weeks from all adult cows present within the herd on that day between October 2014 and June 2015. This included both lactating and dry cows. Data were obtained for each cow, regarding days in milk, milk yield and SCC from information held in the National Milk Recording (NMR, Chippenham, UK). Any additional treatments, including administration of antibiotic for CM, was collected from farm's computer database (C21, Westfailia, GEA, USA). At the time of sampling, body condition scoring (BCS) was performed, using the 5 point scale with 0.50 increments (AHDB, 2014a) (Appendix 1) and the type of housing environment was recorded for each individual cow.

Study 2 was a cohort study, in which faecal sampling of a cohort of 46 housed cows occurred every Monday, Wednesday and Friday for four consecutive weeks, between February and March 2016. The cohort of cows was selected using a random number generator and consisted of 34 randomly selected multiparous cows, of which 10 were dry cows at the start of the sampling period, as well as 12 randomly selected first lactation cows, of which 3 were dry cows at the start of the sampling period. The 46

cows were assessed individually and given a cleanliness score for the udder and legs, using the AHDB scoring system (AHDB, 2014b) (Appendix 2 and the housing location of each cow recorded. Weekly BCS was performed on the Monday of each visit, using the 5 point scale described for Study 1 (Appendix 1); however 0.25 increments were used, in order to increase the sensitivity of the BCS over time. An individual cow SCC test was performed on milk samples collected by an external laboratory (NMR, Chippenham) on the second day of the study. Daily temperature and humidity recordings were taken from the local weather station, Nottingham Weather Centre, which is approximately 20 miles North West of the dairy farm and were used as an indicator of weather conditions on the farm during the sampling period.

#### 3.2.5.2 Data Analysis

All of the data collation and descriptive statistics were performed in Microsoft Excel. Data categorization for each of the measured variables are outlined in Table 3.2. Information was categorised and collated for each of the cows at each sampling time point which included previous shedding and potential future shedding. Calculations were performed to determine whether *S. uberis* had been detected in samples taken prior to and/or after this sampling time point, as well as whether the faecal samples from each cow had been positive for *S. uberis* at one, two or three previous sampling time points. These were denoted as a binary indicator; 0 was no,

1 was yes and 999 denoted the inability to calculate the parameter due to missing of data.

Other calculated parameters included changes in BCS between two sampling dates, which was denoted as a binary indicator; 0 was no, 1 was yes and 999 denoted the inability to calculate the parameter due to missing of data. The SCC value in the same month that faecal sampling was recorded and was categorized as a binary indicator for SCC >200,000 cells/ml and for SCC <50,000 cells/ml (no was 0, yes was 1). These values were used as a SCC >200,000 cells/ml has been classified as infected (McDermott, Erb and Natzke, 1982; Dohoo and Leslie, 1991) and SCC< 50,000 cells/ml have been identified as cows at increased risk of clinical mastitis (Beaudeau *et al.*, 2002). The incidence of a clinical mastitis case was recorded for each time point between sample collection and was categorized as a binary outcome for whether there was a case of clinical mastitis (no was 0, yes was 1), as well as recorded as a binary outcome for whether there was a case of *S. uberis* clinical mastitis (confirmed by culture) (no was 0, yes was 1).

#### 3.2.5.3 Statistical analysis

Multilevel logistic regression models were used to evaluate the association between different variables and the outcome of whether *S. uberis* was detected (yes or no) in the faecal samples, with model building carried out in MLWiN version 2.31 (Rabash *et al.,* 2012). Initial model building was performed by forward selection and explanatory variables were retained in

the model where the estimated coefficient was greater than twice the standard error (such that the 95% confidence interval for the estimate did not include zero); all rejected variables were re-offered to the final model and retained if they now met these criteria. This equated to a significance of $P<0.05$.

The model used for analysis was a 2-level hierarchical model, in order to account for correlations between repeated samples within individual cows, with individual sampling points set as the lowest level unit of data.

Model building occurred by forward selection. A variety of potential categorical explanatory variables were added to the model sequentially and coefficients and standard error of coefficients estimated. The variables tested are shown in Table 3.1 and Table 3.2 for each of sampling periods.

The model took the conventional form:

$$\textit{S. uberis}\ \text{detected}_{ij} \sim \text{Bernoulli}\ (\pi_{ij})$$

$$(\text{logit}=\pi_{ij}) = \alpha + \beta_1 X_{ij} + \beta_2 X_j + u_j$$

$$[u_j] \sim N\ (0, \Omega_u)$$

where subscripts i and j denoted the ith sampling point of the jth cow, respectively. πij was the probability of detection of *S. uberis* in the faeces for the ith sampling point of the jth cow, α the intercept value and $X_{ij}$ and $X_j$ were explanatory covariates at sampling point and cow levels respectively, with $\beta_n$ being the corresponding coefficients, $u_j$ was the random effect to

account for residual variation between cows (assumed to be normally distributed with mean = 0 and variance = $\sigma^2$ v).

Significant variables for the samples taken between October 2014 and June 2015 were converted into a probability of infection using the following equation:

$$\text{Ln}\left(\frac{P}{1-P}\right) = \text{cons} + (\beta X)$$

$$P = \left(\frac{\exp \text{ logit}}{1 + \exp \text{ logit}}\right)$$

**Table 3.1: Categorization of measured variables for detection of *S. uberis* in faeces for the logistic regression model in Study 1.**

| Variable | Variable Category | Category |
|---|---|---|
| **Body Condition Score** | ≤2.0 | 1 |
| | 2.5 | 2 |
| | 3.0 | 3 |
| | ≥3.5 | 4 |
| **Loss of ≥0.5 BCS since last sample** | No | 0 |
| | Yes | 1 |
| **Season** | April to October (outdoor housing) | 1 |
| | November to March (indoor housing) | 2 |
| **Housing environment** | Pasture | 1 |
| | Straw yard | 2 |
| | Cubicles | 3 |
| **Lactation Number** | Primiparous | 1 |
| | Multiparous | 2 |
| **Days in Milk** | <50 | 1 |
| | 50 to 99 | 2 |
| | 100 to 149 | 3 |
| | 150 to 200 | 4 |
| | 200 to 249 | 5 |
| | 250 to 299 | 6 |
| | >299 | 7 |
| **Milking Status** | Milking cow | 1 |
| | Dry cow | 0 |
| **SCC > 200,000 cells/ml** | No | 0 |
| | Yes | 1 |
| **SCC <50,000 cells/ml** | No | 0 |
| | Yes | 1 |
| ***S. uberis* detected in the sampling visit prior** | No | 0 |
| | Yes | 1 |
| ***S. uberis* detected in the sampling visit after** | No | 0 |
| | Yes | 1 |
| ***S. uberis* detected in the sampling visit before or after** | No | 0 |
| | Yes | 1 |
| **One previous test where *S. uberis* has been detected** | No | 0 |
| | Yes | 1 |
| **Two previous tests where *S. uberis* has been detected** | No | 0 |
| | Yes | 1 |
| **Three previous tests where *S. uberis* has been detected** | No | 0 |
| | Yes | 1 |

**Table 3.2: Categorization of measured variables for detection of *S. uberis* in faeces for the logistic regression model for the Study 2**

| Variable | Variable Subset | Category |
|---|---|---|
| **Housing** | Cubicles | 1 |
| | Straw yard | 2 |
| **Milking Status** | Dry cow | 0 |
| | Milking cow | 1 |
| **Parity** | Parity 1 | 1 |
| | Parity 2 | 2 |
| | Parity 3 and above | 3 |
| **Body Condition Score** | ≤2.0 | 1 |
| | 2.25 to 2.5 | 2 |
| | 2.75 to 3.0 | 3 |
| | 3.25 to 3.5 | 4 |
| | >3.5 | 5 |
| **Leg cleanliness** | <10% surface area dirty (scores 0 and 1) | 1 |
| | >10% surface area dirty (scores 2 and 3) | 2 |
| **Udder cleanliness** | <10% surface area dirty (scores 0 and 1) | 1 |
| | >10% surface area dirty (scores 2 and 3) | 2 |
| **Shed in previous test** | No | 0 |
| | Yes | 1 |
| | Missing | 999 |
| **Shed at all in previous two tests** | No | 0 |
| | Yes | 1 |
| | Missing | 999 |
| **Shed at all in previous three tests** | No | 0 |
| | Yes | 1 |
| | Missing | 999 |

## 3.3 Results

### 3.3.1 Study 1: A longitudinal study investigating the herd level prevalence of *S. uberis* in bovine faeces

#### 3.3.1.1 Prevalence of Detection of *S. uberis*

Faecal samples were taken every 8 weeks resulting in a mean of 216 cows sampled per time point and a total of 1,080 cows sampled over a period of 32 weeks. The overall herd prevalence of detection of *sub0888* positive faecal samples was 195 out of 1,080 samples (Table 3.3). The individual cow sample results are given in Appendix 3. There was an increase in prevalence of *S. uberis* positive samples from cows during the winter housing months with a mean of 29.3% of positive faecal samples, compared to the summer months at pasture where a mean of 11.2% was recorded.

There were a total of 133 cows tested on all five sampling occasions. Of those cows, 34% (n=45) did not test positive for *S. uberis* at any time point. There were no cows that were positive on all five sampling occasions, with one animal being positive on four occasions, two animals positive on three occasions and twenty four animals positive on more than two occasions (Figure 3.1). From the overall herd prevalence occurring at each time point, a calculation was made to estimate the proportion of the cows that would be predicted to be negative for all sampling time points, if each cow had an equal chance of becoming positive at each sample time point (Table 3.4).

That is, each cow was assumed to have a probability of detection equal to the observed herd prevalence for that time point. The results showed that after five sampling time points 36% of the cows would not have tested positive for *S. uberis* and that no cows should have tested positive at all five time points.

**Table 3.3: The *S. uberis* faecal detection rate in dairy cows over a 10 month period (Study 1).***

| | October 2014 | December 2014 | February 2015 | April 2015 | June 2015 |
|---|---|---|---|---|---|
| **Milking cows' environment** | Pasture | Housed | Housed | Pasture | Pasture |
| **Number of cows tested** | 226 | 209 | 202 | 219 | 224 |
| **Number of cows tested 0888 positive** | 23 | 53 | 67 | 27 | 25 |
| **Percentage of herd testing positive (%)** | 10.2 | 25.4 | 33.2 | 12.3 | 11.2 |

*Detection was based on a species specific PCR with all cows in the herd at each time point being sampled.

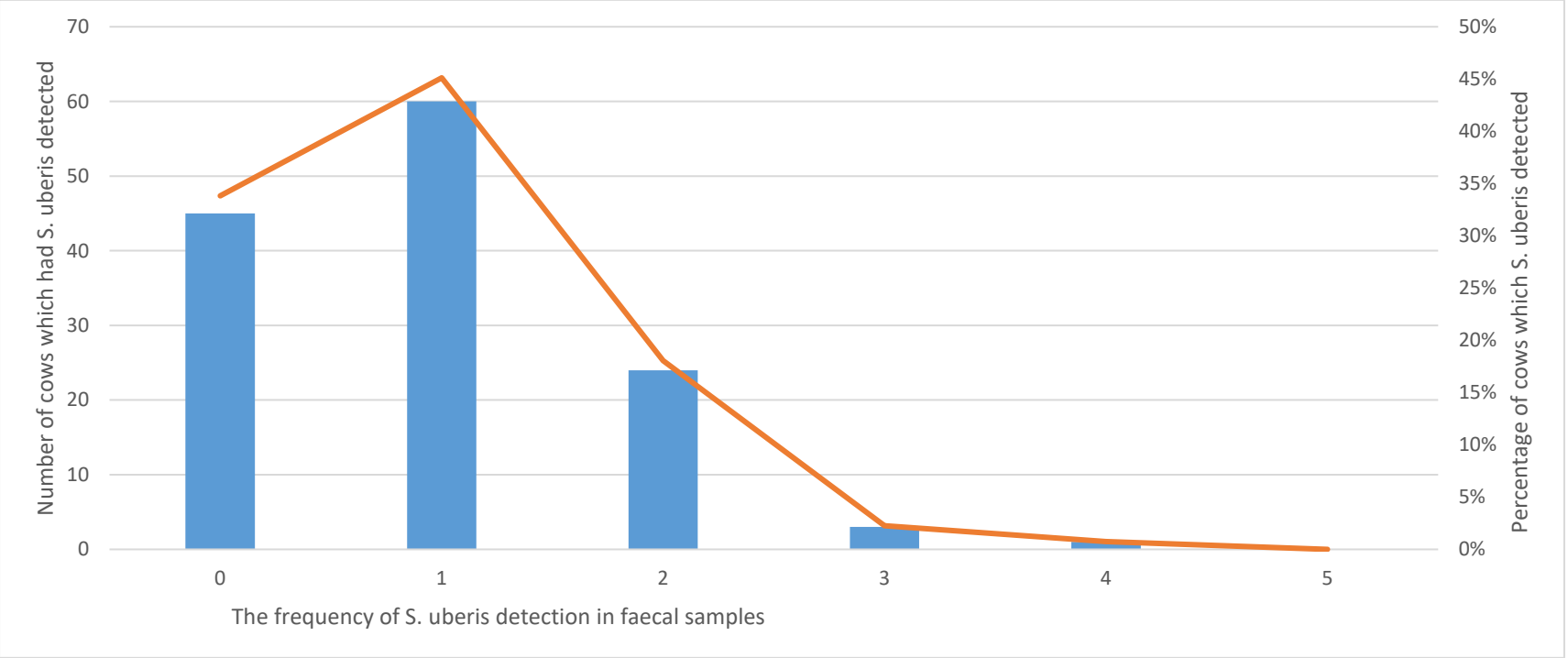



**Figure 3.1 Prevalence of *S. uberis* positive faecal samples over the five sampling points in herd cattle (Study 1)**

Graphical representation of the presence of *S. uberis* within faeces detected by *sub0888* PCR at all five sampling points over a period of 10 months at 8 weekly intervals (n=133). The x axis represents the number of times a cow had *S. uberis* detected within their faeces over the five sampling time points. The blue bars represent the number of animals which had *S. uberis* detected using the species specific *sub0888* PCR. The red line represents the percentage of cows which had *S. uberis* detected within faeces. The number of cows shown by the bars and on the left hand y axis and as a percentage shown by the orange line on the right hand y axis.

**Table 3.4: Summary of the probability of the frequency of detection of *S. uberis* in faeces occurring due to chance in Study 1.**

| Sampling time point | Detection rate of *S. uberis* negative cows | Detection Rate of *S. uberis* in the faeces | If the chance of detection was random and was equal to the observed herd prevalence of detection for each sampling time point* | | | |
|---|---|---|---|---|---|---|
| | | | Percentage of cows that would never have *S. uberis* detected | Number of cows which would never have *S. uberis* detected | Percentage of cows that would always have *S. uberis* detected | Number of cows which would always have *S. uberis* detected |
| 1 | 89.8% | 10.2% | 90% | 119 | 10% | 14 |
| 2 | 74.6% | 25.4% | 67% | 89 | 3% | 3 |
| 3 | 68.8% | 33.2% | 46% | 61 | 1% | 1 |
| 4 | 87.7% | 12.3% | 40% | 54 | 0% | 0 |
| 5 | 88.8% | 11.2% | 36% | 48 | 0% | 0 |

*Calculations are based on the herd prevalence *S. uberis* detection within faeces for each time point. The calculation of the chance of detection was based on the risk of a cow becoming infected at each time point being equal to the base level of infection (i.e. at the observed herd prevalence of detection) and thus the chance of detection being random.

#### 3.3.1.2 Statistical Modelling: The Impact of Different Variables effecting whether *S. uberis* is detected in their faeces

Results of the final multilevel logistic regression model with *S. uberis* detection as the outcome are provided in Table 3.5. There was an increase in odds of detecting *S. uberis* in the faeces of cows (OR = 2.8) during the winter/housing season compared to the summer/outside season (Table 3.5). The non-significant variables included whether the cow had previously had *S. uberis* detected in their faeces, SCC, BCS loss and housing type. In addition there was an increased odds of *S. uberis* detection in the faeces of cows which were in early lactation (0-49 DIM), whether cows had a BCS ≥3.5 and cow parity.

These results were then transformed into probabilities of detection, which are summarized in Table 3.6. The impact of BCS is highlighted in Figure 3.2, which shows the probability of detection of *S. uberis* detection in faeces increasing when BCS increases to >3.5, when compared to BCS 2.5 in cows, regardless of housing and DIM. Results highlighted that regardless the BCS and parity, the probability of detection of *S. uberis* in faeces was highest when cows were housed in comparison to being at pasture.

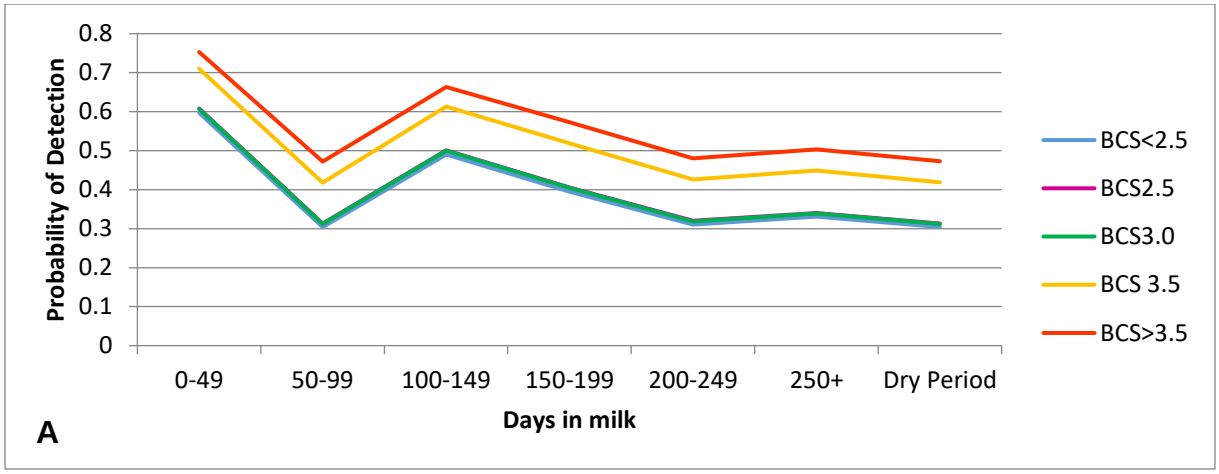
The impact of parity is highlighted in Figure 3.3, which shows the probability of detection of *S. uberis* detection in faeces increasing for primiparous, compared to multiparous cows, regardless of cow location and BCS. The probability of detection of *S. uberis* at the same lactation

time point and at the same BCS was increased if the heifer was housed indoors, rather than outdoors. For example a heifer with a BCS 2.5 at 168 DIM had a probability of detection of detection of *S. uberis* of 0.181 if the heifer was outdoors at pasture, compared to 0.406 if the heifer was housed.

**Table 3.5: Parameter estimates from the final multilevel logistic regression model for detection of *S. uberis* in faeces when investigating whole herd prevalence.**

| Model Term* | Number of Samples | Odds Ratio | Confidence Interval | P |
|---|---|---|---|---|
| **Outdoor Season (October, April and June)** | 669 | Reference | | |
| **Inside Season (December and February)** | 411 | 2.82 | 1.95-4.07 | P<0.05 |
| **0-49 DIM** | 151 | Reference | | |
| **50-99 DIM** | 111 | 0.30 | 0.16-0.59 | P<0.05 |
| **100-149 DIM** | 145 | 0.57 | 0.33-0.99 | P<0.05 |
| **150-200 DIM** | 135 | 0.42 | 0.24-0.75 | P<0.05 |
| **200-249 DIM** | 126 | 0.28 | 0.14-0.53 | P<0.05 |
| **≥250 DIM** | 148 | 0.33 | 0.18-0.58 | P<0.05 |
| **Dry Cows** | 164 | 0.29 | 0.15-0.56 | P<0.05 |
| **BCS ≤2.0** | 145 | 0.56 | 0.33-0.99 | P<0.05 |
| **BCS 2.5** | 281 | 0.54 | 0.34-0.86 | P<0.05 |
| **BCS 3.0** | 295 | 0.59 | 0.37-0.94 | P<0.05 |
| **BCS ≥3.5** | 226 | Reference | | P<0.05 |
| **Parity 1** | 268 | Reference | | |
| **Parity 2 and above** | 682 | 0.689 | 0.480-0.988 | P<0.05 |

*Non-significant factors omitted included change in BCS, milking status (dry or milking), somatic cell count, previous and future faecal detection of *S. uberis* and housing (cubicles or straw yards). DIM = days in milk; BCS = body condition score.



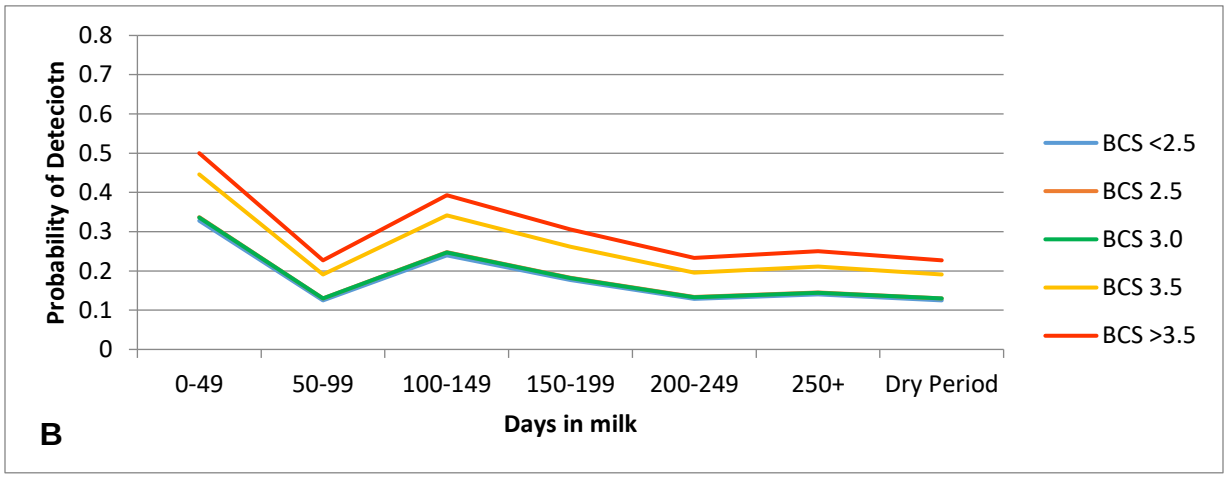
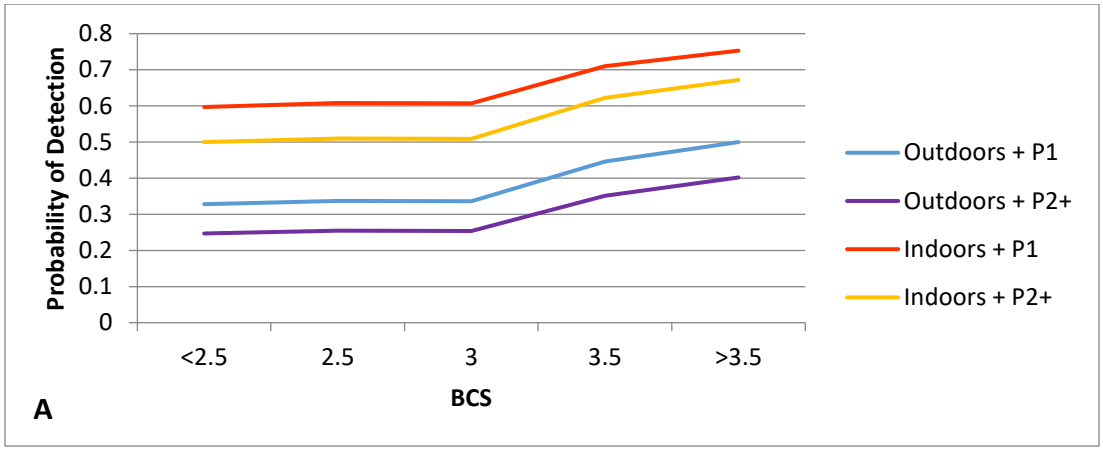




**Figure 3.2 The probability of detection *S. uberis* in faeces at different parities during the housing period according to different body condition scores or lactation time points.**

The combined effect of the significant variables from the logistic regression model were transformed into the risks of the probability of detection of *S. uberis* in the faeces of dairy cows and presented in graphical format. The x axis indicates the stage of lactation, in terms of days in milk. Each of the lines indicates the Body Condition Score (BCS) of the animals , scored on a scale of 1 to 5, based on the AHDB scoring system. This indicates an increased probability of detection of *S. uberis* in animals which had an increased BCS and were early in lactation.

**A:** Represents first lactation heifers during the housing period, with the probability of detection of detection of *S. uberis* in their faeces of 0.753 in the first 50 days of lactation if the BCS is >3.5, in comparison to a probability of detection of 0.608 if the BCS is 2.5.

**B:** Represents multiparous cows during the housing period, with the probability of detection of detection of *S. uberis* in their faeces of 0.672 in the first 50 days of lactation if the BCS is >3.5, in comparison to a probability of detection of 0.510 if the BCS is 2.5.



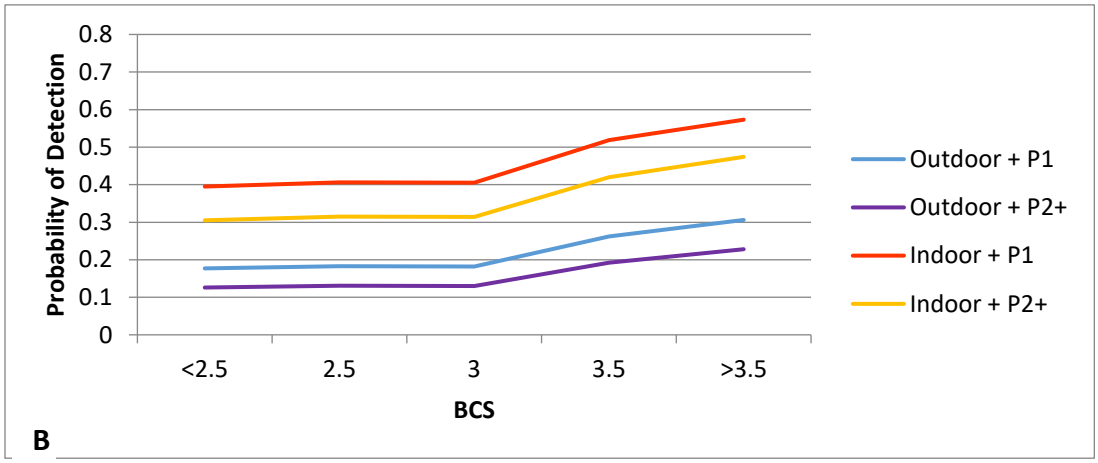




**Figure 3.3 The probability of detection of *S. uberis* in faeces at two different stages of lactation from in cows according to body condition score and housing conditions.**

The combined effect of the significant variables from the logistic regression model were transformed into the risks of the probability of detection of *S. uberis* in the faeces of dairy cows. P1 refers to a lactation of 1, P2+ refers to a lactation 2 and above, with housing differentiated as indoor (housed) and outdoor (grazing season). The x axis indicates the body condition score (BCS) of the cows at the time of sampling and lines indicate whether the animals were P1 (red and blue) or P2+ (purple and yellow) and if the animals were housed indoors (red and yellow) or outdoors grazing (blue and purple). The graph indicates that grazing animals with higher BCS (based on the 5 point scale) have an increased probability of *S. uberis* detection in their faeces, with the risk higher in first lactation heifers than multiparous cows.

**A:** Represents cows that were 0-49 days in milk with a probability of detecting *S. uberis* for cows of BCS of 3.5 and cows >3.5

**B:** Represents cows that were 150-199 days in milk with a probability of detecting *S. uberis* for cows of BCS of 3.5 and cows >3.5

**Table 3.6: Probability of combined significant variables for *S. uberis* detection within a faecal sample from the multilevel logistic regression model.**

| Variables | Parity 1 | Parity 2 | Variables | Parity 1 | Parity 2 |
|---|---|---|---|---|---|
| **S1+D1+B1** | 0.33 | 0.25 | **S2+D1+B1** | 0.60 | 0.50 |
| **S1+D1+B2** | 0.34 | 0.26 | **S2+D1+B2** | 0.61 | 0.51 |
| **S1+D1+B3** | 0.34 | 0.25 | **S2+D1+B3** | 0.61 | 0.51 |
| **S1+D1+B4** | 0.45 | 0.35 | **S2+D1+B4** | 0.71 | 0.62 |
| **S1+D1+B5** | 0.50 | 0.40 | **S2+D1+B5** | 0.75 | 0.67 |
| **S1+D2+B11** | 0.13 | 0.09 | **S2+D2+B1** | 0.30 | 0.23 |
| **S1+D2+B2** | 0.13 | 0.09 | **S2+D2+B2** | 0.31 | 0.23 |
| **S1+D2+B3** | 0.13 | 0.09 | **S2+D2+B3** | 0.31 | 0.23 |
| **S1+D2+B4** | 0.19 | 0.14 | **S2+D2+B4** | 0.42 | 0.33 |
| **S1+D2+B6** | 0.23 | 0.17 | **S2+D2+B5** | 0.47 | 0.38 |
| **S1+D3+B1** | 0.24 | 0.18 | **S2+D3+B1** | 0.49 | 0.39 |
| **S1+D3+B2** | 0.25 | 0.18 | **S2+D3+B2** | 0.50 | 0.40 |
| **S1+D3+B3** | 0.25 | 0.18 | **S2+D3+B3** | 0.50 | 0.40 |
| **S1+D3+B4** | 0.34 | 0.26 | **S2+D3+B4** | 0.61 | 0.52 |
| **S1+D3+B5** | 0.39 | 0.30 | **S2+D3+B5** | 0.66 | 0.57 |
| **S1+D4+B1** | 0.18 | 0.13 | **S2+D4+B1** | 0.40 | 0.31 |
| **S1+D4+B2** | 0.18 | 0.13 | **S2+D4+B2** | 0.41 | 0.32 |
| **S1+D4+B3** | 0.18 | 0.13 | **S2+D4+B3** | 0.41 | 0.31 |
| **S1+D4+B4** | 0.26 | 0.19 | **S2+D4+B4** | 0.52 | 0.42 |
| **S1+D4+B5** | 0.31 | 0.23 | **S2+D4+B5** | 0.57 | 0.47 |
| **S1+D5+B1** | 0.13 | 0.09 | **S2+D5+B1** | 0.31 | 0.23 |
| **S1+D5+B2** | 0.13 | 0.09 | **S2+D5+B2** | 0.32 | 0.24 |
| **S1+D5+B3** | 0.13 | 0.09 | **S2+D5+B3** | 0.32 | 0.24 |
| **S1+D5+B4** | 0.20 | 0.14 | **S2+D5+B4** | 0.43 | 0.33 |
| **S1+D5+B5** | 0.23 | 0.17 | **S2+D5+B5** | 0.48 | 0.38 |
| **S1+D6+B1** | 0.14 | 0.10 | **S2+D6+B1** | 0.33 | 0.25 |
| **S1+D6+B2** | 0.15 | 0.10 | **S2+D6+B2** | 0.34 | 0.26 |
| **S1+D6+B3** | 0.14 | 0.10 | **S2+D6+B3** | 0.34 | 0.26 |
| **S1+D6+B4** | 0.21 | 0.15 | **S2+D6+B4** | 0.45 | 0.35 |
| **S1+D6+B5** | 0.25 | 0.18 | **S2+D6+B5** | 0.50 | 0.41 |
| **S1+D7+B1** | 0.13 | 0.09 | **S2+D7+B1** | 0.30 | 0.23 |
| **S1+D7+B2** | 0.13 | 0.09 | **S2+D7+B2** | 0.31 | 0.24 |
| **S1+D7+B3** | 0.13 | 0.09 | **S2+D7+B3** | 0.31 | 0.23 |
| **S1+D7+B4** | 0.19 | 0.14 | **S2+D7+B4** | 0.42 | 0.33 |
| **S1+D7+B5** | 0.23 | 0.17 | **S2+D7+B5** | 0.47 | 0.38 |

S1: outdoor/summer, S2: indoor/winter. D1: 0-49 DIM, D2: 50-99DIM, D3: 100-149 DIM, D4: 150-199 DIM, D5: 200-249 DIM, D6: >249 DIM, D7: Dry period. B1: BCS≤2.0, B2: BCS 2.5, B3: BCS 3.0, B4: BCS 3.5, B5: BCS>3.5.

Analysis of the probability of detections were used to check the fit of the model for each season This was based on calculating the mean BCS, parity and DIM for all cows sampled during the grazing period (n=669) and cows sampled during the housing period (n=411). For the samples obtained during the grazing outdoor period (October 2014, April 2015 and June 2015), the mean BCS was 2.90 and mean parity was 2.71, with a mean DIM of 184 days. The calculated probability of detecting *S. uberis* in the faeces of an average cow in the herd during the grazing season was calculated to be 13%. This was close to the observed prevalence of 11.3%, with 73 faecal samples out of the 661 faecal samples obtained during this period positive for *S. uberis* detection. For samples obtained from cows during the indoor housed period (December 2014 and February 2015), the mean BCS was 2.76 and mean parity was 2.86, with a mean DIM of 175 days. The calculated probability of detecting *S. uberis* within faeces of an average cow in the herd during the housed period was calculated to be 31%. This was again close to the observed prevalence of 29.2%, with 120 faecal samples out of the 411 faecal samples obtained during this period positive for *S. uberis* detection.

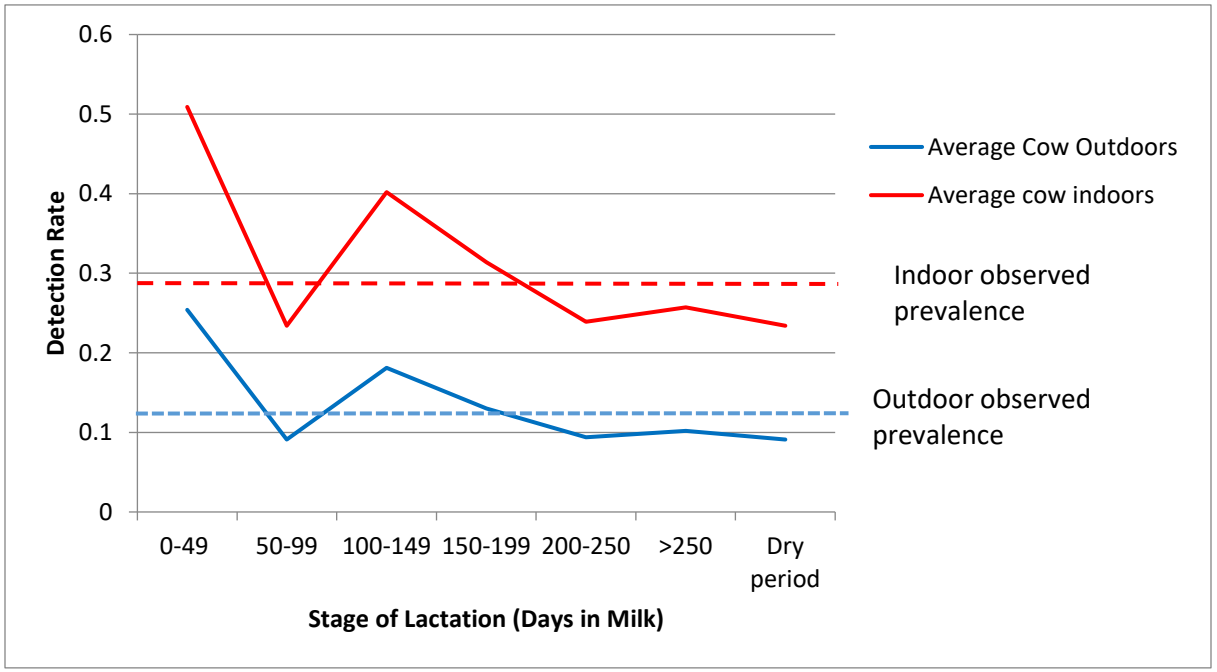



**Figure 3.4 Comparison of the predicted probability and observed prevalence of *S. uberis* detection during the housing or grazing period over the lactation period.**

Graphical representation of the predicted risk of detecting *S. uberis* in the faeces in an average cow within the herd when housed outdoor or grazing (solid blue line) and when housed indoors (solid red line) over the course of the lactation period (x axis). The mean prevalence of the observed detection of *S. uberis* during the outdoor period of 11.3% (dotted blue line) and indoor period of 29.2% (dotted red line). .

#### 3.3.1.3 Incidence of Clinical Mastitis

The clinical mastitis (CM) incidence was recorded by the farm staff over the 12 month period and is highlighted in Figure 3.5. The incidence is based on farm staff identification and treatment of CM, which equated to 55 cases of CM over the 12 months. The incidence rate of CM decreased during Study 1, initially starting at 62 cases per 100 cows per year and ending at 19 cases per 100 cows per year. This decrease was potentially linked to management changes made on the farm, after consultation with a veterinary expert because of the high number of clinical mastitis cases in the previous winter.

A summary of the clinical mastitis bacteriology results is shown in Figure 3.5, with the detailed bacteriology results found in appendix 4. There was a mixed aetiology of mastitis on the farm, with a total of 47 samples tested for bacteriology (submission rate of 85% of all CM cases). Of the 47, 12 were culture positive (25.5%) for *S. uberis*, of which only half (n=6) were cultures of only *S. uberis*. The next most common pathogen cultured was *E. coli* (n= 8, of which 4 cases were mixed cultures) followed by coagulase negative Staphylococci (CNS) (n =7, of which 6 cases were mixed cultures).

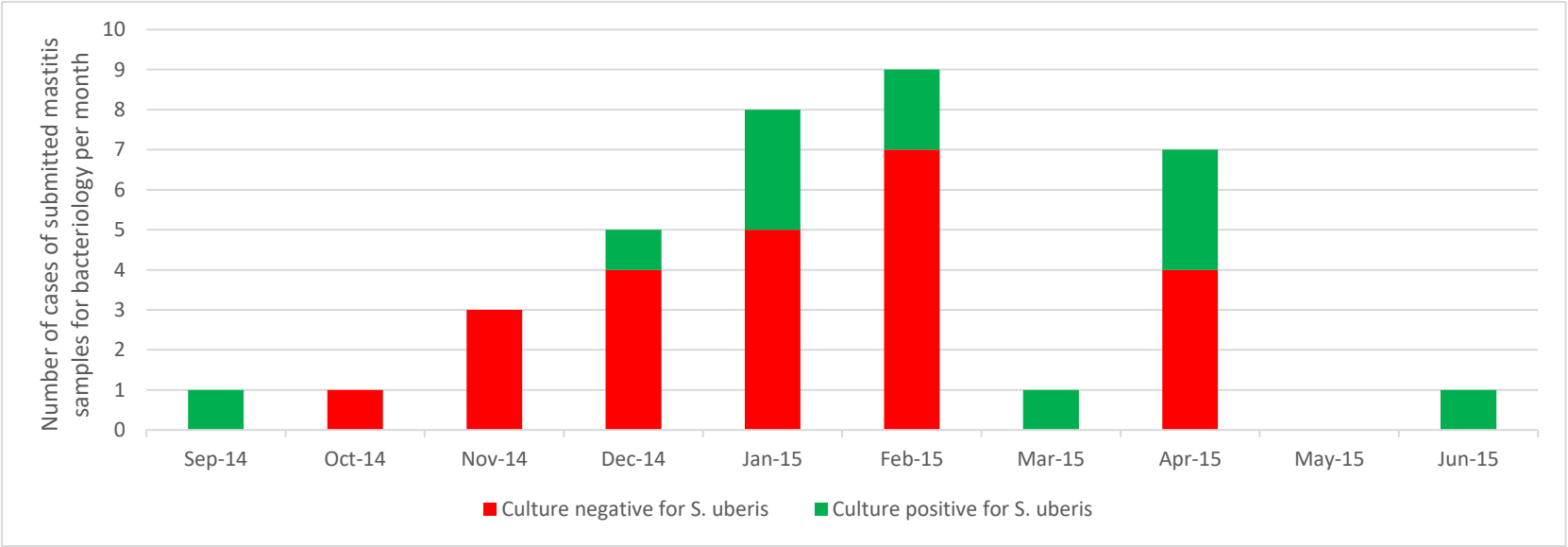



**Figure 3.5 Number of clinical mastitis cases per month and associated pathogens.**

The number of clinical mastitis cases detected and recorded by the farm staff and results of the bacteriology of the submitted mastitic milk. A total of 47 samples were submitted for bacteriological culture, of which 36 had an identifiable cow and sampling date. The red bars indicate bacteriology results which were negative for *S. uberis* and the green bars the number of *S. uberis* samples. The highest incidence of *S. uberis* detection correlated with the winter months when cows were housed indoors.

### 3.3.2 Study 2: A cohort study investigating the prevalence of detection of *S. uberis* in bovine faeces

#### 3.3.2.1 Prevalence of Detection of *S. uberis* over a 4 week period

Faecal samples were taken from a cohort of 46 randomly selected cows, which were housed, every Monday, Wednesday and Friday for a period of four weeks from the start of February 2016. The mean prevalence of detection of *S. uberis* in faecal samples was 30.1%, with a range of 13.0 to 54.3% (Figure 3.6, Table 3.7). The median detection prevalence rate was 28.2% with the 25$^{th}$ percentile rate at 19.6% and the 75$^{th}$ percentile at 36.4%).

The weather recordings taken from the local weather station were analysed to investigate whether the temperature or humidity of the day was associated with the faecal detection of *S. uberis* (Table 3.7). There was no apparent association between the detection *S. uberis* and changes in weather conditions, such as increased rainfall, humidity or abnormal temperature fluctuations. During Study 2, there were no changes in yard management, such as frequency of cleaning of straw yards or dietary alterations that accompanied the changes in prevalence.

During the four week sampling period, 9% of 46 cows sampled never tested positive for *S. uberis* (

Figure 3.7). Fifteen percent of the cohort were positive on more than 50% of sampling occasions, and no cows tested positive more than nine times in the four week sampling period. The

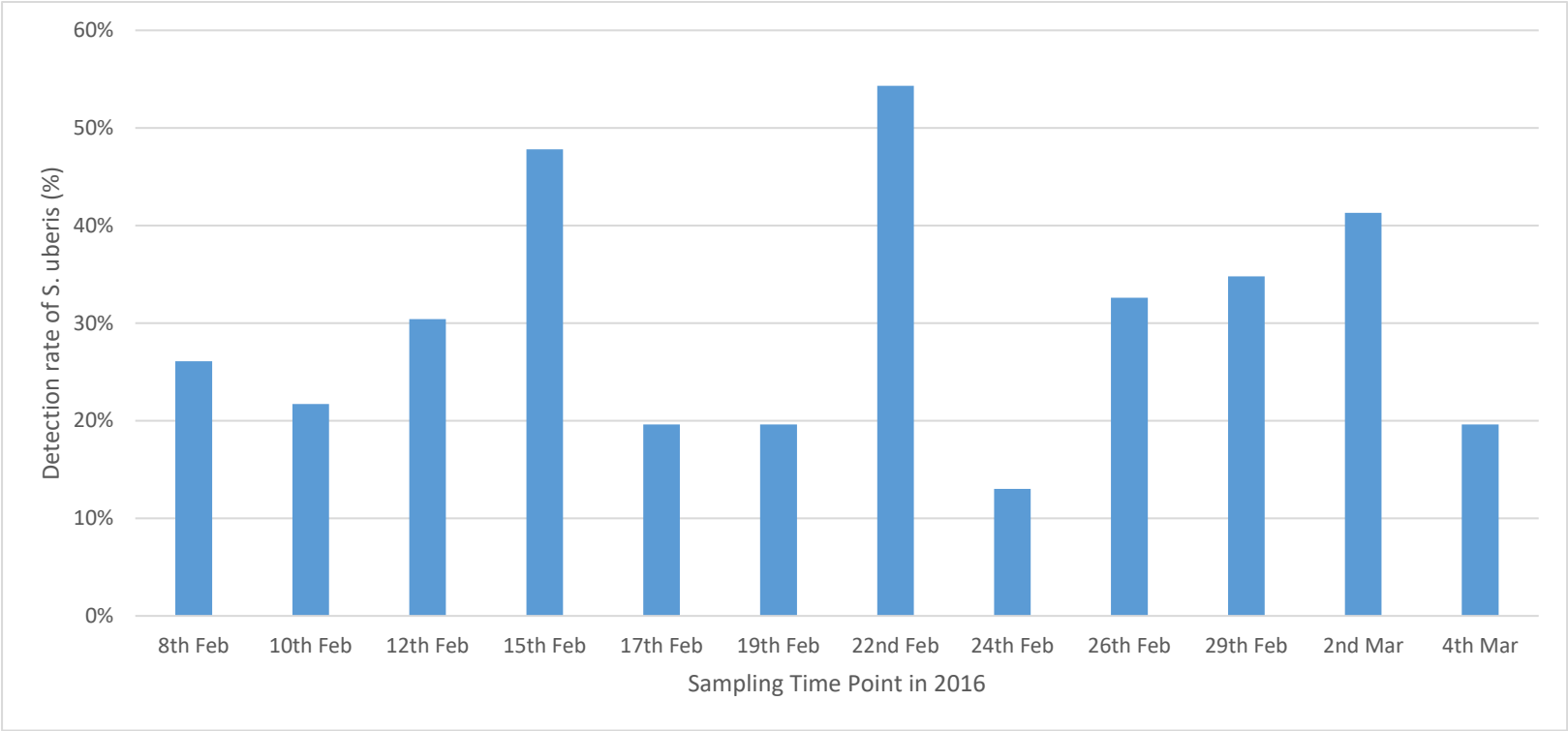



**Figure 3.6 Detection rate of *S. uberis* in the faeces of a cohort of indoor housed cows.**

A total of 46 cows were sampled over 12 time points (x axis), during the indoor housing period corresponding to colder climatic conditions. Faecal samples were cultured and the presence of *S. uberis* determined using *sub0888* specific PCR. The detection rate of *S. uberis* varied from 13% to 54.3%, with no apparent trend or pattern over the time course of sampling. .

**Table 3.7: Faecal detection rate of *S. uberis* from a cohort of 46 housed cows in Study 2.**

| | 8th Feb | 10th Feb | 12th Feb | 15th Feb | 17th Feb | 19th Feb | 22nd Feb | 24th Feb | 26th Feb | 29th Feb | 2nd Mar | 4th Mar |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Number of cows tested** | 46 | 46 | 46 | 46 | 46 | 46 | 46 | 46 | 46 | 46 | 46 | 46 |
| **Number of samples in which *S. uberis* was detected** | 12 | 10 | 14 | 22 | 9 | 9 | 25 | 6 | 15 | 16 | 19 | 9 |
| **Detection rate of *S. uberis* in faeces (%)** | 26.1% | 21.7% | 30.4% | 47.8% | 19.6% | 19.6% | 54.3% | 13.0% | 32.6% | 34.8% | 41.3% | 19.6% |
| ***Mean ambient temperature (◦C)** | 6.3 | 4.4 | 2.6 | 2.0 | 3.7 | 4.1 | 5.7 | 1.8 | 2.9 | 2.4 | 4.0 | 2.5 |
| **Range of ambient temperatures (◦C)** | 5.1-8.2 | 2.7-7.1 | -0.1-5.8 | -0.3-5.7 | 2.6-4.4 | -0.5-8.2 | 2.9-8.2 | -2.7-6.9 | 2.1-4.9 | -2.0-6.7 | 2.0-6.4 | 1.0-4.2 |
| **Mean humidity (%)** | 83.4 | 76.5 | 89.8 | 77.4 | 90 | 87.7 | 76.5 | 73.7 | 65.6 | 83.1 | 82.1 | 87.2 |
| **Precipitation (mm)** | 10 | 0 | 0.2 | 0.2 | 11.6 | 0.2 | 2.0 | 0 | 0 | 0 | 2.8 | 13.2 |

*Weather conditions for each day are given as retrospective values taken from Nottingham Weather Centre data set available for the specified date.

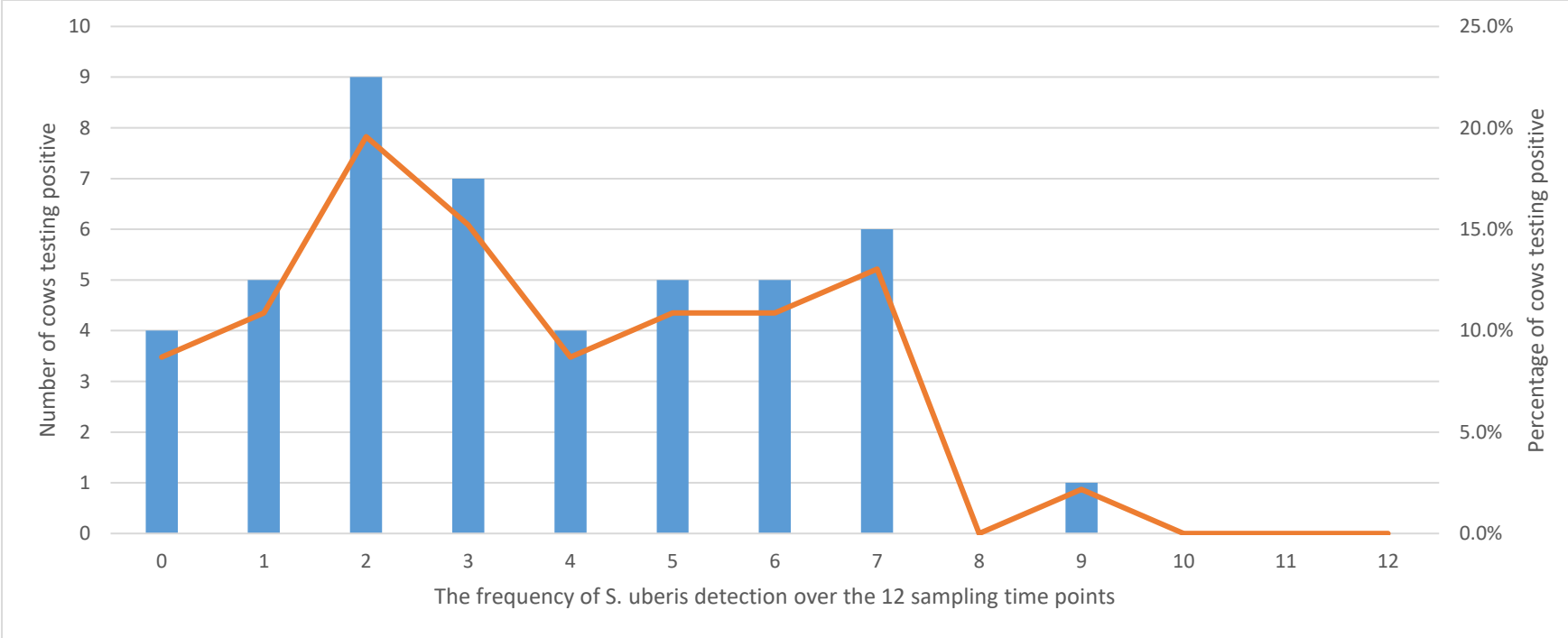



**Figure 3.7 Frequency of *S. uberis* faecal detection from indoor housed cohort cows in Study 2.**

Samples were collected over a period of 33 days in February and March 2016 and were tested with a species specific PCR for *S. uberis* targeting *sub0888*. The number of cows from which *S. uberis* was detected within their faeces are shown by the bars and read off the left hand y axis. The percentage of cows in which *S. uberis* detected in their faeces are shown by the orange line and read off the right hand y axis. *S. uberis* could not be detected in the faeces of cows at all 12 sampling time points; with the maximum number of times of detection found to be at 9 sampling time points for 1 cow. There were a total of four cows from which *S. uberis* was never detected.

median number of samples in which *S. uberis* was detected was three out of the 12 samples for an individual cow (mode =2, mean = 3.7).

#### 3.3.2.2 Statistical modelling: The Impact of Different Variables effecting whether *S. uberis* detection

Results of the multilevel logistic regression model are shown in Table 3.8. Observed non-significant variables included parity, days in milk, whether the cow was milking or not, whether the cow had been previously positive once at the previous test or in the previous two tests or whether the cow had ever tested positive in previous time points tested. There was an increase in odds of detecting *S. uberis* in the faeces of cows if animals were housed in a loose straw yard, compared to those housed on straw cubicles. There was also an increased odds of detection of *S. uberis* if the cows had tested positive at least once in the previous three tests, compared to if *S. uberis* had not been detected.

#### 3.3.2.3 Clinical Mastitis

Over the four week study period, there were 12 reported cases of CM in 12 different cows; of which 3 cows were part of the tested cohort of cows. This is the equivalent to 60 cases of CM per 100 cows per year. The presence of *S. uberis* was confirmed in four cases of CM, with one case occurring in a cow which was part of the cohort. The bacteriology results indicate that the main pathogens in this herd at this time point were environmental pathogens *S. uberis* and *E. coli*.

**Table 3.8: Parameter estimates from the final multilevel logistic regression model for S*. uberis* detection in faeces in Study 2.**

| Model Term* | Number of Samples | Odds Ratio | Confidence Interval | P |
|---|---|---|---|---|
| **Cubicle housing** | 326 | Reference | | |
| **Straw housing** | 226 | 1.77 | 1.13-2.76 | P<0.05 |
| ***S. uberis* was not detected in the previous three tests** | 159 | Reference | | |
| ***S. uberis* has been detected at least once in the previous three tests** | 255 | 2.28 | 1.37-3.77 | P<0.05 |

* non-significant variables which were also tested included parity, milking status (dry or milking), body condition score, leg cleanliness, udder cleanliness, detection of *S. uberis* in the previous immediate test or previous two tests or if the cow had ever tested positive in previous time points.

## 3.4 Discussion

The main aim of this chapter was to investigate the prevalence of detection of *S. uberis* in the faeces of dairy cows in a herd in the United Kingdom, over a long time period (study 1) and within a cohort of cows over a shorter time period during the housing period (study 2). Study 2 was designed after the longitudinal study (study 1) showed an increased rate of both *S. uberis* faecal detection and environmental clinical mastitis cases during the housing period. The aim of Study 2 was to address the question of whether the variability identified in long term detection rates was reflected e in the shorter term, as well as to investigate the potential impact of cow and management factors.

### 3.4.1 The prevalence of *S. uberis* detection in faeces within the herd over time

The mean herd level detection rate of *S. uberis* within cattle faeces over the 10 month longitudinal period was 18.2%, with a range between 10.2% and 33.2%, and the highest detection rate corresponded to the February 2015 sampling time point when cattle were housed indoors over winter. The detection levels were similar to the prevalence of *S. uberis* reported from a grazing herd in New York, which involved testing 107 samples between March and December 2003 (Zadoks *et al.*, 2005). Similar to the study presented here, the New York study identified a large range of detection rates observed between the sampling time points, however the whole herd was not sampled, only cows before and after calving. In

contrast, the mean detection level in the current study was three times lower than the mean detection level reported in a study of 100 grazing dairy cows in New Zealand, which involved testing 1600 samples collected from July 2003 to November 2004. There are multiple factors which may explain the differences in detection rates of *S. uberis* between the studies, including cattle genetics, geographical and climate differences, the study design and methods used for detection of *S. uberis*.

### 3.4.2 The impact of seasonality and cow location on the detection of *S. uberis* in bovine faeces

In this research, there was a significant seasonal trend in faecal shedding of *S. uberis* (Study 1), with an increased odds of detection rate in the winter months (December and February). A seasonality effect was reported in the New York Study, with an elevated detection rate reported in May to September in the warmer months, in complete contrast to this study and no significant impact of season in the New Zealand study (Lacy-Hul*bert et al*, 2005; Zadoks *et al.,* 2005a). A significant seasonal trend in the detection of *S. uberis* from different sites on and within the cow has previously been reported, which coincided with an increased detection rate during the winter months, in comparison to spring and autumn in another UK-based study (Buddle *et al.,* 1989), which mirrored the increased detection over similar time points in this study. However another larger study in New Zealand reported no obvious trends in terms of seasonality in the detection of *S. uberis* on the surface teats (Lacy-Hulbert *et al.,* 2005).

Therefore the exact impact of seasonality on the detection of *S. uberis* within the faeces and from different locations of a cow is currently unclear and may reflect differences in the environment, diet or meteorological conditions.

The wide range of detection rates which occurred over the 10 month period in Study 1 was partially explained by the housing environment of cows, with the mean prevalence in detection during the grazing period season and during the housing period found to be 11.2% and 29.3% respectively. The detection rate of *S. uberis* in the faeces of grazed dairy cows in Study 1 was double the mean prevalence detected in the New Zealand study, but was approximately half of the mean prevalence detected in the New York study. The only other study which has reported prevalence of *S. uberis* in faeces investigated 17 dry cows housed in a loose straw yard (Bramley *et al.*, 1978) with a reported prevalence of 32%. Given the time of this study and use of a culture based differentiation method, it is unlikely that *S. uberis* could have been distinguished from *Enterococcus* species and some *Streptococcus* species such as the SEBEC complex, (as discussed in section 2.4). This is the first reported study to the author's knowledge that has investigated detection rates of *S. uberis* of the faeces in housed cattle, with similar detection rates of *S. uberis* over the housing period in both Study 1 and Study 2 of 29.3% and 30.1% respectively. This suggests that the housing period resulted in a repeatable increase in the level of detection of *S. uberis* in cattle faeces in this particular herd.

The wide range of detection rates of *S. uberis* between the sampling time points was explained to some extent by the living environment of the cow. The variation between detection rates during the grazing season was 2.1% over the three grazing sampling time points. This differs from the New Zealand study, where a variation of 8.5% was detected, as well as the New York study, where a variation of 41% was detected between time points (Lacy-Hulber*t et al*, 2005; Zadoks *et al.,* 2005a). This suggests that the potential for external factors to influence the detection rates of *S. uberis* whilst the cows are grazed in this herd may have only small impacts on the detection rate of *S. uberis*. There was a variation of 7.8% between the two sampling time points during the housing period in Study 1 and 34.8% between the 12 sampling time points during the housing period in Study 2, which may suggest that there is the potential for external factors to have larger impacts on the detection rates of *S. uberis* and that the detection rate of *S. uberis* might be less stable. Another factor which may have impacted on the variation in detection rates is the stochastic effect of the swab sampling methodology, as Study 2 involved analysis of more samples per cow (12 samples per cow), in comparison to Study 1 (5 samples per cow).

The major risk factor identified in Study 1 for an increased odds of detection of *S. uberis* in cattle faeces was the living environment. The living environment was categorized as whether the cows were housed in either the loose straw yards or straw cubicles, or whether the cows were at

pasture. The impact of indoor housing the herd was an increased odds of detecting *S. uberis* in the faeces of 2.8, compared to cows out at pasture. These differences in detection rates may reflect potential differences in the ability of *S. uberis* to survive and replicate in the two different environments, in terms of the both actual numbers of replicating bacteria and stability within the environment over time. One study reported that *S. uberis* could not be detected on previously ungrazed pasture, however within 24hours of the introduction of cows to the pasture, the average number of *S. uberis* was found to be 28cfu/g at the level of the grass and a further 151 cfu/g at the level of the subsoil in winter. These were similar to the levels of *S. uberis* detected on pasture within 24 hours after cattle effluent was spread on the clean pasture, with no detection prior to spreading (Lopez-Benavides *et al.*, 2007). The detection rate of *S. uberis* on the soil was found to be longer in the warmer winter months, compared to spring and summer months. This indicates that pasture management may affect the survival of *S. uberis* on the pasture, which in turn could affect the prevalence of *S. uberis* detected in the faeces, assuming that *S. uberis* is transmitted faeco-orally between the environment and the cow.

There are very few published studies regarding the survival of *S. uberis* on different bedding substrates and within different housing systems. There was a difference in the odds of detecting *S. uberis* in the faeces in the cohort study between cows housed in the loose straw yard and the straw cubicles, with an increased odds of detection of 1.77 if the cows were

housed in loose straw yards (Table 3.8). This may reflect the differences between the cleaning of the housed environment, or the design of the housing system. The location of a cow when housed was not significant in the longitudinal study (study 1), whilst it was significant in the cohort study (study 2). This may reflect the shorter time period of sampling (four weeks compared to 10 months) or the increased number of sampling time points during housing in the cohort study of 12 sampling time points, compared to two sampling time points in the longitudinal study, in which the housing environment did not have a significant impact. The impact of bedding substrate has been reported to impact on the risk of *S. uberis* clinical mastitis, with a decreased odds of IMI due to *S. uberis* if cows are housed on sawdust bedding in comparison to straw (OR = 0.47, n = 111 cases). Another study comparing loose straw yards (n=12 cows) and cubicles (n=12 cows) showed that cows in straw yards had significantly increased SCC and a greater incidence of clinical mastitis, in comparison to cubicle yards, which was suggested to be related to the increased cleanliness of the cows in the cubicle housing (Fregonesi and Leaver, 2001). This may be related to the increased risk of the cow lying in faeces in a loose straw yard, compared to cubicle yard, where the cubicles are designed to prevent the cow from defecating in the area in which the cow lies down. Therefore further research is required into the impact of design of housing, in terms of structure, bedding substrates and environmental conditions for the replication and survival of *S. uberis*, given these studies suggest that persistence of *S. uberis* in straw bedded housing systems may be an

additional factor affecting the *S. uberis* faecal detection and could also be an additional a risk factor for *S. uberis* clinical mastitis.

The differences in the detection rates between the housed and grazed cows may also reflect differences in space availability for the cows. During the housing period, the available space per cow usually decreases when compared to grazing, which could result in a potential increased risk of the udder coming into contact with the environmental reservoir of *S. uberis*. The impact of space allowance for cows has been investigated in terms of effect on cow comfort, behaviour and overall production (Fregonesi and Leaver, 2002; O'Connel*l et al.*, 2010; Irrgang *et al.*, 2015) but to the author's knowledge there are very few studies published on the impact of space allowance on disease transmission in dairy cows. The impact of space allowance on hygiene scores in housed dairy cattle has been investigated, and in one study it was found that increasing the stocking density rate from 100% to 142% in a cubicle shed for a period of 14 days did not significantly alter the hygiene scores of the cows (Krawczel *et al.*, 2012). High hygiene scores, indicating dirty cows, have been reported to result in a greater risk of an elevated SCC (Schreiner and Ruegg, 2003) and given the few studies that have investigated this potential link, more research is required to investigate the impact of cleanliness on IMI.

The housing of cattle also has the potential to increase exposure cows to certain areas which may yield higher levels of *S. uberis* contamination including feed trough and collecting yards. Areas with a high volume of

cow traffic and lying areas have been found to have an increased probability of *S. uberis* detection from within the environment (Zadoks *et al.,* 2005a; Lopez-Benavides *et al.*, 2007) which suggests cattle management plays an important role in influencing the level of *S. uberis* within the environment. Previous research has shown that cubicle housed cows mainly defecate in feed alleys and whilst occupying a cubicle, which could potentially result in a decreased risk of contamination of the udder by faeces containing *S. uberis* (Villettaz Robichaud *et al.*, 2011). Whilst a research study has shown that housed cattle will try to avoid bodily contact with fresh faeces in the majority of instances (Whistance *et al.*, 2007), it may become more difficult for cows to do this in housed conditions compared to grazing, where the cows would generally have more available space. This is based on the assumption that the transmission of *S. uberis* to the mammary gland is through the faecal contamination of the teat.

The differences in *S. uberis* detection rates between housed and grazed cows may also reflect differences in the diet, which could affect the microbiota of the gastrointestinal tract, and alter the amount of *S. uberis* within the rumen and gastrointestinal tract. The main differences within the diets of these cows was the inclusion of fresh grass for the grazed cows, as well as a decrease in the amount of the partial mixed ration, in relation to the housed cows. The differences between the housed and grazed diets would lead to alterations in the amounts of different carbohydrates in the diets, and therefore different concentrations and ratios of volatile fatty acids

(VFA) produced from these carbohydrates by the rumen microbiome, changing the microbial population due to nutrient availability and environmental changes such as pH. *S. uberis* has previously been isolated from rumen samples (Cullen and Little, 1969) and therefore it is likely that the rumen environment and the diet of the cow could impact *S. uberis* survival and replication within the rumen. This has been highlighted in calf nutrition studies, where diets containing higher levels of fibre resulted in reduced shedding of *E. coli* (Tkalcic *et al.*, 2003) and another showing the numbers of the rumen commensal bacteria *Streptococcus bovis* (S. *bovis*) dramatically increasing when the rumen environment became more acidic (Herrera *et al.*, 2009). The impact of the diet on the faecal microbiome has been shown to increase the abundance of Fibrobacteraceae and Erysipelotrichaceae species as well as Firmicutes in cows fed a total mixed ration (TMR), compared to pasture fed cows (de Menezes *et al.*, 2011). These findings were similar to another study in beef cattle where the bacterial community composition correlated significantly with starch concentrations, largely reflected in changes in the Bacteroidetes, Proteobacteria, and Firmicutes bacterial populations (Shanks *et al.*, 2011) indicating the potential for altering the population of *S. uberis* due to dietary changes.

The other dietary impact which may impact *S. uberis* survival in the gastrointestinal tract and faeces is the effect on the rate of rumination and peristalsis with the amount of physically effective fibre in the diet reported

to increase the rate of rumination and to impact on the diurnal rumination pattern at high concentrations (Salfer *et al.*, 2018). The composition of the diet has also been linked to the rate of peristalsis of the gastrointestinal rate, with rumination time increasing as the percentage of dietary forage is increased (Maekawa *et al.*, 2002). The rate of peristalsis will also effect the consistency of the faeces, as increased peristalsis results in a decreased time for digestion and absorption, especially absorption of water. Therefore it is likely that the rate of rumination and peristalsis will have an impact on the faecal microbiota and that alterations in transit time within different parts of the gastrointestinal tract could have an impact on *S. uberis* survival.

### 3.4.3 The relationship between the detection rate of faecal *S. uberis* and the incidence of clinical mastitis

The relationship between the detection rate of *S. uberis* in the faeces and whether the cow had a clinical or subclinical IMI, caused by *S. uberis* or any other pathogen was investigated in both Study 1 and Study 2, with no significant correlation found in these studies. This may suggest that there is no predisposition or protection against *S. uberis* or any other IMI pathogens if a cow has *S. uberis* detected within the faeces. However there were only 55 cases of clinical mastitis on the farm, of which 12 were confirmed cases of *S. uberis* within the 10 month time period of Study 1. A sample size of over 33 cases of *S. uberis* would be required to determine a difference in the detection rates of *S. uberis* in the faeces of 10%, with a

significant probability (P<0.05) and a power of 80%. Given the low number of *S. uberis* infections confirmed on the farm, Study 1 was therefore under powered to identify any significant effect on detection of *S. uberis* in faeces and correlation with clinical mastitis cases. Similarly for the cohort study, 50 cases of *S. uberis* IMI would have been required to identify a significant effect. Whilst it appears likely that IMI rates increase during indoor housing of cattle, in comparison to those at pasture, further research is required to explore the relationship between *S. uberis* detection in cow faeces and *S. uberis* mastitis.

### 3.4.4 The impact of cow factors on the detection rate of *S. uberis* in bovine faeces

It is likely that study design provided another difference between the studies presented and those previously investigating the prevalence of *S. uberis* detection. In this longitudinal study (study 1), the whole herd was sampled at every time point and every animal selected as part of the cohort study was sampled at every time point. This included cows of varying stages of lactation, which differs from the New York study, which only sampled cows at drying off and calving (Zadoks *et al.,* 2005a), and the New Zealand study which did not sample cows for the first two months post calving (Lacy-Hulbert *et al.*, 2005). The statistical model developed for the longitudinal study highlighted that there was an increased odds of detecting *S. uberis* in cow faeces at the start of the lactation cycle. This may partially explain why the prevalence of detection of *S. uberis* in the

faeces of cows was higher in the New York study (Zadoks *et al.,* 2005a) and could be related to a number of factors including alterations in metabolism, immune response or gastrointestinal tract conditions in response to the physical demand of milking. This phenomenon has been reported with faecal shedding of *E. coli,* with lactating cows having an increased chance of shedding *E. coli* in their faeces, compared to non-lactating cows, as well as an increased detection rate of faecal salmonella if a cow is ≤60 days in milk (Fitzgerald *et al.*, 2003). The apparent decreased probability at 50 to 99 DIM and then apparent increased probability at 100 to 149 DIM was repeatable for different parity cows (Figure 3.2) and different cow locations (Figure 3.3), which occurs after the peak lactation of the cow. There is no immediate biological explanations as to the cause in this alteration of probability of detection, and therefore further research into whether this is a repeatable finding in other herds, as well as to a potential explanation is required.

In addition, it was found that multiparous cows had a decreased odds of detection of *S. uberis* compared to primiparous cows (odd ratio 0.69) in the longitudinal study which may be related to the energy demands placed upon the primiparous cows as these animals are yet to achieve a mature body weight, and require energy for this as well as milk production (Coffey *et al.,* 2006; Wathes *et al.*, 2007). The impact of parity has not previously

been reported on the detection rate of *S. uberis* in bovine faeces, nor on the prevalence of IMI due to *S. uberis.*

The impact of the metabolism and energy demands of the cow could also be a potential reason why over conditioned cows with body condition scores of 3.5 or above, had an increased odds of *S. uberis* detection. The use of BCS as a management tool to assess stored fat body can provide an indirect measurement energy balance. Over conditioned cows have been shown to have an increased risk of negative energy balance around the time of calving (Garro *et al.,* 2014; Macrae *et al.*, 2019), through mobilization of body fat stores and can lose weight when there is a high demand for energy for milk production. Alterations in BCS in individual cows between sampling time points was monitored in both studies, and found to be non-significant for *S. uberis* detection. This could be related to a lack of power within the study or it could suggest there may be another factor related to over conditioning of cows which results in an increased odd of *S. uberis* detection in faeces. This could relate to differences in dietary intake, as thinner cows tend to eat more than over conditioned cows, with one study reporting a difference of 24% in dry matter intake between a group of thin and over conditioned cows (Bines and Morant, 1983). Other metabolic changes which have been reported between thin and over conditioned cows including an enhanced rate of absorption of fatty acid precursors in thinner cows (Bines and Morant, 1983), an increased chance of insulin resistance and less consistency in the rates of

lipid, protein and carbohydrate metabolism (Weber *et al.*, 2016). The differences in the metabolism between cows with different BCS values would likely impact on the gastrointestinal environment and factors, such as the decreased rate of fatty acid precursor absorption, which may result in a more favourable environment for *S. uberis* survival and replication, or may reflect changes in the gastrointestinal microbiome for digestion. Whilst studies have not been published on the impact of cow BCS on gastrointestinal microbiomes, there are studies investigating the impact of obesity on the gastrointestinal microbiome in human studies. It has been shown the gastrointestinal microbiome has an association with the regulation of body weight, as the composition of the gut microbiome can result in differences in caloric extraction of ingested food substances (Ley *et al.*, 2005), with a correlation found in both mice and humans between the changes of Firmicutes species and obesity and percentage weight loss (Ley, 2010). However, whilst there is an impact of body weight on the gastrointestinal microbiome, causal relationships remain unclear.

The other cow related factor which had a significant impact on the *S. uberis* detection rate in the cohort study was detection in previous samples. This was in contrast to the lack of significance found in the longitudinal study where the sampling time points were further apart. This suggests that the gastrointestinal tract environment may provide favourable conditions for *S. uberis* survival and replication which may be last for a period of days, but not weeks. A previous study has investigated

the frequency of *S. uberis* detection within individual cows and found of 32 cows which were faecal sampled multiple times, only one showed *S. uberis* detection multiple times. The study also performed ribotyping on 17 isolated *S. uberis* faecal strains , with 59% of samples yielding multiple ribotypes and the authors concluded that their study did not support the role of persistent *S. uberis* faecal shedding cows in the spread of *S. uberis* through a dairy herd (Zadoks *et al.,* 2005a). In contrast another study reported the potential presence of cows which persistently shed *S. uberis* in faeces, with seven cows in a herd showing multiple *S. uberis* detection that contributed to 56% of the total monthly positive *S. uberis* samples, suggesting these cows may have a role in propagating *S. uberis* environmental contamination (Lacy-Hulbert *et al.*, 2005). A limitation of these studies was that the strains of *S. uberis* were not identified and therefore it is unknown whether it was the same strain/strains of *S. uberis* detected at each of the time point, which would indicate whether there may be gastrointestinal colonisation of the gut or potentially different strains of the bacteria cycling through the gastrointestinal tract.

### 3.4.5 The role of persistent shedders within the herd

There were a number of cows in both of the current studies from which *S. uberis* was never detected over the sampling time periods and which could potentially represent a group of cows which could be immune/resistant to colonisation by *S. uberis*. The role of potential *S. uberis* resistant cows has not been reported or discussed previously

within the literature, and theoretically, these animals could act as means of diluting the environmental contamination by *S. uberis*. The small sample size of genetically related cows in Study 1 meant it was not possible to investigate whether the genetics or familial relationships had an impact of the detection of *S. uberis* in study 1.

### 3.4.6 Study design implications

Another major difference between the studies discussed above and the current studies was the method of detecting *S. uberis* from the faeces. Other studies used culture based methods using colony morphology to identify *S. uberis* colonies, with a secondary molecular method used to confirm bacterial identity. The validity of these methods and the ability to compare the detection rates of *S. uberis* in the faeces between the studies is discussed in Chapter 2. With different detection methods used, it is difficult to compare studies as there will be different detection thresholds between the studies. The study in New Zealand grazing cattle used a rectal swab to obtain faecal material, in a very similar method used in the studies presented here, and similar detections rates were observed between the grazing seasons (Lacy-Hulbert *et al.*, 2005). The New York grazing study used 5 grams of faeces for culture and bacterial detection, which was shown to have an undetermined impact on the probability of identifying *S. uberis* within the faecal samples (section 2.3.3.2).

There were limitations to the longitudinal and cohort studies which were conducted, which included the inability to sample all the same cows at

each time point in Study 1, due to the logistics of using a commercial dairy farm, where cows were sold, culled and bought into the milking herd over the period of the ten months. There were also cows which were occasionally housed elsewhere at the start of the dry period, which resulted in some cows being absent from some sampling time points. The potential for selection bias through weighted sampling of certain groups of cows within a population was avoided in Study 1 by sampling all of the cows present in the herd at that time point, and by ensuring a representative range of parities and stages of lactation were present within Study 2. In these two studies, a faecal swab was used to collect samples due to the logistics of sample collection, testing a maximum of 0.2g of faeces per sample. Thus it is possible that the detection rates reported for both studies were underestimated compared to the true prevalence of *S. uberis* within the FVF.

Another limitation of this study was the inability to perform strain typing on the samples where *S. uberis* was detected. This was not possible due to the inability to isolate *S. uberis* as in pure culture (section 2.3.4). This meant that it was not possible to determine the number of different strains of *S. uberis* present within the faeces of a single cow, how those strains compared over time within one cow and between cows, as well as how the faecal strains compared to those attributed to IMI within the herd. This information may have helped to ascertain how important the presence of *S. uberis* in the faeces of cows is in terms of contamination of the

environment and the potential to cause IMI. Further research is required to develop a robust method of *S. uberis* identification from complex bacterial species found within cattle faeces.

Other limitations of the studies include the inability to generalize these to other dairy farms, as the studies were conducted on one herd in the UK and therefore would need to be repeated on different farms, which have different management and housing systems in order to understand farm and cow-related variation. The study was only performed over one year and not repeated, therefore it is unknown whether the impact of season would be repeatable. Therefore the results of these studies may not applicable to the population of dairy cows within the UK.

### 3.4.7 Conclusion

This chapter has shown that the detection of *S. uberis* in bovine faeces can be influenced by different management and cow factors, including the living environment of the cows. Further work is required to elucidate the underlying biological reasons as to why there was increased probability of detection of *S. uberis* in the faeces of cows when housed, compared to when at pasture, as well as to whether the impact of living environment is seen in different herds and across multiple housing systems. The impact of the management system has been hypothesed to potentially be due to a change in diet influencing the survival and potential replication of *S. uberis* in the gastrointestinal tract and faeces of the cow, differences in stocking density increasing the probability of faecal-oral transmission of *S. uberis*,

as well as the ability of *S. uberis* to survive in the different environments. The impact of stocking density has not been directly investigated, however there are suggestions within the literature that areas of high cow volume have higher probabilities of detecting *S. uberis* (Zadoks *et al.,* 2005a; Lopez-Benavide*s et al*, .2007)*,* which suggests that stocking density would impact on the probability of detection of *S. uberis.* The impact of *S. uberis* to survive within the environment has been investigated on pasture and in slurry (Lopez-Benavides *et al.*, 2007), however the survival of *S. uberis* on different bedding substrates used in the housed environments of cows is unknown; this will be discussed further in Chapter 4.

# Chapter 4. Survival of *S. uberis* on different bedding substrates

## 4.1 Introduction

*S. uberis* has been reported to be one of the major pathogens causing mastitis in dairy cows, both in extensive grazed herds (McDougall, 1998; Compton *et al.*, 2007) and indoor more intensive herds (Ward *et al.*, 2002; Unnerstad *et al.*, 2009). The risk factors for *S. uberis* in both systems worldwide have been investigated at the cow level, with risk factors such as very severe hyperkeratosis of the teat end, parity, breed and stage of lactation being reported to increase the odds of *S. uberis* clinical mastitis (Zadoks *et al.*, 2003; Breen, Green and Bradley, 2009; Taponen *et al.*, 2017). However there is limited research into the impact of different environments on the risk of *S. uberis* mastitis. Research investigating the risk factors for dry period origin clinical mastitis in 52 British dairy farms indicated there was an increased odds of clinical mastitis related to factors such as frequency of bedding application, frequency of scraping housing passageways and also housing design (Green *et al.*, 2007). These findings were in agreement with a study of 15 dairy herds in Ireland that highlighted a significantly greater prevalence of *S. uberis* within herds with suboptimal housing, compared to those with adequate housing (Barrett *et al.*, 2005). Herds were assigned to the suboptimal housing category if deficiencies were observed in cubicle number and cleaning, calving areas, exposure to

wind and rain, and prolonged periods spent on straw-bedded yards. These results suggest that environmental conditions are likely to have an impact on the survival of different mastitis pathogens and could potentially be manipulated to decrease the survival time and prevalence of the different bacteria.

The bedding conditions of housed dairy cows, including the substrate used, has been reported to be associated with the probability of mastitis (Rowbotham and Ruegg, 2016), for example, it has been shown that the use of sawdust has been associated with a decreased risk of *S. uberis* clinical mastitis (Unnerstad *et al.*, 2009). It has been postulated that this is due to reduced potential for direct contact between bacterium and the teats of the dairy cow (Hogan and Smith, 2012). Most bedding substrates which are used are organic by-products, such as straw and sawdust and have the potential to allow bacteria to survive and replicate, thus leading to a high environmental infection pressure. Different faecal streptococcal species have been isolated from different bedding substrates, with a recent study highlighting that there were $10^5$ to $10^9$ Cfu/g of Streptococcus spp and / or Enterococcus spp found in the bedding of dairy cows in the UK (Bradley *et al.*, 2018). This was also shown across nine commercial dairies in the USA (Hogan *et al.*, 1989). However, it is difficult to differentiate *S. uberis* from other environmental and faecal Streptococcal species, such as *Streptococcus bovis* and *Enterococcal*

species. It is therefore unknown as to whether these findings include *S. uberis* and, if so, in what proportions.

Other conditions, such as temperature and humidity, have been shown to have an impact on the quantity of *S. uberis* recovered from pasture based environments (Lopez-Benavide *et al.,* 2005), indicating that such conditions could also influence the rate of survival and/or replication of *S. uberis* within a housed environment. Differences between environmental conditions could have resulted in differences in the survival and replication of *S. uberis* in the loose straw yard and straw cubicle housing, resulting in different levels of environmental contamination of *S. uberis*, leading to different infection pressures. This could be one of the potential explanations for the significant difference in the detection rate of *S. uberis* in the faeces of cows in a loose straw yard, compared to cubicle housing. Information regarding the replication and survival of *S. uberis* in different environmental conditions could be used to indicate high risk environmental contamination and increased probability of mastitis due to *S. uberis*.

*S. uberis* is a nutritionally fastidious organism, requiring carbohydrates as a source of energy and between 10 and 13 amino acids to grow, which may or may not be present in different bedding substrates (Smith *et al.*, 2002). *S. uberis* strains have evolved different methods to survive environments which may be nutritionally challenging, as well environmental challenges. For example, it has been postulated that the ability of *S. uberis* to survive in the environment may be enhanced by

the presence of a hyaluronic acid capsule, as it would serve as a means of protection from desiccation (Oliver, Almeida and Calvinho, 1998). A typical methodology to investigate individual factors in a bacterium is to use strains containing mutations in the gene of interest, which may impact on the phenotype of the bacterium and its survival or virulence (Maloy, 2001).

The aim of this chapter was to investigate the ability of nine strains of *S. uberis*, five isolated form cases of bovine mastitis (including the strain 0140J), and four mutant strains of *S. uberis* each with a single genetic lesion, (derived from strain 0140J) to survive and replicate on three different bedding substrates.

This chapter will firstly describe a pilot study, which was designed to optimise the methodology including how to best recover *S. uberis* and appropriate sampling time points. It will then describe the experiment investigating the ability of five field isolates of *S. uberis* to survive and potentially replicate on different types of bedding, including bedding substrates which were not used by cows compared with used bedding material. The last section compares the survival of isogenic mutants of reference strain 0140J on clean bedding, to identify genes which may be important for *S. uberis* survival on bedding substrates.

## 4.2 Materials and Methods

All reagents used in this study were purchased from either Sigma-Aldrich (Dorset, UK) or Thermo Fischer Oxoid (Basingstoke, UK) unless otherwise stated. The esculin blood agar plates were purchased from Cherwell Laboratories (Bicester, UK).

### 4.2.1 Bacterial Strains and Growth Conditions

The *S. uberis* strains used were 0140J, EF20, Ab71, C6344 and 6780 (Hossain et al., 2015) (Table 4.1), as well as *S. uberis* strain 0140J mutants , including hasA (Ward *et al.*, 2001), vru (Egan *et al.*, 2012), srtA (Egan *et al.*, 2010; Leigh *et al.*, 2010) and mtuA (Smith *et al.*, 2003) (Table 4.2). Single colonies were selected from esculin blood agar, which had previously inoculated from frozen stocks (in THB containing 25% glycerol) of each *S. uberis* strain and cultured overnight 1ml BHI at 37°C. The overnight culture was centrifuged (13,000g x 3 minutes) and the cell pellet resuspended in 1 ml of saline, diluted 100 fold prior to inoculation of the bedding material with 1 volume of the diluted bacterial suspension (1ml to 3ml). The size of the initial inoculum was determined, in each case, by serial dilution and plate counts of viable bacteria on esculin blood agar.

**Table 4.1: Strains of *S. uberis* selected for investigating survival on different bedding substrates**

| *S. uberis* Strains | Genome Sequence Size (base pairs) | Year of Isolation | Isolated from | MLST Sequence Type |
|---|---|---|---|---|
| C6344 | 1907431 | 2002 | Mastitis (clinical) | ST5 |
| EF20** | 1933244 | 1970 | Mastitis (clinical) | ST55 |
| AB71 | 1849250 | 2002 | Mastitis (clinical) | ST11 |
| 6780 | 1960858 | 2000 | Mastitis (clinical) | ST29 |
| 0140J* | 1852352 | 1973 | Mastitis (clinical) | ST1 |

*Clinical virulent reference isolate (Ward *et al.*, 2009)

** Non-virulent isolate (Hossain *et al.*, 2015)

**Table 4.2: Strains of *S. uberis* inactivated mutants used to investigate the potential to survive on different bedding substrates.**

| Mutant Strain of *S. uberis* | Mutated Gene* | Coding Sequence annotation | Reference |
|---|---|---|---|
| **vru** | Sub0144 | Gene regulator | Egan et al, 2012 |
| **HasA** | Sub1697 | Hyaluronan synthase | Ward *et al.*, 2001 |
| **mtuA** | Sub0473 | Manganese transport protein | Smith *et al.*, 2003 |
| **srtA** | Sub0881 | Sortase A | Egan *et al.*, 2010; Leigh *et al.*, 2010 |

*gene number indicated in Ward et al 2009

### 4.2.2 Bedding samples

Bedding samples were collected from two dairy farms, one in Leicestershire and one in Derbyshire. Unused samples (referred to as clean) were collected from the on-farm storage areas, sawdust and straw was stored under cover, but sand was exposed (uncovered). Composite samples of bedding materials were collected from five cubicles in the lactating cow sheds for the samples of used sawdust and used sand. Approximately 200g of bedding material was collected from the top 5cm layer from approximately 0.5m inwards from the curb of the cubicle. These samples were then comingled and thoroughly mixed. A composite sample of straw was collected from five different areas in the lactating cow loose yard as above and were comingled, thoroughly mixed and chopped to approximately 2-3cm in length.

The bedding samples were autoclaved at 121°C for 15 minutes. One gram samples of each of the autoclaved bedding materials was mixed with 3ml of BHI, incubated overnight at 37°C and plated onto esculin blood agar to confirm sterility. All of the bedding samples used in each of the experiments were aliquoted as 0.5g per sample for straw and sawdust and 1.5g per sample for sand in 50ml sterile tubes (Falcon, Fischer Scientific, Leicestershire). Triplicate samples were analysed for all bedding types for each strain of *S. uberis* tested.

### 4.2.3 Sample Processing

Recovery of *S. uberis* from the bedding samples was performed by the addition of 9 volumes of maximum recovery diluent (1g peptone, 8.5g sodium chloride per litre) and incubation at room temperature for 10 to 20 minutes. The level of viable bacteria recovered was determined by 10 fold serial dilution of the recovered bacterial media, plating 50µl of each serial dilution onto esculin blood agar and incubation overnight at 37°C. Plate counts were performed after 24h incubation, with counts only being recorded if 5 or more colonies were present for each serial dilution

Single colonies were picked from esculin blood agar at the last time point at which *S. uberis* colonies appeared. These were cultured overnight in 1.5ml of BHI at 37°C and stored at -20°C following the addition of glycerol (25% v/v). Each culture was subsequently grown, DNA extracted and PCR conducted (see section 2.2.3.1 and 2.2.6) using *sub0888* primers for the confirmation of the presence *S. uberis.* Twelve colonies of each *S. uberis* mutant were selected and PCR conducted using the primers listed in Table 4.3 to confirm the genetic lesion of each mutant strain.

If no *S. uberis* were present on the plate count, 500µl of the maximum recovery diluent was added to 5ml of BHI and incubated overnight at 37°C. The overnight culture was subsequently plated onto esculin blood agar and cultured overnight at 37°C to determine the presence of any bacteria; identification of *S. uberis* was based on colony morphology.

### 4.2.4 Experimental Design

The pilot study was carried out using the sampling process discussed below, with a single sample analysed at each sampling time point. The sampling time points were 0 hours, 3 hours, 6 hours, 24 hours, 48 hours and 7 days. The initial inoculum of *S. uberis* in saline added to each sample was 3ml, with 27ml of maximum recovery diluent used for recovery of *S. uberis.*

The studies investigating the field isolates of clinical mastitis (0140J, EF20, Ab71, C6344 and 6780) and the isogenic mutants (vru, HasA, mtuA, srtA and 0140J) used an initial inoculum of *S. uberis* in saline added to each sample was 1ml, with 9ml of maximum recovery diluent used for the recovery of *S. uberis,* based on the findings of the pilot study. The sampling time points for the field isolates of clinical mastitis were 0 hours, 24 hours, 7 days, 14 days, 21 days, 28 days and 35 days, with individual triplicate samples of clean and used bedding substrates analysed at each sampling time point. The sampling time points for the isogenic mutants were 0 hours, 24 hours, 48 hours, 7 days, 14 days, 21 days and 28 days, with individual triplicate samples of clean bedding substrates analysed at each sampling time point.

### 4.2.5 Statistics

All data was captured and analysed in Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA). The 95% confidence interval of the mean of the triplicate samples was calculated at each time point in order to

assess whether the mean recoverable amount of *S. uberis* for each bedding substrate at each time point was within the confidence intervals of another bedding substrate and/or another sampling time point. A significant difference in the amount of recoverable *S. uberis* between two means was determined by the absence of overlap between the error bars of the 95% confidence interval of the mean.

**Table 4.3: Oligonucleotide primer sequences for identification of *S. uberis* mutants by PCR**

| Target | Sense Primer | Antisense Primer | Product Size (base pairs) |
|---|---|---|---|
| *S. uberis* 0140J *mtuA* | 5’-TTGCTTCCCATCTCCAAAT-3’ | 5’-CGAAACTTCTCCAAAAGCA-3’ | 2927 |
| *S. uberis* 0140J *vru* | 5'-CATCGAAGGGGGTCTCATTTA-3' | 5'-TTGAGAAAGCTGTCCCTGCT-3' | 692 |
| *S. uberis* 0140J *hasA* | 5’-GGTGGAGCGTGCTGCTCA-3’ | 5’-CAAAACGAATAGATGTATCAATCC -3’ | 550 |
| *S. uberis* 014J *srtA* | 5’-TGGTTGAAGCAGAAGCTGAA-3’ | 5’-GGCCACATGATGGATAGCAT-3’ | 950 |

## 4.3 Results

### 4.3.1 Pilot study to test recovery of *S. uberis* from bedding substrates

A pilot study to investigate the ability to recover *S. uberis* strain 0140J from different unused bedding substrates was undertaken, to inform the duration for subsequent more detailed studies. Each bedding sample was inoculated 3ml of saline containing ~ 7 x $10^4$ cfu *S. uberis* strain 0140J. *S. uberis* was recovered in pure culture from all bedding substrates in similar amounts (log 3 cfu) and remained similar over the period of 0 hours to 6 hours. The number of bacteria recovered from both straw and sand at 24 and 48 hours increased (Figure 4.1). *S. uberis* was recovered from sawdust at all time points within the first 48 hours of sampling, however it was not recovered on sawdust at 7 days. *S. uberis* was recovered from sand and straw at all sampling time points, however it was recovered at approximately $10^3$ cfu for time points 0 to 6 hours, which was less than the starting concentration of $10^4$ cfu. At 48 hours, *S. uberis* was recoverable at a similar concentration to the starting concentration. However at 7 days, *S. uberis* was only recoverable on sand and straw at a concentration of $10^4$ and $10^6$ cfu respectively.

The results from the pilot study highlighted that the methodology was robust for the recovery of *S. uberis* from the three bedding substrates. It also indicated that sampling the bedding substrates between the periods of 3 to 24 hour was of little value and therefore based on these

results it was decided to sample at time points 0 hours and 24 hours as the first two time points in subsequent experiments. It was also decided to decrease the inoculating volume to 1ml as it was hypothesised that the potentially low recovery of *S. uberis* may be related to the large inoculating volume of saline.

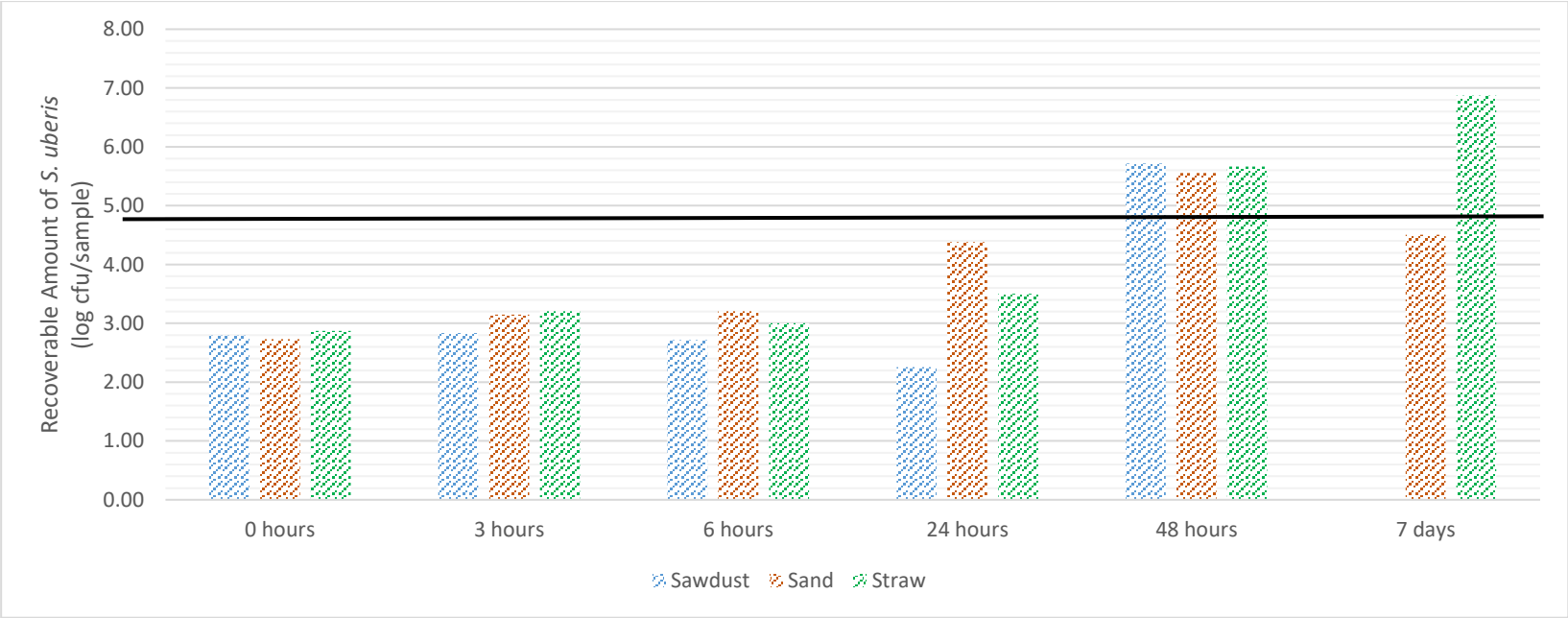



**Figure 4.1: Recovery of *S. uberis* on three bedding substrates at different time points.**

*S. uberis* 0140J was inoculated on sterilised sand, straw or sawdust in 3ml of saline and incubated at room temperature for various different time periods. The black line indicates the initial number of *S. uberis* used to inoculate bedding samples. The recoverable amount of *S. uberis* was based on the number of colonies which were grown on esculin blood agar after overnight incubation at 37°C. The bars indicate the survival of *S. uberis* on unused sawdust bedding (blue), sand (red) and straw (green)

### 4.3.2 Investigation of *S. uberis* to survival on three bedding substrates

The experiment was designed to investigate survival (and or growth) of different strains of *S. uberis* obtained from cases of bovine mastitis (Hossain *et al*., 2015) on commonly use bedding materials. In addition, the bedding substrates used included unused ("clean") samples and samples of used bedding materials. None of the five strains were recovered at or after 7 days incubation on clean or used sawdust, nor did any of the strains replicate on sawdust.

All five strains were successfully recovered for up to 35 days on at least one bedding substrate. *S. uberis* strains EF20, Ab71, C6344 and 6780 were recovered from the clean straw samples until Day 35 (

Figure 4.2, Figure 4.3, Figure 4.4, Figure 4.5 and Figure 4.6), whilst strain 0140J was only recovered up to Day 7. *S. uberis* strain 0140J was the only strain to be recovered from the clean sand samples at Day 35 (Figure 4.2), all other strains were recovered for at least 14 days post inoculation. There was a significant increase in the number of bacteria recovered on the clean straw bedding for strain C6344 at Day 1, 7, 14, 21, 28 and 35, in comparison to Day 0. This is suggestive of replication of strain C6344 on clean straw.

All five strains of *S. uberis* were recovered from the used sand and the used straw bedding samples up to day 35 post inoculation, with the exception of strain 6780, which was only detected after additional culture of the recovered samples in BHI at 37°C overnight (Figure 4.7).

This may reflect the lower starting concentration of *S. uberis* strain 6780 used ($10^3$ cfu/ml, compared to $10^5$ to $10^6$ cfu/ml for the other four strains). There was a significant increase in the number of *S. uberis* recovered for strains 0140J, EF20, Ab71 and C6344 at least one sampling time point over the 35 day time period on used straw, indicating replication of these strains on this bedding substrate. There was a significant increase in the number of *S. uberis* recovered for strains 0140J, C6344 and 6780 over the 35 day time period on used sand. *S. uberis* was recovered in greater numbers from used bedding samples compared to clean bedding samples at each sampling time point. The maximum recovery for the different strains was typically between $10^7$ and $10^8$ cfu for used bedding samples, whereas that for clean bedding was between $10^5$ and $10^6$ cfu.

This study was conducted over two different sections, with the first part investigating the survival on clean bedding and the second part investigating the survival on used bedding. The temperature was recorded using a temperature data logger during the 35 days of both studies. The median temperature was 22.0°C (mean 22.1°C), with a $25^{th}$ to $75^{th}$ percentile of 21.5°C to 22.5°C (range 19 to 29.5°C) for the clean bedding study. The median temperature was 22.0°C (mean 22.2°C), with a $25^{th}$ to $75^{th}$ percentile of 21.5°C to 22.5°C (range 19 to 27.5°C) for the clean bedding study.

Samples in which *S. uberis* was not recovered using the standard methodology, which had a detection threshold of 100 cfu, were enriched using BHI. This was performed in order to detect whether there was a

concentration of viable *S. uberis* still present but a concentration of less than 100cfu, which was the detection threshold of the standard method. This indicated that all five strains were still present on used sand bedding at low concentration at day 35 and all strains except 6780 were present on used straw at day 35. Strains Ab71, EF20, C6344 and 0140J were also present on clean sand at day 35, whilst strains Ab71, EF20, C6344 and 6780 were present on clean straw at day 35 (Figure 4.7).

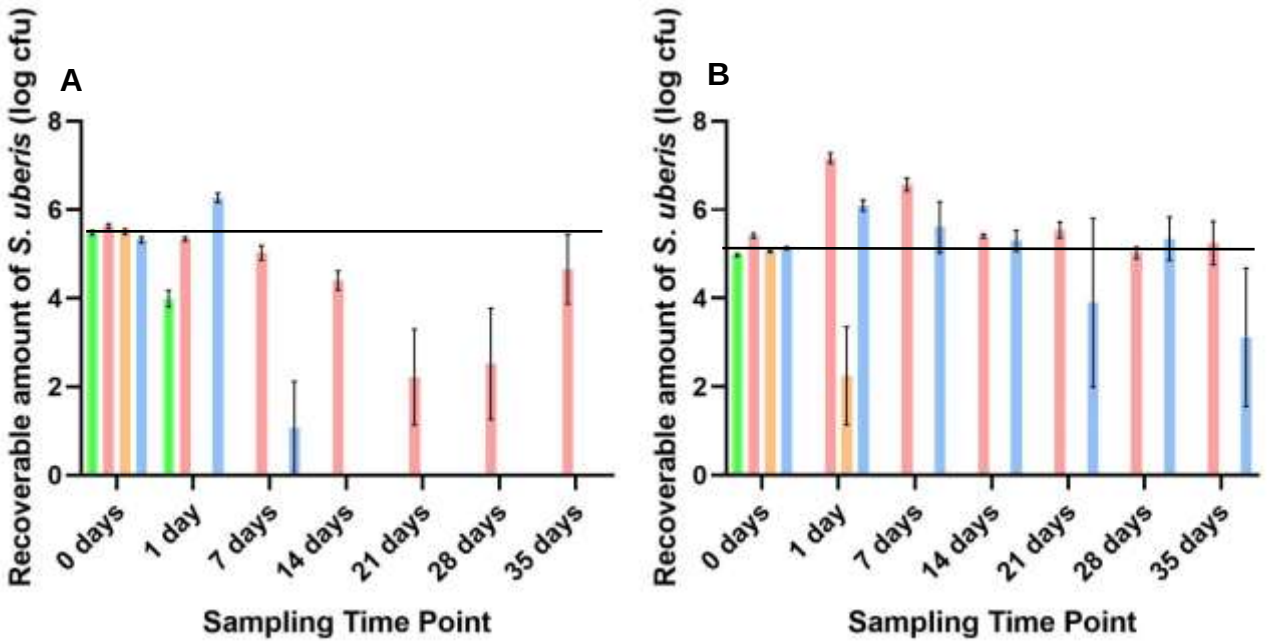



**Figure 4.2: Recovery of *S. uberis* strain 0140J on different clean and used bedding substrates**

The black line indicates the initial number of *S. uberis* used to inoculate bedding samples. The recoverable amount of *S. uberis* was based on the number of colonies which were grown on esculin blood agar after overnight incubation at 37°C. The bars indicate the survival of *S. uberis* on sawdust bedding (orange), sand (pink), straw (blue) and no bedding (green). The error bars represent the 95% confidence interval of the mean.

A: The mean survival of *S. uberis* strain 0140J on different clean bedding substrates over time (starting inoculum of log 5.6 cfu)
B: The mean survival of *S. uberis* strain 0140J on different used bedding substrates over time (starting inoculum of log 5.2 cfu)

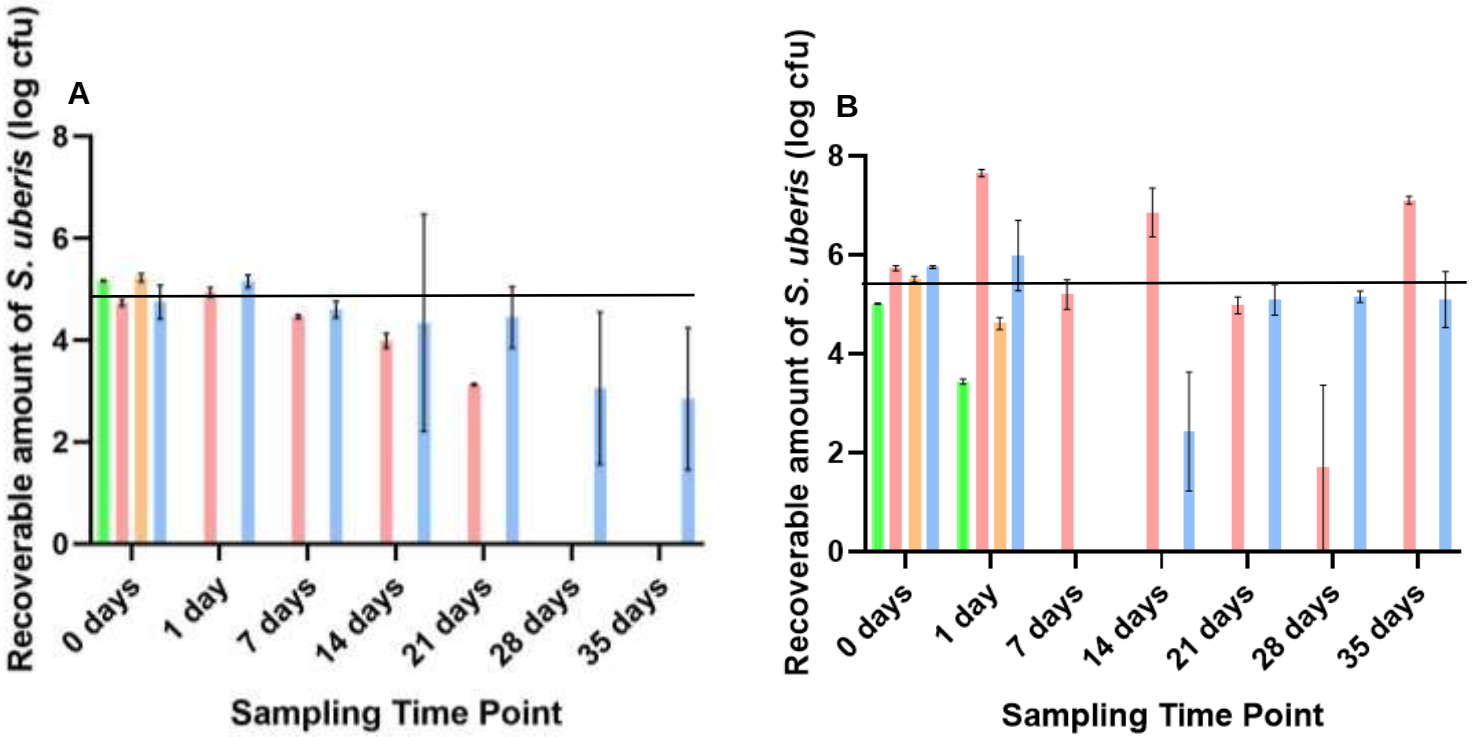



**Figure 4.3 Recovery of *S. uberis strain* EF20 on different clean and used bedding substrates**

The black line indicates the initial number of *S. uberis* used to inoculate bedding samples. The recoverable amount of *S. uberis* was based on the number of colonies which were grown on esculin blood agar after overnight incubation at 37°C. The bars indicate the survival of *S. uberis* on sawdust bedding (orange), sand (pink), straw (blue) and no bedding (green). The error bars represent the 95% confidence interval of the mean.

A: The mean survival of *S. uberis* strain EF20 on different clean bedding substrates over time (starting inoculum of log 5.5 cfu)
B: The mean survival of *S. uberis* strain EF20 on different used bedding substrates over time (starting inoculum of log 5.3 cfu)

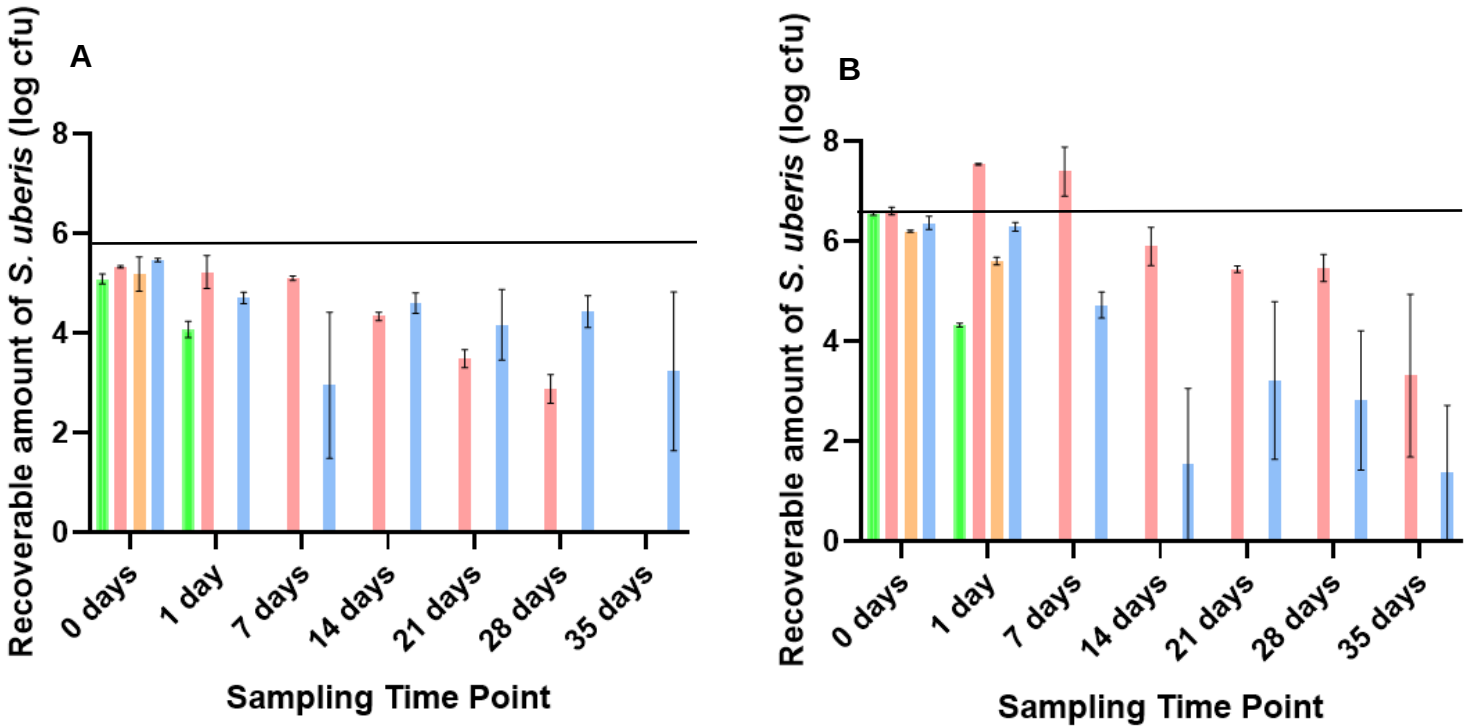



**Figure 4.4: Recovery of *S. uberis* strain Ab71 on different clean and used bedding substrates**

The black line indicates the initial number of *S. uberis* used to inoculate bedding samples. The recoverable amount of *S. uberis* was based on the number of colonies which were grown on esculin blood agar after overnight incubation at 37°C. The bars indicate the survival of *S. uberis* on sawdust bedding (orange), sand (pink), straw (blue) and no bedding (green). The error bars represent the 95% confidence interval of the mean.

A: The mean survival of *S. uberis* strain Ab71 on different clean bedding substrates over time (starting inoculum of log 5.9 cfu)
B: The mean survival of *S. uberis* strain Ab71 on different used bedding substrates over time (starting inoculum of log 6.5 cfu)

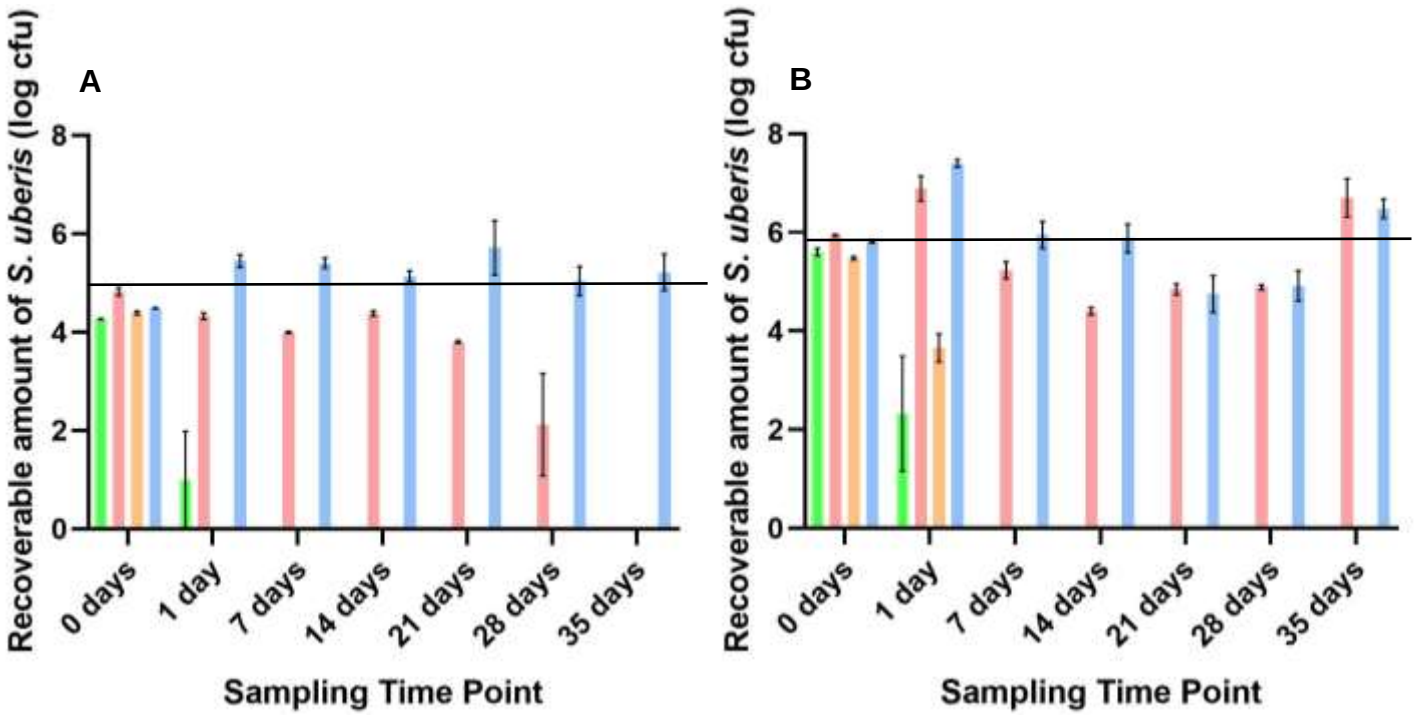



**Figure 4.5: Recovery of S. uberis strain C6344 on different clean and used bedding substrates**

The black line indicates the initial number of *S. uberis* used to inoculate bedding samples. The recoverable amount of *S. uberis* was based on the number of colonies which were grown on esculin blood agar after overnight incubation at 37°C. The bars indicate the survival of *S. uberis* on sawdust bedding (orange), sand (pink), straw (blue) and no bedding (green). The error bars represent the 95% confidence interval of the mean.

A: The mean survival of *S. uberis* strain C6344 on different clean bedding substrates over time (starting inoculum of log 5.0 cfu)
B: The mean survival of *S. uberis* strain C6344 on different used bedding substrates over time (starting inoculum of log 5.8 cfu)

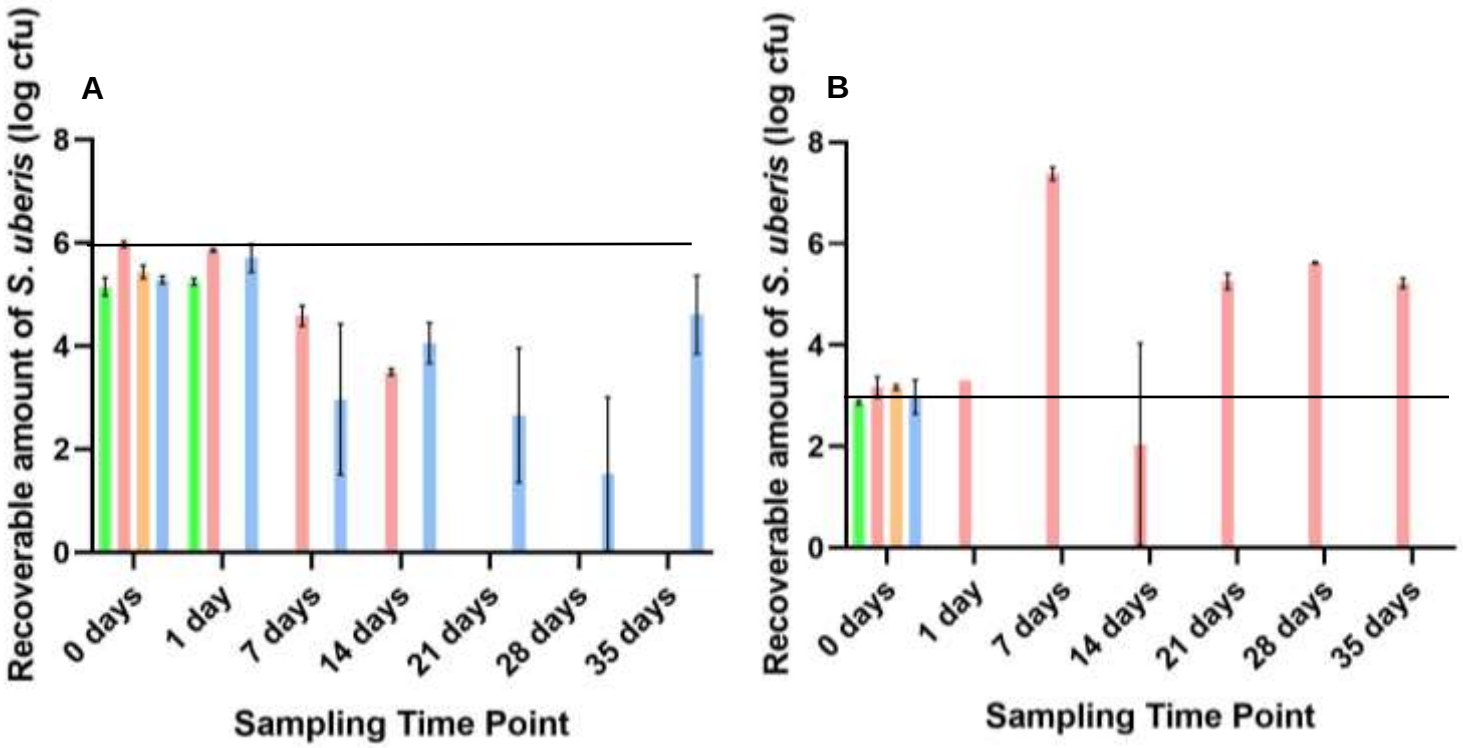



**Figure 4.6: Recovery of *S. uberis strain* 6780 on different clean and used bedding substrates**

The black line indicates the initial number of *S. uberis* used to inoculate bedding samples. The recoverable amount of *S. uberis* was based on the number of colonies which were grown on esculin blood agar after overnight incubation at 37°C. The bars indicate the survival of *S. uberis* on sawdust bedding (orange), sand (pink), straw (blue) and no bedding (green). The error bars represent the 95% confidence interval of the mean.

A: The mean survival of *S. uberis* strain 6780 on different clean bedding substrates over time (starting inoculum of log 6.0 cfu)
B: The mean survival of *S. uberis* strain 6780 on different used bedding substrates over time (starting inoculum of log 3.0 cfu)

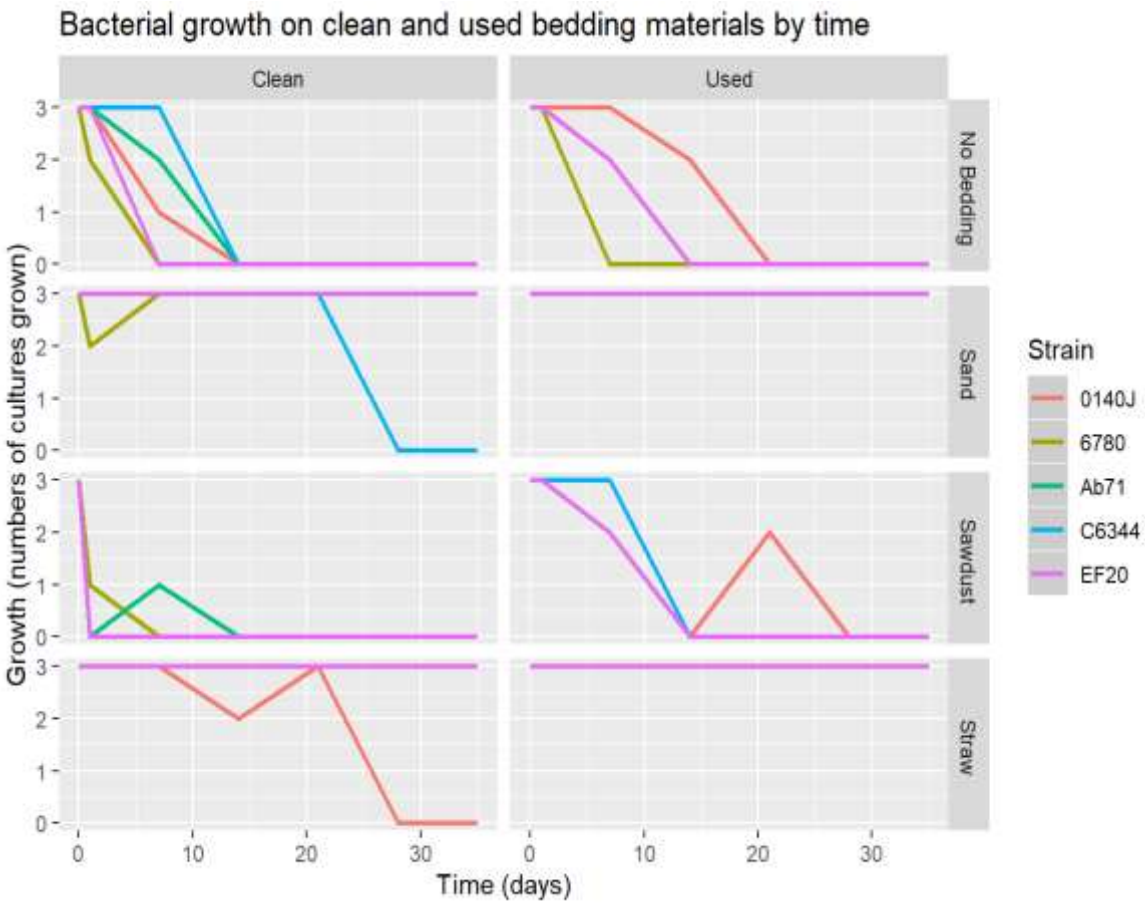



**Figure 4.7 Presence of *S. uberis* confirmed after overnight enrichment of bedding sampling with brain hearts infusion.**

Overnight culture of the triplicate bedding samples at each sampling time point was performed at 37°C, with the addition of 5ml BHI. The presence of *S. uberis* was confirmed by plating 5µl of the overnight culture onto blood esculin plates and incubating the plates overnight at 37°C. The identification of *S. uberis* was based on colony morphology. This was done to identify the presence of *S. uberis* which may be present at level below the detection threshold of the standard recovery method of 100cfu.

There were contemporaneous controls of tubes without bedding for the clean bedding and used bedding samples as the experiments were undertaken at separate time points and therefore under different environmental conditions.

### 4.3.3 The survival of different *S. uberis* mutants on different clean bedding substrates

This experiment was designed to investigate the impact of the absence of individual gene products in isogenic mutants of strain 0140J on the survival and potential replication on different bedding types compared to the wildtype strain 0140J. None of isogenic mutants or strain 0140J replicated on any of the clean bedding substrates over the 28 day sampling time period. None of the isogenic mutants were recovered from the sawdust bedding after the initial sampling time point of 0 hours, whilst strain 0140J was only recovered at the first two sampling points, up to 24 hours.

The wildtype strain 0140J was not recovered from clean straw after day 2 but it was recovered from clean sand at the last sampling time point of 28 days. Samples of which *S. uberis* was not obtained using the standard methodology were enriched by the addition of BHI. This indicated that strain 0140J was present on clean straw until Day 14, but at concentrations below the detectable limit of 100cfu (Figure 4.11).

The mutant mtuA was recovered at Day 28 on both clean sand and on clean straw (Figure 4.8). The mutant vru was recovered up to 14 days on both clean sand and straw (Figure 4.9). The enrichment with BHI indicated that mutant vru was present on clean sand at Day 28 and on clean straw until Day 21.

The strain srtA were recovered up to 14 days on both clean sand and clean straw (Figure 4.9) and was present on both bedding substrates

after enrichment until Day 28. The mutant hasA was recovered up to 14 days on sand and 7 days on straw (Figure 4.8). Enrichment using BHI indicated that hasA was present on clean straw and sand at Day 28 of sampling, but was not present on sawdust after Day 2.

The mutants had the same survival time on the clean sawdust as the wildtype 0140J, however there was a decreased survival time of the isogenic mutants in comparison to 0140J on clean sand bedding, except for mtuA. On clean straw bedding, there was an increased survival time of isogenic mutants compared to strain 0140J. The temperature was recorded during the 35 days of the study. The median temperature was 20.5°C (mean 20.5°C), with a $25^{th}$ to $75^{th}$ percentile of 19.0°C to 22.0°C (range 14.5 to 27.0°C).

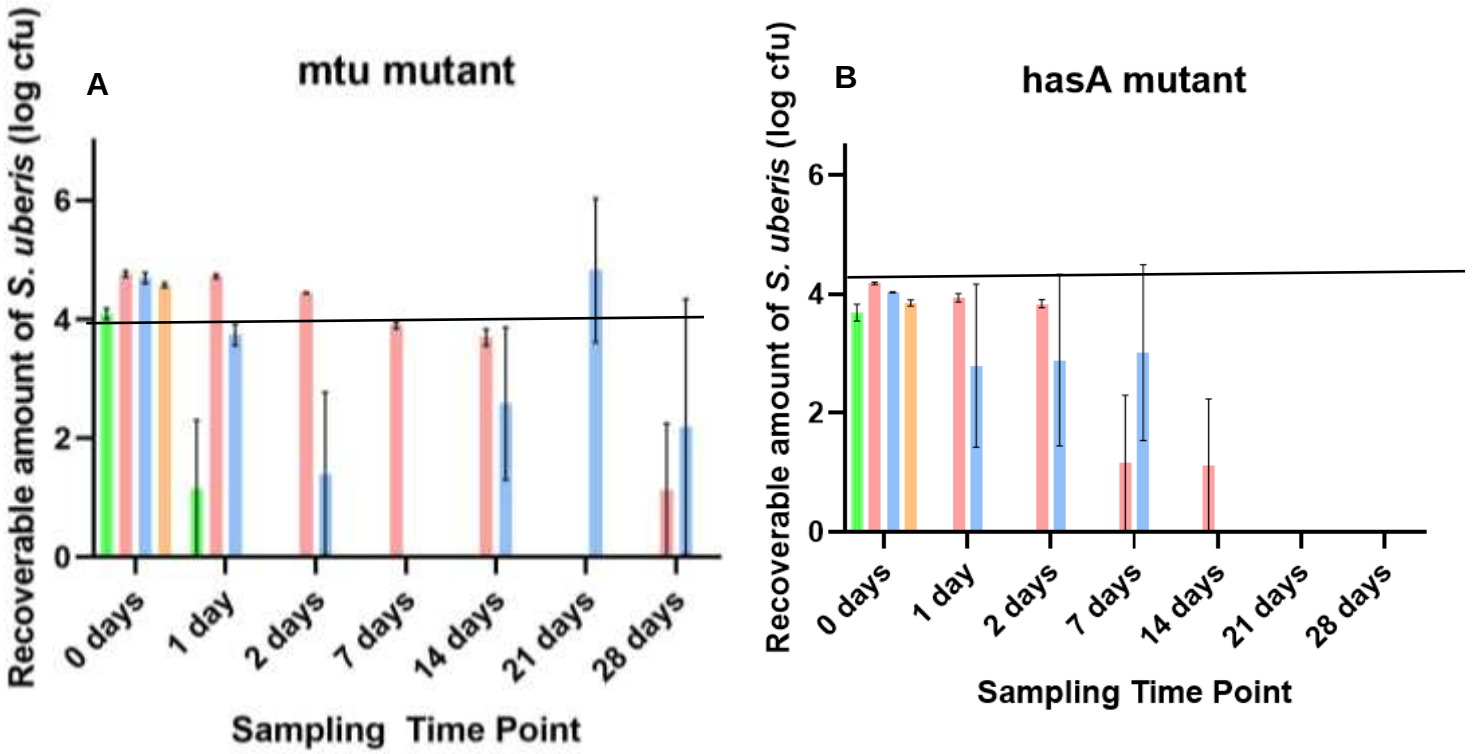



**Figure 4.8 Recovery of isogenic mutants mtuA and hasA on different clean bedding substrates**
The black line indicates the initial number of *S. uberis* used to inoculate bedding samples. The recoverable amount of *S. uberis* was based on the number of colonies which were grown on esculin blood agar after overnight incubation at 37°C. The bars indicate the survival of *S. uberis* on sawdust bedding (orange), sand (pink), straw (blue) and no bedding (green). The error bars represent the 95% confidence interval of the mean.
A: The mean survival of *S. uberis* strain mtuA on different clean bedding substrates over time
B: The mean survival of *S. uberis* strain hasA on different used bedding substrates over time

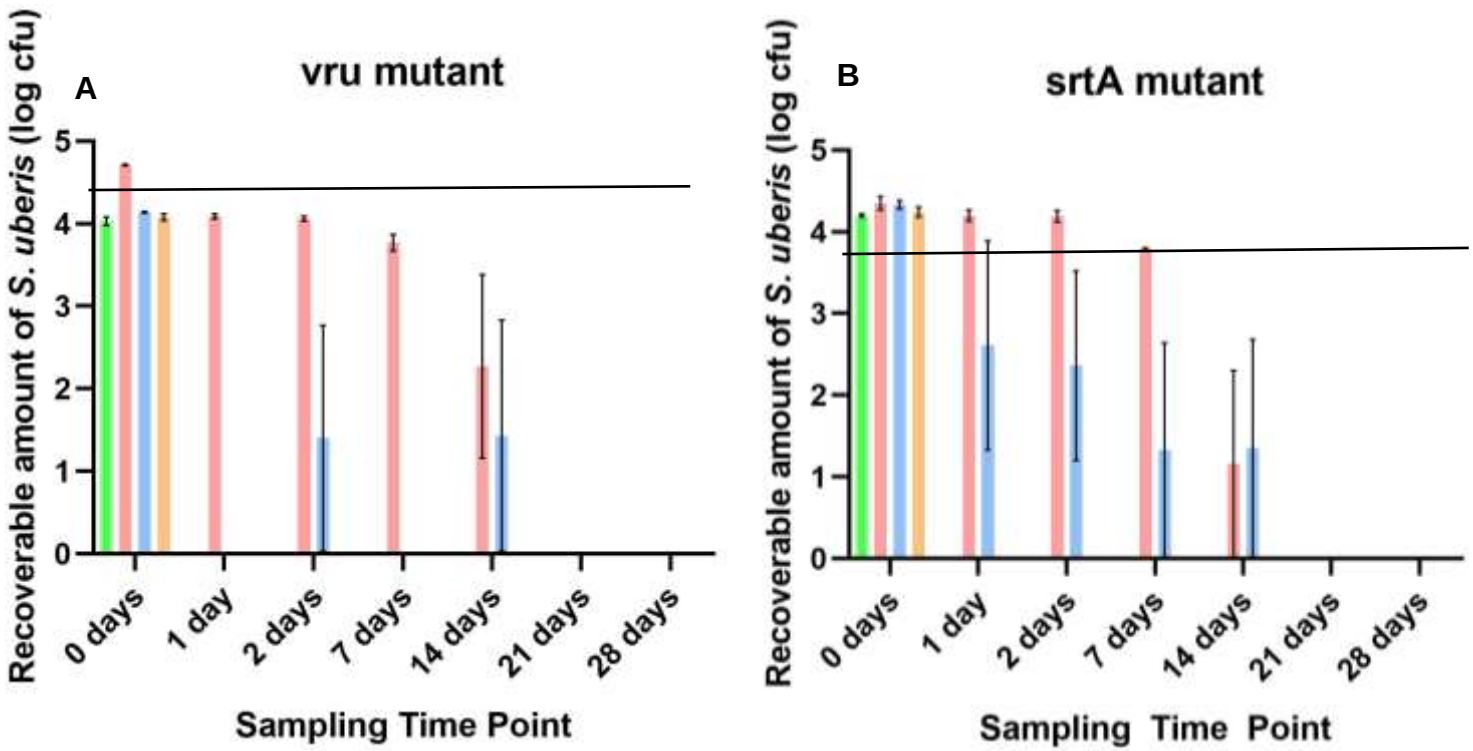



**Figure 4.9 Recovery of isogenic mutants srtA and vru on different clean bedding substrates**

The black line indicates the initial number of *S. uberis* used to inoculate bedding samples. The recoverable amount of *S. uberis* was based on the number of colonies which were grown on esculin blood agar after overnight incubation at 37°C. The bars indicate the survival of *S. uberis* on sawdust bedding (orange), sand (pink), straw (blue) and no bedding (green). The error bars represent the 95% confidence interval of the mean.
A: The mean survival of *S. uberis* strain srtA on different clean bedding substrates over time
B: The mean survival of *S. uberis* strain vru on different used bedding substrates over time

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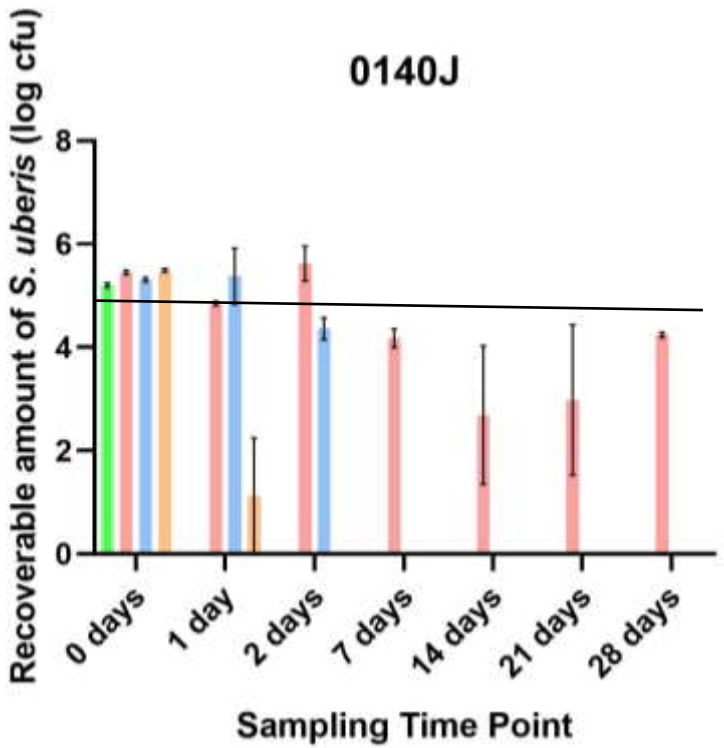


**Figure 4.10 Recovery of wildtype strain 0140J on different clean bedding substrates**

The black line indicates the initial number of *S. uberis* used to inoculate bedding samples. The recoverable amount of *S. uberis* was based on the number of colonies which were grown on esculin blood agar after overnight incubation at 37°C. The bars indicate the survival of *S. uberis* on sawdust bedding (orange), sand (pink), straw (blue) and no bedding (green). The error bars represent the 95% confidence interval of the mean.

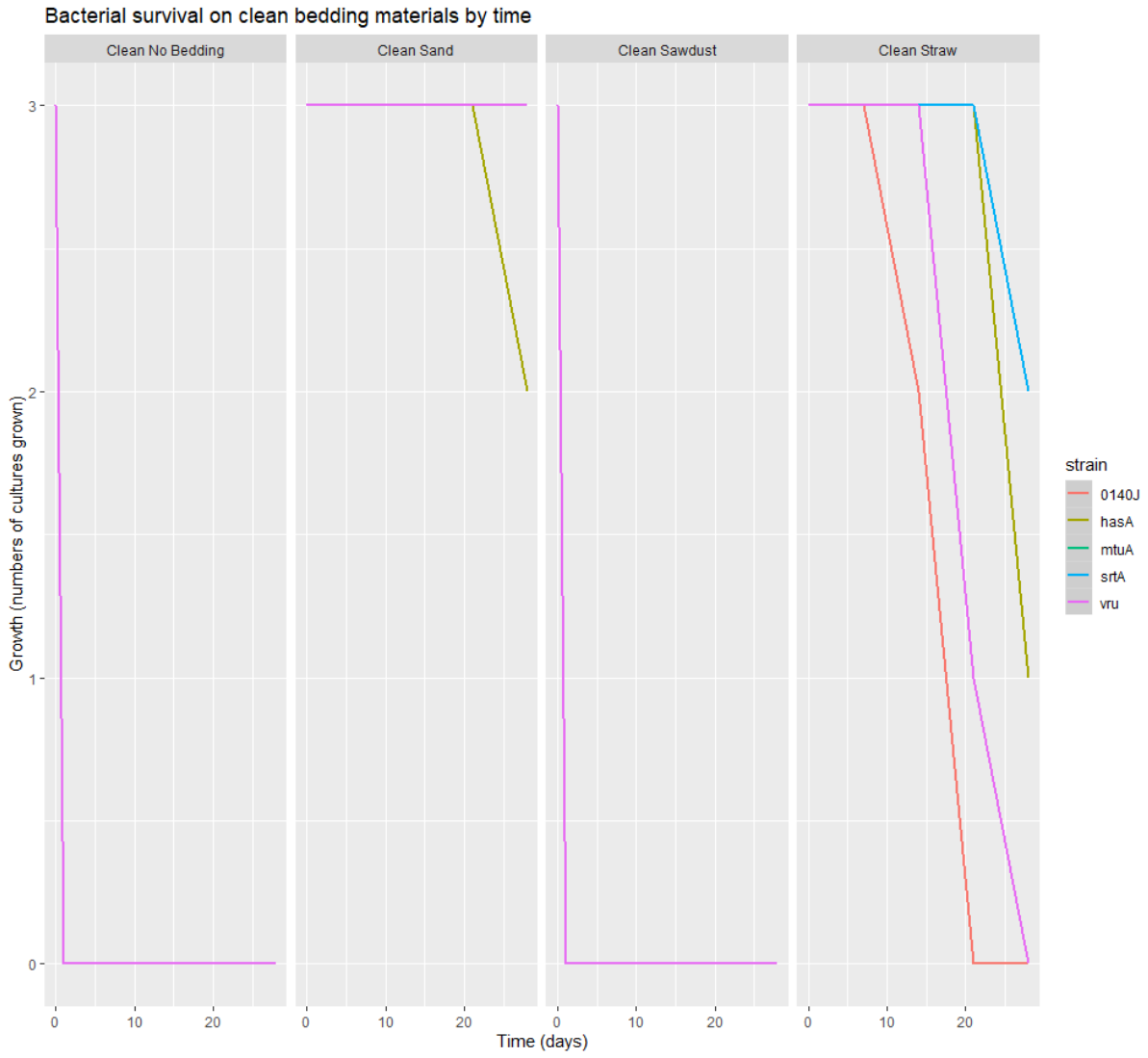


**Figure 4.11 Presence of *S. uberis* confirmed after overnight enrichment of bedding sampling with brain hearts infusion.**

Overnight culture of the triplicate bedding samples at each sampling time point was performed at 37°C, with the addition of 5ml BHI. The presence of *S. uberis* was confirmed by plating 5µl of the overnight culture onto blood esculin plates and incubating the plates overnight at 37°C. The identification of *S. uberis* was based on colony morphology. This was done to identify the presence of *S. uberis* which may be present at level below the detection threshold of the standard recovery method of 100cfu.

## 4.4 Discussion

The aim of this research was to investigate the ability of nine strains of *S. uberis*, five isolated from cases of bovine mastitis, including the strain 0140J, and four mutant strains of *S. uberis*, derived from strain 0140J, each with a single genetic lesion, to survive and replicate on three bedding substrates.

### 4.4.1 Survival of *S. uberis* strains on sawdust

The results indicated that none of strains of *S. uberis* survived for 7 days in either clean or used sawdust samples, which suggests that the bacteria may require an exogenous input of nutrients from other sources, or that there is something related to sawdust which is inhibitory to the survival of *S. uberis*. These findings align with clinical findings that reported that the use of sawdust bedding was associated with a lower risk of *S. uberis* mastitis (Unnerstad *et al.*, 2009). Prior research has shown the presence of *Streptococcus* species on sawdust could be dependent upon the time period in which it has been present in the cubicle housing. One study reported a recovery rate of $10^3$ cfu/g Streptococci from sawdust immediately after it had been placed in the cubicle, increasing to more than $10^8$ cfu/g after the bedding had been in place for 48 hours and Streptococci were recovered at levels greater than $10^8$ cfu/g six days after the fresh sawdust was applied to the housing (Hogan and Smith, 1997). However the Streptococci species were detected using the TKT/FC agar (BBL Microbiology Systems, Cockeysville, MD), which would not discriminate between *S. uberis* and *Enterococcus* species or *Streptococcus bovine – equinus* complex

(SBSEC). TKT/FC was originally developed to differentiate *S. uberis* and *Streptococcus agalactiae* from milk samples, based on differential haemolysis and hydrolysis of esculin (Smith and Johnson, 1972); however, both *S. uberis* and *Enterococcus spp* typically show similar properties (haemolysis negative, esculin hydrolysis positive) and similar colony morphologies.

The impact of different types of sawdust (derived from different wood) on the survival of *S. uberis* has not been reported in the literature. However research into the impact of the growth of *E. faecium* on different types of sawdust has shown that there were significant differences between the potential antibacterial effects of different types of sawdust, with *E. faecium* not recoverable after 24 hours on sawdust made from pine or larch, but it was still recoverable after 7 days on beach or poplar sawdust (Milling *et al.*, 2005). Previous research has shown that *E. faecium* has similar growth requirements to *S. uberis* (section 2.3.4.3) and therefore it is reasonable to suggest that there may be similar antibacterial effects on *S. uberis*. The overnight growth of *Streptococcus equi* and *Streptococcus zooepidemicus* on different sawdust substrates and chopped straw indicated that both species of *Streptococcus* had a lower recovery rate on the sawdust bedding substrates, in comparison to the straw bedding, with lowest recovery from pine derived sawdust (Yarnell et al., 2017). This may suggest that sawdust derived from coniferous tress may have greater antibacterial properties than deciduous trees, which agrees with research investigating the antibacterial effects of essential oils from conifer trees

(Hong *et al.*, 2004; Lee *et al.*, 2009). The sawdust used in this study was made from a combination of silver birch and great redwood. To the author's knowledge, there is no published literature on the antibacterial effects of either silver birch or great redwood sawdust, but this research suggests that it may have similar antibacterial properties as pine and larch sawdust, which correlates with the findings of this study.

The inability of *S. uberis* to survive on sawdust differs from *E. coli*; a highly prevalent mastitis pathogen in the UK. *E. coli* strain 0157 has been reported to survive in used sand and sawdust for more than 100 days (LeJeune and Kauffman, 2005). This may be related to different nutritional requirements of the bacteria, or differential resistance to sawdust derived inhibitor compounds. As the addition of potential nutrients, in the form of manure in the used bedding, did not have a significant impact on the survival rate of any of the strains of *S. uberis*. It is reasonable to hypothesise that it is an inhibitory property (as opposed to a lack of nutrient) of sawdust that results in a decreased survival of *S. uberis*.

One factor that may inhibit the survival of *S. uberis* is pH; the pH of sawdust has been reported to be pH 4 when clean increasing to ~pH 6.5 when contaminated during use (Milling *et al.*, 2005). The acidity of sawdust may have the potential to decrease survival of *S. uberis*, but it is also the bedding which is most likely to have the largest impact of the addition of alkaline urine and faeces on the pH of the microenvironment. Further research is required into the potential properties of sawdust that may inhibit the survival of *S. uberis*, as it could be utilised as a natural

**Table 4.4: Summary of significant growth of *S. uberis* field isolates over the 35 day sampling time period**

| Strain of *S. uberis* | Clean Sawdust | Clean Sand | Clean Straw | Clean Control (no bedding) | Used Sawdust | Used Sand | Used Straw | Used Control (no bedding) |
|---|---|---|---|---|---|---|---|---|
| **0140J** | - | - | + (Day 1) | - | - | + (Day 1) | +(Day 1, 7) | - |
| **EF20** | - | - | - | - | - | - | + (Day 1, 14, 35) | - |
| **Ab71** | - | - | - | - | - | - | + (Day 1, 7) | - |
| **C6344** | - | - | + (Day 1, 7, 14, 21, 28,35) | - | - | + (Day 1, 35) | + (Day 1, 35) | - |
| **6780** | - | - | - | - | - | + (Day 7, 21, 28, 35) | - | - |

+ represents significant growth compared to the recoverable amount at time point 0 hours ($P<0.05$);

- represents significant decrease in the amount of *S. uberis* recovered over the entire 35 days ($P<0.05$).

control method for reducing the concentration and survival of *S. uberis* in the housed environment of cows.

### 4.4.2 Survival of *S. uberis* strains on sand

Differences in survival time on clean sand were identified between the mastitis strains in this study. Strain 0140J was detected at levels of approximately 10,000 cfu/ml at Day 35, whilst the other strains did not survive on clean sand for more than 28 days at levels >100 cfu/sample. None of the strains increased in number on the clean sand. In contrast, all strains of *S. uberis* were recovered from used sand at day 35, with strains 0140J, C6344 and 6780 having increased in number over the sampling time period. Survival of streptococci and streptococcal-like organisms on sand has been reported from dairy farms in Wisconsin, USA, levels of $10^6$ cfu/g reported in new sand beds and $10^7$ cfu/g in deep recycled sand beds. These findings are in line with another study, conducted in the UK, where levels of $10^6$ cfu/g of *Streptococcus*/*Enterococcus* were detected in used sand bedding (Bradley *et al.*, 2018). One difficulty comparing the findings of these studies to those in this study is confidence that field studies were truly counting *S. uberis*, as on many bacteriological media it is very difficult to differentiate *S. uberis* from the other Streptococci and Enterococci (including modified Edwards media, Edward's media containing oxolinic acid and colistin KF streptococcus agar containing 2,3,5 triphenyltetrazolium chloride and TKT/FC agar) (Hogan and Smith, 1997; Ward *et al.*, 2002; Hogan *et al.*, 2007; Rowbotham and Ruegg, 2016; Bradley *et al.*, 2018). Therefore it is not possible to know with

certainty if *S. uberis* was present in these two studies and if it was present, at what concentration. The findings in the current study are similar to those that investigated the survival of enterococci in seawater and on sand (Hartz *et al.*, 2008). The study reported that enterococci showed increased survival on sand, in comparison to seawater, with survival on sand lasting for the duration of the sampling period (16 days). It was concluded that enterococci were able to replicate in the sand utilising associated organic matter as a source of nutrients, resulting in the hypothesis that sand could be an important reservoir for metabolically active organisms.

### 4.4.3 Survival of *S. uberis* strains on straw

In comparison to sawdust, the field isolates of *S. uberis* survived well on clean straw, with the exception of strain 0140J which was no longer recovered without enrichment after 7 days. All other strains were still recoverable on clean straw at day 35. None of the strains, except for strain C6344 increased in number at any time point (Table 4.4).

Straw bedding has been associated with higher prevalence of *S. uberis* mastitis (Unnerstad *et al.*, 2009). The survival of *S. uberis* has previously been reported on wheat straw, with *S. uberis* detected four weeks after the initial inoculation at a concentration which was >1,000 times less than the (unstated) starting concentration (Mackey and Hinton, 1990). This agrees with the findings of this study where strains 6780, C6344, Ab71 and EF20 were present at day 35, at a recovery rate which was lower than day 0.

Species differences between streptococci have been noted in terms of recovery rates from chopped straw, with *S. zooepidemicus* ($10^7$ cfu/ml) having higher recovery rates after 24 hours than *S. equi* ($10^4$ cfu/ml), following inoculation of each at $10^5$ cfu/ml (Yarnell *et al.*, 2017). This suggests that there are differences in survival and potential replication between different *Streptococcus spp* and could be related to different nutritional requirements or environmental adaptations between different streptococci species.

The reported composition of wheat straw, is approximately 57% carbohydrates, of which 44% is cellulose, 46% xylan and 27% lignin (Jurak *et al*., 2015). *S. uberis* has a diverse set of genes for carbohydrate transport and utilization within the genome, which provides *S. uberis* with the capacity to survive in complex host and environmental niches (W*ard et al*., 2009). This is partially related to *S. uberis* containing an expansion of glycoside hydrolyase family proteins 1, which catalyse the hydrolysis of glycosidic bonds between two or more carbohydrates or between a carbohydrate and non-carbohydrate moiety. This suggests that *S. uberis* may have the ability to utilise a wide range of different carbohydrates which are therefore unlikely to be a limiting factor for survival of *S. uberis* on clean straw.

Another aspect of the nutritional requirement of *S. uberis* is the need for certain amino acids. Previous investigations has revealed that some of the amino acids essential for growth of *S. uberis* differs between strains

(Kitt, 1998). The amino acid composition of wheat straw has been reported within the literature and is shown in Table 4.5 (Oosting *et al.,* 1995). Wheat straw theoretically contains all of the amino acids essential for growth for *S. uberis*, except for L-cysteine, the absence of which in a chemically defined medium was shown to significantly impact the growth of some strains of *S. uberis* (including strains 0140J and EF20, which were also used in this project) (Kitt, 1998). However, as strain EF20 survived better than strain 0140J on straw it is unlikely that the absence of cysteine alone can explain differences in survival on straw detect in this study.

**Table 4.5: Amino acid composition of wheat straw and comparison of essential amino acids for *S. uberis* strains 0140J and EF20**

| Amino Acids in Wheat Straw* | Essential for *S. uberis* 0140J# | Essential for *S. uberis* EF20# |
|---|---|---|
| Alanine | Undetermined | Undetermined |
| Arginine | Yes | Yes |
| Asparatic Acid | No | Undetermined |
| Cystine | Yes | Yes |
| Glutamic Acid | Yes | Yes |
| Glycine | No | No |
| Histidine | Yes | Yes |
| Isoleucine | Yes | Yes |
| Leucine | Yes | Yes |
| Lysine | Undetermined | Undetermined |
| Methionine | Yes | Yes |
| Phenylalanine | Yes | Yes |
| Proline | No | No |
| Serine | No | Yes |
| Threonine | No | Undetermined |
| Tryptophan | Yes | Yes |
| Tyrosine | Yes | Yes |
| Valine | Yes | Yes |

*From Hoseney, 1994

# From Kitt, 1998

Strains 0140J, EF20, Ab71 and C6344 were all recovered at Day 35 from used straw bedding and there was evidence of replication of these strains on used straw over the sampling time period. It is likely that the replication of the *S. uberis* strains on used bedding samples is due to the potential nutrient availability. One major difference between clean and used bedding is the presence of bovine faeces and urine on used bedding. Whilst the composition of the faeces is highly dependable upon many factors, including diet and individual cow factors, the composition of faeces has been reported to be 13.2% crude protein, 31.4% crude fibre, 5.4% ash, and 44.8% acid detergent fibre (on a dry matter basis) (Lucas *et al.,* 1975). This is different from the composition of straw, which is approximately 4% crude protein, 42% crude fibre, 6.6% ash and 45.1% acid detergent fibre. The addition of faecal matter to the bedding will increase the availability of protein. As discussed previously, the limited availability of certain amino acids has the potential to prevent growth and, possibly, survival of *S. uberis* in a strain dependent manner. Therefore, addition of faeces to the microenvironment of the bedding could have an impact on the survival and or replication of *S. uberis*. As discussed previously, addition of faeces and urine to bedding is likely to alter the pH of bedding material. Fresh cow faeces has been reported to have a pH of 8.5 (pH 7.4 if composted) (Irshad *et al.*, 2013) and bovine urine of pH 8. The pH of straw has been reported to be pH 7.5 to pH 9.5, with the deeper layers of the straw bed having a higher pH (Ward *et al.*, 2002), consequently

the impact of urine and faeces on the pH on straw bedding is likely to be minimal.

### 4.4.4 Replication of *S. uberis* on bedding substrates

There was a potential plateau effect demonstrated in terms of recoverable amounts of *S. uberis* with the field isolates on the bedding, where in this study the highest recovery rates were between $10^7$ to $10^8$ cfu for used bedding, suggesting it may have reached the maximal number of bacteria, which can be supported on this medium. The plateau effect observed may be due to either nutrient exhaustion or due to the ability of *S. uberis* bacterium to limit the cell growth to a certain density through cross cell signals. The levels of detection on both the used and clean bedding substrates was higher than that previously reported for pasture levels, which were between 1.7 and 660 cfu/g for paddocks (Lopez-Benavides *et al.*, 2007a). These differences may reflect differences in the sampling and diagnostic techniques, differences in the microenvironment including the presence of other microbes or differences in the strains of *S. uberis* present, as well as a comparison of in vitro versus in vivo experimental studies. It may also reflect the impact of other environmental factors such as ultraviolet light and temperature, which could impact on the survival and ability to replicate in different environments. The impact of different concentrations of *S. uberis* within the environment of the cow on the risk of the cow developing *S. uberis* mastitis is unknown, as currently the dose required to cause an intramammary infection with *S. uberis* in dairy cows is unknown. When directly infused into the udder, a

concentration of 1000 cfu of *S. uberis* can result in clinical mastitis (Hossain *et al.*, 2015); however direct infusion would bypass many of the defence mechanisms of the cow against intramammary pathogens, such as the teat sphincter. Research has shown that if a cow's teat is dipped in a concentration of $10^8$ cfu of *S. uberis,* there is a 50% infection rate of *S. uberis* (personal comms, Leigh) Therefore the significance of the ability of *S. uberis* to replicate to levels of up to $10^8$ cfu on used straw and sand are currently unknown in terms of the impact on the probability of transmission to the mammary gland.

### 4.4.5 Variation in the ability of different strains to survive and replicate on bedding substrates

Another reason for the difference in the survival and ability to replicate on the different bedding substrates of the different *S. uberis* strains could be related to genomic differences between the five strains of *S. uberis,* which have previously been reported (Hossain *et al.*, 2015). Differences between genomes of the five clinical strains was analysed using the genome comparator in the *S. uberis* BIGSdb (Jolley and Maiden, 2010), using *S. uberis* strain 0140J as the reference strain (Accession number AM946015). The analysis showed that there were only 124 coding sequences which were identical in all strains. There were 40 coding sequences which were present in strain 0140J, but absent from the other four strains. It is possible that there are differences in the exact alignment of these 40 coding sequences which are present in all five strains, which may affect the efficacy or function of the protein which they encode. By comparing the genomes to strain

0140J, it was not possible to investigate the potential roles or impacts of the additional genes which the other four mastitis strains may have, but are absent in strain 0140J. Therefore the process was repeated using the New Zealand strain NZ01 as the comparator genome (Accession number CP022435). There were five genes which were present in NZ01, EF20, C6344, Ab71 and 6780, which were not present in 0140J (Table 4.6). Potentially the most interesting of the five genes is the LysR family transcriptional regulator, which are reported to have the function of regulating a diverse set of genes, including those involved in virulence, metabolism, quorum sensing and motility (Maddocks and Oyston, 2008). However there are six other reported LysR transcriptional regulators present in all five mastitis strains, therefore the impact of the absence of the LysR transcriptional regulator at CGZ53_08260 is currently unknown.

As with all bacterial species, there are a large number of coding sequences within the *S. uberis* genome for which the function and role is unknown (Ward *et al.*, 2009; Hossain *et al.*, 2015; Hutchison *et al.*, 2016) and therefore it is highly possible that, coding sequences of as yet unknown function, account for differences in survival between strains.

**Table 4.6: Genes which were identified as absent from *S. uberis* strain 0140J but identified in comparative strains***

| Locus | Product | Sequence length (base pairs) | Genome position |
|---|---|---|---|
| CGZ53_04530 | hypothetical protein | 870 | 869803 |
| CGZ53_06745 | shikimate kinase | 567 | 1332705 |
| CGZ53_08250 | hypothetical protein | 333 | 1615307 |
| CGZ53_08255\|citC | [citrate (pro-3S)-lyase] ligase | 1047 | 1615858 |
| CGZ53_08260 | LysR family transcriptional regulator | 108 | 1617080 |

* Comparison performed using genome comparator tool on the *S. uberis* BIGSdb database and *S. uberis* strain NZ01 and identified to be present in EF20, Ab71, C6344 and 6780.

### 4.4.6 The role of genetic mutations in survival of *S. uberis* on different bedding substrates

The reason to investigate the survival of isogenic mutants of strain 0140J was to observe if there were any differences between the mutant strains compared to the wildtype strain 0140J, which might be attributed to the mutations present within their genomes. In addition, each of the genes investigated for bedding survival in the isogenic mutants were also present in the *S. uberis* EF20, Ab71, C6344 and 6780 strains, with varying levels of similarity in the gene sequences or regions upstream of the promoter region in the case of *vru* (Hossain *et al.*, 2015). Neither the wildtype strain 0140J nor the isogenic mutants replicated on the clean bedding substrates, which was similar to four out of the five field isolates of *S. uberis.*

There was a shorter survival time period on clean sand when compared to the wildtype strain 0140J, which was used as a positive control. However, all four isogenic mutants were present at Day 28, indicated by the ability to detect the strains after enrichment using BHI. The isogenic mutants had the same survival time period on sawdust bedding to 0140J, which was not surprising considering that the other four field isolates did not survive for 24 hours on sawdust bedding, without enrichment. The survival time period was longer for the mutant strains on clean straw, when compared to strain 0140J. The reason is to why this occurred is currently unknown.

The mutant strains were chosen for particular attributes, including their potential role in virulence, as well as availability. The hasA mutant lacks the ability to produce the hyaluronic acid capsule, which has been postulated to be related to the ability of *S. uberis* to resist phagocytosis (Leigh and Field, 1994). Capsule production is dependent on the Has operon, which is a cluster consisting of the *hasAB* gene and the *hasC* genes (Ward *et al.*, 2001), however bacterial pathogenicity in terms of mastitis was not affected by mutation of *hasA*, despite being over-represented in strains obtained from clinical mastitis (Field *et al.*, 2003). This led to other hypothesis regarding the purpose of the capsule, which includes the potential to prevent desiccation and promote environmental survival (Oliver, Almeida and Calvinho, 1998). The non-capsulated mastitis strain EF20 was used as a field isolate and did not appear to have a decreased survival time on any of the bedding types, when compared to the other four field isolates (Figure 4.3). The isogenic hasA mutant had a decreased survival time on sand, but an increased survival time on straw in comparison to strain 0140J; however it was present at day 28 after enrichment with BHI, suggesting that the lack of capsule did not influence bacterial survival.

The mtuA mutant has a mutation which results in the absence of the active transport system for manganese uptake. This results in the mutant only being able to gain exogenous manganese via passive diffusion and not being able to grow in conditions of low manganese, such as those found within milk directly affecting its replicative ability

and survival within the mammary gland (Smith et al., 2003). The mtuA mutant survived at levels >1,000 cfu/ml for 28 days on clean straw, which was an increased survival time in comparison to wildtype strain 0140J. MtuA survived for 21 days on clean sand, which was not dissimilar to the survival time on clean sand from wildtype strain 0140J. This suggests that clean straw and clean sand bedding environment provided adequate exogenous manganese, for replication and survival of *S. uberis*.

The vru mutant has an insertional mutation into the promoter region of the *vru* gene. Experimental challenge of dairy cattle with the vru mutant showed reduced ability to colonize the mammary gland and an inability to induce clinical signs of mastitis compared with the wild-type strain. The vru mutant has been shown to alter the expression of a number of coding sequences, including known and putative virulence factors, lactoferrin binding protein (Lpb), sclB, plasminogen activating factor (PauA) and hasA (Egan et al., 2012). Despite this, the vru mutant was able to survive in the clean sand and straw environment for 14 days. SclB has been shown to be involved in adhesion to fibroblasts and can bind host thrombin-activatable fibrinolysin (Leigh et al., 2010) Lbp is hypothesised to enable the *S. uberis* bacteria to acquire iron from the glycoprotein lactoferrin which is present within the milk (WeiHuan and Oliver, 1999). The virulence factor PauA also interacts with the host by activation of plasminogen (Rosey et al>, 1999). As all most of these virulence factors involve interactions with the host's immune system, it

is unlikely that these virulence factors are required for the survival of *S. uberis* in the environment.

The srtA mutant has an insertional mutation which disrupts the process by which surface proteins are anchored to the cell wall, due to the lack of the sortase A enzyme. Whilst there was a decreased survival time on clean sand and an increased survival time on clean straw compared to strain 0140J, the disruption of the formation of covalent attachment of surface anchored proteins did not appear to a large impact on the survival of mutant srtA, especially considering mutant srtA was detectable at day 28 after enrichment. The srtA enzyme substrates are still produced and likely to present around cell membrane, or secreted into the surrounding environment due to the presence of signal peptides on each substrate and are unable to be anchored to the cell wall peptidoglycan in the absence of the srtA transpeptidases. These substrates include lbp and sclB which are linked to the gene regulator vru and the sub1154/C5a peptidase like protein, all of which been shown to be essential for causing mastitis (Ega*n et al*., 2010; Leigh *et al*., 2010). However srtA itself and the additional 6 proteins that it anchors to the cell wall appear to be non-essential for survival of *S. uberis* on sand and straw.

### 4.4.7 Conclusion

The results from the experiment investigating the survival of the mutants suggests that none of the genes which were investigated has a

significant role in the survival of *S. uberis* on bedding, under the conditions tested. This was not surprising for the strains vru and srtA, as the mutated genes in these strains have been shown to be involved in virulence and interaction with the bovine host. The lack of capsule for both strains hasA and EF20 was interesting as it possibly suggests that the capsule has a different role than previously hypothesised as the presence of a capsule appeared to have a limited impact on the survival on different bedding substrates. *S. uberis* was still able to access manganese ions at a high enough concentration via passive diffusion from the environment, with limited restrictions on the growth of the mtuA mutant in bedding material. These findings, alongside the different survival rates on different bedding substrates for the mastitis strains, indicates that the environmental survival mechanisms of *S. uberis* strains are related to unknown components of the *S. uberis* genome and further research into the investigation of these mechanisms is required.

Whilst these results show that *S. uberis* has the ability to survive on straw and sand bedding up to 35 days in a laboratory setting, this may not be true for an field setting for several reasons. The bedding which was used had been autoclaved prior to use, to ensure that there were no other culturable bacteria present. This was because it would have been very difficult to differentiate the inoculated *S. uberis* strains from other bacterial species (particularly Enterococcus) which might have been present. However the impact of autoclaving the bedding has unknown consequences as it may have potentially altered the chemical

composition of the organic bedding, for example any proteins present would have been denatured and may have become more accessible for the *S. uberis* bacteria to utilise for metabolism. It would have also removed the microbiome, which would also have an impact on the ability of *S. uberis* to survive and potentially replicate. Previous research has shown antagonist interactions between other faecal pathogens, such as *Enterococcus faecium* (section 2.3.4.3) and non-aureus staphylococci (Wuytack *et al.,* 2019), do occur and are likely to impact on the survival of *S. uberis* in the faeces. Previous research also reported a synergistic relationship *S. uberis* and *E. coli* in uterine infections, which may also be applicable for the relationship between the two bacteria in faeces (Wagener *et al.*, 2014). The impact of autoclaving sand and growth of *E. coli* and *Enterococci* has shown a decrease in the concentration of bacteria recovered, in comparison to unsterilized sand (Hartz *et al.*, 2008). It was postulated that this was due to predation and competition with indigenous bacteria in the non-sterilised sand.

Another aspect of this study which may not be transferrable to the field setting is the temperatures at which the experiments were performed. The median temperature in the lab was 22°C for the field isolates and 20.5°C for the isogenic mutants. However whilst these temperatures differ from the air temperatures generally found on UK farms, research has shown that the temperature of the deeper layers of a straw yard usually are above 20.0°C (Figure 4.12) (Ward *et al.*, 2002). To the author's knowledge, there are no published studies reporting the

temperature of deep sand cubicles at different depths, nor of the temperature of sawdust cubicles. Sawdust is usually used as a bedding material on top of mattresses in a cubicle system in dairy cow housing. Therefore there is only a thin surface layer of the bedding applied and thus it is unlikely to reach temperatures above 20°C, due to the nature of its use and thus the temperatures used for the experiments are unrealistic for sawdust bedding in the field.

The results from this study indicated that there are significant differences of survival of *S. uberis* strains on different bedding substrates, with the survival and replication of *S. uberis* strains being significantly inhibited on sawdust, compared to straw and sand. There appeared to be strain differences in terms of ability to survive and replicate on straw and sand between strains, which suggested the presence of multiple genetic mechanisms which influence the survival of certain strains in different environmental conditions. The ability of *S. uberis* to survive on all bedding substrates was increased if the bedding substrate had been used by cows and that *S. uberis* strains have the ability to replicate on used bedding substrates, which suggested that the management of bedding could have a crucial role in the environmental ecology of *S. uberis.* The impact of bedding management on the presence of *S. uberis* within the environment was further highlighted as this study has shown that *S. uberis* strains can survive for at least 35 days on straw and sand, which is longer than the reported survival time of 14 days on pasture (Lopez-Benavides *et al*., 2007a).

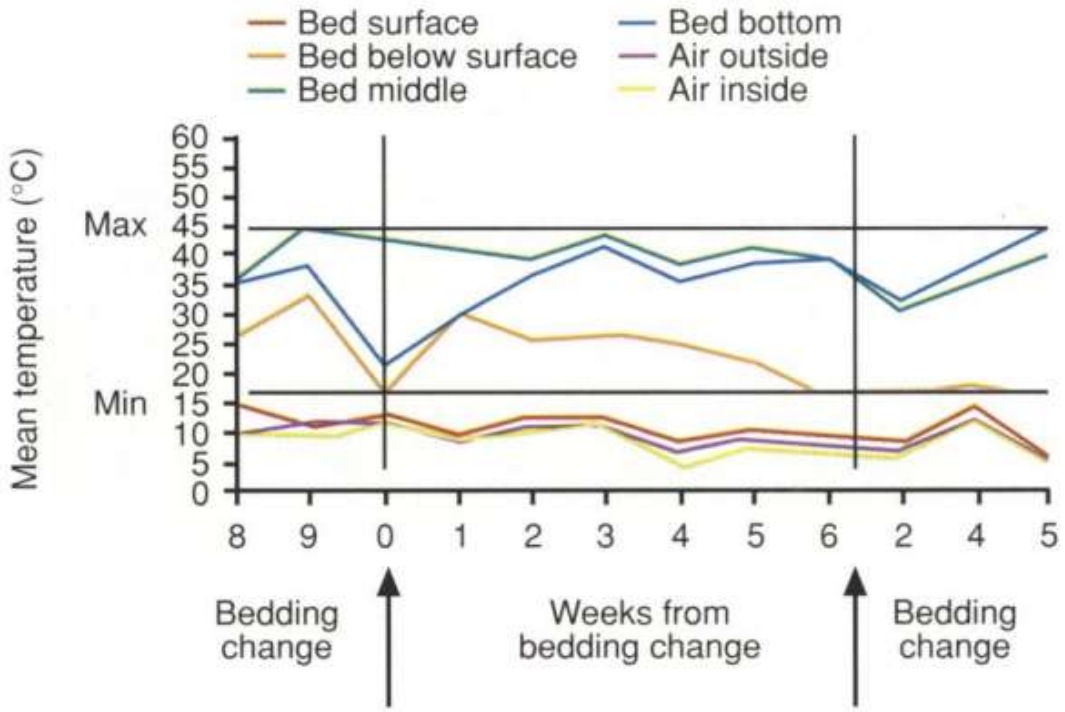


**Figure 4.12 : Mean temperatures on the surface of the straw bed of the early lactation cows**

Temperatures were taken 10 cm below the surface of the straw bed, in the middle of the bed, at the bottom of the bed, in the air outside the house, and in the air inside the house. The horizontal lines show the boundaries between the zones of temperature at which Escherichia coli and *S. uberis* survive (below 1 5°C), multiply (15 to 45°C) or die (over 45°C) (from Ward et al, 2002)

# Chapter 5. Discussion

The purpose of this research was to advance the understanding of bovine faecal carriage of *S. uberis* and to characterise different aspects of *S. uberis* within the environment. The research focused on the development of a reproducible PCR based assay and has investigated culture based methodology to explore some of these aspects, including the detection rates of *S. uberis* within cattle faeces over different time intervals and investigating the survival potential of *S. uberis* on bedding substrates used within the housed environment. There were three major themes which were highlighted by this research and warrant further discussion.

## *5.1* Understanding gastrointestinal carriage of *S. uberis*

This research highlighted that when using the PCR based molecular test for the detection of *S. uberis*, which had a high analytical sensitivity of 12 cfu, that *S. uberis* could be detected in bovine faeces from the majority cows within a herd. Whilst the method did not quantify the amount of *S. uberis* present, it could be extrapolated that if a cow produced an average of 50kg of faeces per day, then at the lowest level of detection, it may be the equivalent of a cow shedding at least 500,000 cfu per day, based on the assumption that *S. uberis* is homogenously spread within bovine

faeces. The use of swabs for sample collecting will have reduced the sensitivity of the detection of *S. uberis* from bovine faeces, due to the reduction in the quantity of faeces analysed per cow, thus resulting in the potential for false negative samples and an underestimation of the detection rate, however the additional resources required to culture larger faceal volumes were not practical in terms of sample collection and processing.

Given the PCR assay determined the presence of *S. uberis* by the detection of DNA and not viable *S. uberis* colonies, the quantity of viable *S. uberis* present within faecal samples is unknown using, as is the quantity of *S. uberis* within the environment required to result in a significant increase in the probability of *S. uberis* mastitis. The impact of detecting of low levels of *S. uberis* DNA within a small faecal sample in this research and the relevance to *S. uberis* mastitis is uncertain, however the correlation between increased detection of other bacterial species, such as *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in faeces has been reported to increase the odds of detection and also quantity of MAP within the environment (Sm*ith et al*., 2011). This suggests that the quantity of bacterial shedding could have an important role in transmission of disease which are traditionally categorised as environmental, such as *S. uberis* mastitis.

The infectious dose and related virulence capacity of *S. uberis* is also likely to vary from strain to strain, as indicated by the variation in virulence

between strains 0140J and EF20, both of which were isolated from cases of mastitis (Hossain *et al.*, 2015), although EF20 has never reproduced clinical disease in experimentally challenged dairy cows. Thus, the impact of different levels of environmental contamination and faecal shedding of different strains of *S. uberis* is unknown. The exact infectious dose required for different bacteria in different situations is difficult to determine as it has been reported to be affected by various different factors such as host genetics, immune response and age (Buchanan *et al.*, 2000; Leggett *et al.*, 2012; Mortier *et al.*, 2013), as well as strain type, with only some strains having a dose dependent effect for some diseases (Kinnula *et al.*, 2015; Vidlak and Kielian, 2016). The infectious dose of the gastrointestinal bacteria *E. coli* has been reported to be as low as 10cfu (Besser *et al.*, 2001; Cornick and Helgerson, 2004; Leggett *et al.,* 2012). A number of experimental infection studies using *S. uberis* 0140J have successfully induced mastitis with an infective dose of 1000 to 1500 cfu (Field *et al*., 2003; Hossain *et al*., 2015), however the exact infectious dose for this strain could be lower.

## 5.2 Importance of the environmental ecology on the survival and replication of *S. uberis*

One hypothesis is that whilst faecal material seeds the environment with *S. uberis,* the ability of *S. uberis* to survive and potentially replicate within the environment is more important in terms of an increased risk of mastitis. As highlighted within this study, the ability of *S. uberis* to survive for at least 5

weeks on a number of sterilised bedding substrates, may have significant implications for infection potential of individual strains from the environment. The ability to survive and replicate within the environment is likely to be affected by various interactions and despite research suggesting that a large percentage of mastitis cases are due to environmental mastitis pathogens (Table 1.3), very little is known about the impact of the environmental ecosystem of both housed and pasture dairy management systems on the risk of mastitis. Factors which could potentially impact on the environmental survival of *S. uberis* include climatic conditions, management of the environment and interactions within the microbiota of the ecosystem, all of which have been reported to impact on the survival of *E. coli* strains in manure and soil (van Elsas *et al.*, 2011) and impact on the survival time of *E. coli* within bovine environments (range: 25 to >231 days) (Fremaux *et al.*, 2008).

Research has indicated that there are synergistic and inhibitory relationships which occur between bacteria within mixed cultures, including between *S. uberis* and other bacteria (Wagener *et al.*, 2014; Wuytack *et al.*, 2019). The inhibitory relationship between *S. uberis* and *E. faecium* was highlighted in Chapter 2, which potentially has a significant implication for the ability to detect and isolate *S. uberis* from bovine faeces in a robust and repeatable manner, where the competitive environment and reproducibility times for both species may impact on the overall *S. uberis* bacterial numbers. The presence of bacteriophages and other predatory

microorganisms, such as bacterivorous *Vibrio spp*. have been reported to decrease the levels of autochthonous bacteria by 50% within a population of aquatic microbes in seawater (Byappanahalli *et al.*, 2012). Within bovine faeces, one example of predatory microorganisms are enterophages, which directly impact on the survival of *Enterococcus* spp., and have been reported at levels of $10^3$ to $10^5$ PFU/100ml (Purnell *et al.,* 2011). The presence and characterisation of phages in terms of *S. uberis* has previously been reported, including the presence of the phage putative lysin (Ply700) reported to result in rapid calcium-dependent lysis of *S. uberis* in milk (Celia *et al.,* 2008). Therefore, predation of *S. uberis* within bovine faeces and the environment is highly plausible and consequently the survival and replication of *S. uberis* on different bedding substrates in a laboratory setting may not reflect how *S. uberis* interacts within a field setting, due to these potential interactions. The role of synergistic and inhibitory relationships between bacterial species, especially *S. uberis* and *E. faecium,* requires further research, due to the implications for understanding diagnostic test results, as well as the potential use if targeted antibacterial properties are found.

## 5.3 Impact of cow and management factors on the detection of *S. uberis* in bovine faeces

There is the potential for re-seeding of the environment with *S. uberis* from the large quantity of faeces deposited by cattle on a daily basis and

therefore the survival and proliferation of *S. uberis* within the environment may not be a critical factor linked to *S. uberis* mastitis risk. This hypothesis is supported by a study which isolated *S. uberis* at higher concentrations from environmental samples with high levels of cattle presence and a notable decline in bacterial concentrations over time when cattle had been removed from the environment (Lopez-Benavide*s et al*., 2007). This suggests that the presence of cattle and regular seeding of the environment may be more important in terms of *S. uberis* environmental contamination, than its ability to survive and replicate within the environment.

### 5.3.1 Factors that influence shedding of *S. uberis* in faeces

The environment of cattle and individual cattle factors were found to increase the odds of *S. uberis* detection, however a number of additional factors may have also contributed, which were unable to be measured within this study. One of the largest potential factors was the genetic variation of the cattle, which may impact on the presence and survival of *S. uberis* within the gastrointestinal tract. All of the cows within this research study were Holstein-Friesian crosses and whilst sample size was too small to perform any breed or familial analysis, variation between the gastrointestinal and faecal microbiomes of cattle has been reported between cows in some studies (Dowd *et al.*, 2008a; Mao *et al.*, 2015). Genetic variation in terms of breed differences has been shown to have an

impact on the presence of faecal shedding of *E. coli* in beef cattle, however the significance of the differing gut microbiomes and genetics upon this detection was unknown (Jeon *et al.*, 2013). The human gut microbiome has been reported to be affected by numerous factors, with specific human genetics found to contribute to differing microbiomes and impact on the perceived overall gut health (Goodrich *et al.*, 2014; Bonder *et al.*, 2016; Lim *et al.*, 2017). Therefore it is likely that individual and herd genetics has a role to play in *S. uberis* within the gut microbiome of cows and warrants further investigation.

The influence of cattle diet on the gastrointestinal tract environment and the presence of *S. uberis* also warrants further investigation, as research into different bovine diets and the gut microbiota has reported a significant impact of diet on the microbiota, for example a diet containing an increased grain content resulted in increased abundances *Selenomonas ruminantium*, *Streptococcus bovis*, and *Prevotella bryantii* in the rumen (Fernando *et al.*, 2010). The impact of nutrition and its manipulation is likely have a role in the constitution of the gut microbiota in cows, especially considering the host–microbe symbiosis that exists in ruminants, which is crucial for meeting energy and essential nutrient needs of the cow.

Environmental temperature may also play a role, with studies reporting a significant positive association with isolation rates for *Streptococcus suis* (*S. suis*) in pigs, including impacting on the prevalence of key virulence genes within the *S. suis* strains isolated from pigs at different

environmental temperature, as determined by MLST. (Zou *et al.*, 2018). The temperature and humidity within the immediate environment and ultraviolet light intensity have also been reported to impact the survival of Enterococci *spp.*in faecal deposits (Byappanahalli *et al.*, 2012), which suggests that climatic conditions, including temperature, can impact on environmental survival of bacteria and alter the rate of detection of some species of bacteria, as well as the phenotypic expression of genes, in both the animal's environment but also within the animal itself.

Research using stimulated rainfall has also shown that climatic conditions have an impact on dispersal of microorganisms from the faecal deposit, including microorganisms such as *Cryptosporidium parvum* oocytes, which could impact on transmission (Davie*s et al*., 2004). However it has also been hypothesed that the faecal deposit can act as a protective environment for bacterial survival against desiccation (Byappanahalli *et al.*, 2012) and therefore dispersal from the faecal deposit may be detrimental to *S. uberis* survival, which could decrease the probability of *S. uberis* transmission.

Other environmental factors including stocking density have been shown to have a significant impact on the detection of different faecally transmitted bacteria, including an increased prevalence of *Salmonella* detected in pigs stocked at higher densities (Funk *et al.,* 2001), as well as an increased risk of faecal carriage of *E. coli* O157:H7 in cattle housed at higher stocking

densities, independent of group size (Stacey *et al.*, 2007). It is therefore likely that housing conditions including stocking density may impact on the detection and potential quantity of *S. uberis* within bovine faeces.

Aspects of pasture management, such as pasture rotation and application of slurry as fertiliser to pasture, as well as aspects of housed management, including bedding frequency and frequency of removal of old bedding are also likely to impact the survival of *S. uberis* within the dairy cattle environment. Research has shown the presence of *S. uberis* within effluent and its survival for up to 14 days (Lopez-Benavides *et al.*, 2005) and led to the pasture management recommendation of 2 weeks of grazing and 4 weeks of pasture rest, which when implemented was shown to decrease the rate of clinical mastitis (Green *et al.*, 2008). The impact of the timing of environmental management changes, such as changing grazing paddocks and the removal of bedding from housed environments has not been measured in terms of impact of faecal detection of *S. uberis* in terms of quantity and prevalence of *S. uberis* shedding. These types of environmental management strategies could impact on the survival and accumulation of *S. uberis* within the environment and the efficiency of faeco-oral transmission of *S. uberis.*

## 5.4 Limitations of the project

One of the largest limitations of this project was the use of one herd within one area of the UK for epidemiological analysis which means the results seen on this unit may only be representative of that single farm, however

the farm selected for this study represented a typical herd in the UK, in terms of management and herd size. A further limitation was the sampling period, which only occurred over one year for the longitudinal study, and may not be reflective of all years. The significant factors, including the seasonality of detection rate, may not be applicable to the herd in a different year.

The study into the prevalence of *S. uberis* detected in faeces was further limited by the small volume of faeces analysed, when compared to the total volume of faeces produced by a cow per day. The distribution of *S. uberis* within bovine faeces and gastrointestinal tract is currently unknown. If the distribution is not homogenous within a faecal deposit, there is the potential for false negative results if a small percentage of the faeces is tested, despite using a molecular test for detection. However it is not practical to sample large volumes of faeces per cow in an epidemiological study and is a limitation of all studies investigating the prevalence of bacterial pathogens within bovine faeces.

There is the risk that the use of *sub0888* for development of a PCR assay detecting *S. uberis* could result in false negative results, as it is unknown whether *sub0888* is present in all *S. uberis* strains, as the function of the gene and related protein remains unknown. Given the high conservation of the primers used for amplification of this sequence, along with confirmation of the present within all known strains from NCBI, including the environmental isolates from New Zealand (Pullinger *et al*., 2006), then it is

likely that *sub0888* will be conserved and presence in faecal strains of *S. uberis.* This should be confirmed using genome sequencing when single faecal isolates of *S. uberis* have been identified. The inability to isolate faecal strains of *S. uberis* using a selective culture based methodology prevented this, as well as the prevention of comparative studies between faecal and mastitic isolates from the same farm to determine strain epidemiology. This was attributed to the large number of phenotypically similar bacteria present in bovine faeces, as well as potential inhibitory relationships between *S. uberis* and other bacteria present, such as *E. faecium.*

## 5.5 Further Work

### 5.5.1 *S. uberis* residency within the gastrointestinal tract or as a cyclic mastitis pathogen

Further work is required to confirm whether *S. uberis* is principally a gastrointestinal commensal, which contaminates the environment of a dairy cow. It is possible that one of the largest evolutionary drivers for this bacterium is survival within the gastrointestinal tract and environment, and not the pathogenicity and survival in the mammary gland, especially as it is considered an opportunistic mastitis pathogen.

Given the hypothesis that the gastrointestinal tract can act as a reservoir for *S. uberis,* it is currently unknown whether the bacteria is transiently

present or whether it can replicate within the tract and whether certain sites are preferential. The collection and testing of samples from different areas of the gastrointestinal tract at slaughter may also provide an indication of *S. uberis* presence within different sites, however the presence and quantity of bacteria may be influenced by post mortem changes. Analysis of faecal and rumen samples from fistulated cows could be used to compare the presence and quantity of *S. uberis* between the samples, however this study would be limited to investigating only two locations of the gastrointestinal tract so may be of limited use. Both of these studies would be limited in terms of investigating the potential roles of *S. uberis* within the gastrointestinal tract and cow health, as well as the interactions with other microorganisms, but could provide evidence of *S. uberis* niches within the gastrointestinal tract.

One method to further investigate this hypothesis would be to genetically modify *S. uberis* and add additional marker genes, to allow for its discrimination from other bacteria, for example the green fluorescent protein, which has been utilised in studies investigating the presence of *E. coli, Morexalla* sp. and *Pseudomonas* spp. in soil and sludge (Errampall*i et al*., 1999). This methodology involves the insertion of the *gfp* gene into the genome of the bacterium, using a plasmid-based vector, and the detection of the bacterium based upon quantification of fluorescence. The modified *S. uberis* bacterium could be utilised in a longitudinal field study investigating *S. uberis* transmission and spread after oral delivery. The aim

would be to isolate the modified *S. uberis* from the faeces of the cow over time, in order to investigate the bacterial transit time and the survival time within the environment and isolation from common sites, such body surfaces, urinary tract, skin and teat, as well as in milk samples. This would give an indication of the process of faeco-oral transmission of *S. uberis,* in terms of ability to survive the gastrointestinal tract, persistency of detection in faeces and ability to contaminate not only the dairy cow's environment but also individual animals.

### 5.5.2 The role of *S. uberis* within the bovine gastrointestinal tract

The role of *S. uberis* within the gastrointestinal tract is currently unknown. Its presence may have beneficial, detrimental or no impact on the health and production of the cow. One method for evaluating the role of *S. uberis* within the gastrointestinal tract would be through challenging cattle which did not have *S. uberis* present within their gastrointestinal tract orally with *S. uberis* and monitoring animals for alterations in overall health and production, as well as changes to the microbiome within different components of the gastrointestinal tract. However one difficulty would be difficult to identify animals which were truly negative for the presence of the bacteria. It might be possible to perform a randomised control trial investigating the impact of oral dosing neonatal calves with *S. uberis* on subsequent calf health as calves are born without a gut microbiome present. However it is currently unknown at what age calves are potentially

colonised by *S. uberis* and the gut microbiome will have a different function in a calf to adult cow due to many factors including changes to physiology and immunology, dietary differences and management.

Metagenomic and metabolomic studies of environmental and faecal strains of *S. uberis* are needed to identify the key genomic sequences required for survival in the gastrointestinal tract and environment, as well as for infection of the mammary gland and a comparison of the presence and phenotypical expression between strains isolated from different locations may indicate essential conserved coding sequences for survival within different these environments. It may also give an indication as to the role of *S. uberis* in the gut through genome differences and the differences in phenotype.

The quantification of *S. uberis* within a faecal deposit needs to be established and could be performed using culture based methodology or using molecular techniques as a proxy for viable bacteria, for example quantifiable transcriptional analysis of mRNA using qPCR. However both methodologies would require DNA extraction directly from a faecal sample as the impact of the enrichment culture utilised in this study on the quantity of *S. uberis* detected is currently unknown. Research into the epidemiology of different faecal strains of *S. uberis* is also required, in terms of heterogenity between strains and a full genome comparative analysis between faecal, environmental and intramammary strains of *S. uberis.* This

should give an indication as to whether pathogenic strains of *S. uberis* were present in bovine faeces and the environment, as well as the epidemiology of the quantity of *S. uberis* shed.

Once faecal strains can be isolated, a challenge study should be performed to investigate the potential infectious dose required for *S. uberis* mastitis to occur from environmental contamination of the teat. This could be performed by dipping teats with different concentrations of faecal pathogenic *S. uberis* strains. This study would require a large sample size of cows in order to determine a statistical difference between mastitis rates attributed to different infectious doses of different *S. uberis* strains. However it would give an indication as to the level of environmental contamination which is required to impact on the potential risk of *S. uberis* clinical mastitis.

### 5.5.3 Impact of variables on *S. uberis* detection rate in bovine faeces

The impact of different management and cow factors on the detection of *S. uberis* in faeces requires further investigation, including analysis of the effect of different management systems in wider number of UK herds. This longitudinal study could also include dairy and beef herds, as it would allow investigation of different management systems, as well as potential breed differences and housing aspects, such as grazing compared to housed

systems and high compared to low intensity units. Repeating the longitudinal study over multiple time periods would also allow for the investigation of the impact of seasonality and what additional factors may influence this. The impact of the stage of lactation should also be investigated within these studies, to determine whether the apparent decrease in probability of detection of faecal *S. uberis* at 50-99 DIM, in comparison to 0-49 DIM and 100-149 DIM is repeatable in different herds. This should include herds with different management systems, as well as different milk productivity/yields and lactation curves. The increased probability of detection in mid lactation does not appear to be explained with any potential changes in the management of this herd, nor any known biological changes in physiology of the cow at this stage of lactation, suggesting further research is required.

In addition, analysis of the effect of stocking density, using two identical housing situations on the same unit would allow for factors such as diet and different housing designs to be accounted for.

The longitudinal study in Chapter 3 showed that there was an increased odds of detecting *S. uberis* in primiparous cows, in comparison to multiparous cows when sampling over a longer period of time. This could potentially suggest that primiparous have never been infected with *S. uberis* prior to entering the milking herd or may have an age-related increased susceptibility to gastrointestinal *S. uberis.* A study investigating the detection of *S. uberis* over time in a cohort of heifers from birth until the

end of first lactation would allow potential age related factors to be investigated for faecal shedding of *S. uberis,* as well as the time period at which the heifers are exposed to *S. uberis.* The impact of diet should also be investigated and one method could be to replicate the different conditions of the gastrointestinal tract of the cow in vitro, for example through changes in pH and nutritional changes through metabolism of volatile fatty acids, to give an indication of the possibility of *S. uberis* replication and survival within different areas of the gastrointestinal tract. Research has shown that *S. uberis* has the ability to utilise a large variety of different carbohydrates, due to the genome containing a large number of carbohydrate metabolism genes (Ward *et al.*, 2009), which may result in the diet of cattle having a limited impact on the presence and quantity of *S. uberis* in faeces.

Another option is to investigate the impact of different diets on the detection rate of *S. uberis* in faeces as a field trial, using a cohort of cows which are fed different diets, whilst in the same living conditions. However the difference in terms of prevalence of detection of *S. uberis* would need to be large or the study would require a large sample size of cows in order to have sufficient power to be statistically significant.

### 5.5.4 Interactions of *S. uberis* within the environment

Further research into the survival and potential replication of *S. uberis* on different bedding substrates, especially sand and straw, is required as there is opportunity to implement different management strategies to

influence the survival of *S. uberis* within the environment. One aspect would be to investigate the time period which *S. uberis* can survive on either clean and used bedding substrates, including pasture and other potential bedding substrates as in this study, *S. uberis* could be recovered over five weeks after the initial inoculation

Other aspects of the bedding microenvironment could be investigated *in vitro*, including the impact of different temperatures, and the presence of other bacteria, by the addition of both *S. uberis* and different organisms of interest to the bedding substrate with selective media used to differentiate between the species. Another method of investigating the survival of *S. uberis* on different bedding substrates would be a field trial to determine the impact of factors such as the presence of cows, the presence of a live microbiota and environmental conditions. This would currently be difficult to perform due to the inability to currently differentiate *S. uberis* from other faecal *Streptococci* and *Enterococci* and therefore this study may benefit from the use of a strains of *S. uberis* which contained a genetic marker to allow for easy identification.

## 5.6 Conclusion

### 5.6.1 Chapter 2

The objective of this chapter was to develop a robust methodological method of screening bovine faecal samples for the presence of *S. uberis,* as well as isolating single strains of *S. uberis* from bovine faeces for strain typing. The results demonstrated that it was possible to detect starting concentrations of *S. uberis* of 12 cfu in bovine faeces, using PCR which targeted the gene *sub0888.* The results also highlighted issues with identification and isolation of *S. uberis* strains from bovine faeces due to the presence and interaction with a large number of other bacteria present in the microbiota of bovine faeces.

### 5.6.2 Chapter 3

The objective of this chapter was to report the prevalence of faecal shedding of *S. uberis* in a typical UK herd and investigate any factors which impact on the presence of *S. uberis* in bovine faeces. There was no evidence from this research of the presence of a few “super-shedding” cows within a herd and that a large percentage of cows were likely to have *S. uberis* detected in their faeces at least once. This research showed that there was a variation in the prevalence of detection of *S. uberis* in bovine faeces within a herd, which was significantly altered by the cow location in both the short and long term. It indicated that some cow factors, such as

parity, stage of lactation and BCS, significantly affected the odds of detection of *S. uberis* in the long term.

### 5.6.3 Chapter 4

The objective of this chapter was to investigate the potential for *S. uberis* to survive and potentially replicate on different housed bedding substrates. This research showed that *S. uberis* strains are capable of surviving on straw and sand for at least 5 weeks, but only 1 week on sawdust. There was evidence of replication of the strains on the used bedding substrate, which was not evident in this research on the clean bedding substrates, which suggested that the addition of faeces to the bedding provided an enhanced environment for *S. uberis.* There was between strain variation in the survival and replication of different *S. uberis* strains on different bedding substrates, suggesting genetic differences between the strains which have an impact on the ability to survive and replicate of individual strains in different environment.

# Chapter 6. References


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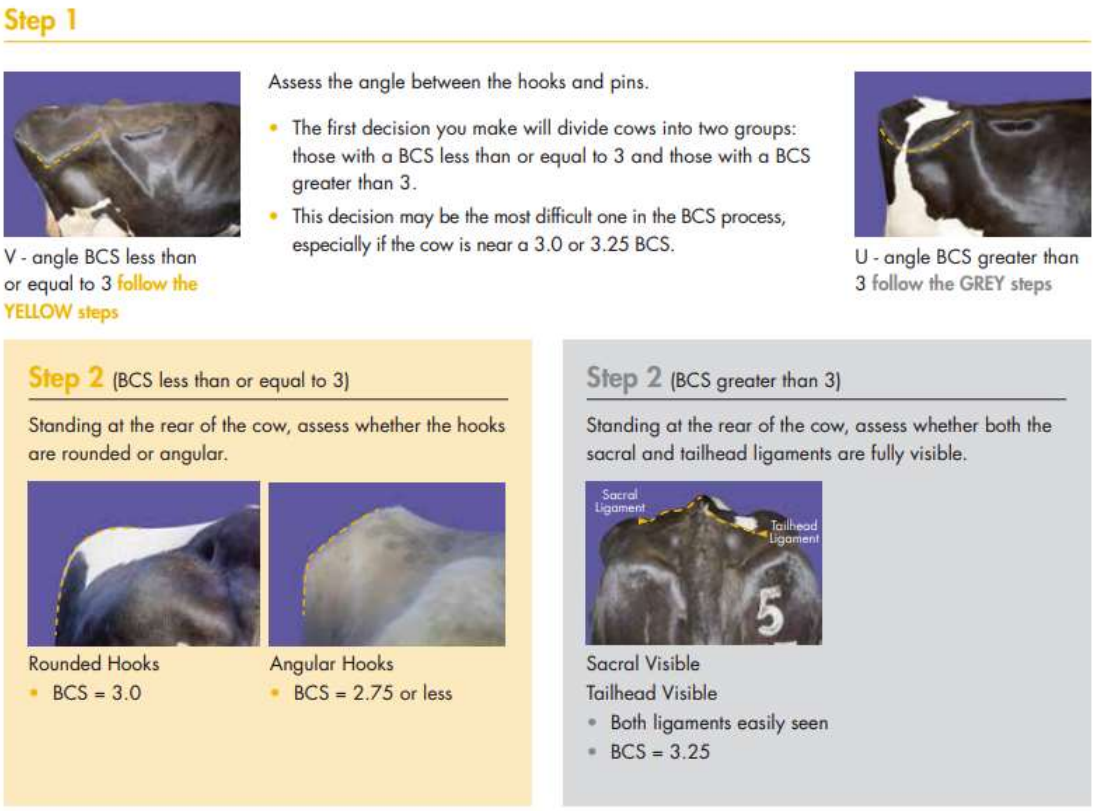
# Chapter 7. Appendices

## Appendix 1: Body Condition Scoring System

The body condition scoring system used was taken from AHDB, 2014, which was based on the scoring system discussed by Edmondson *et al.* in 1989.



## Step 3

Refine the score '2.75 or less' by evaluating the PINS.



Padded Pins

- BCS = 2.75

## Step 4

Now we need to feel the pins to assess the presence of a palpable fat pad (one that is not visible to the eye but can be felt) to refine the score of '2.5 or less'.



Palpable Fat Pad on Pins

- Fat pad present
- BCS = 2.5



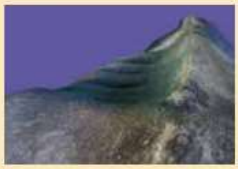
No Fat Pad on Pins

- No fat pad
- BCS = 2.25 or less

## Step 5

Evaluate the visibility of the short ribs.

- Look for the bony ridges of the short ribs.
- Estimate the distance that these ridges are easily seen from the tip of the short ribs to the spine.
- Are the ridges visible half of the distance, three-quarters of the distance, more?



Visible ½ the distance

- Ribs visible halfway to the spine
- BCS = 2.25



Visible ¾ the distance

- Ribs visible ¾ of the distance of the spine
- BCS = 2.0

## Step 6

Cows with sawtooth spine and ribs are severely under-conditioned and will score less than 2.0.

## Step 3

Continue to assess the visibility of the ligaments. The tailhead ligament will become covered in fat first.




Sacral Visible
Tailhead Barely Visible

- Tailhead ligament partly covered in fat
- BCS = 3.50

## Step 4

The tailhead is now completely covered. Assess the visibility of the sacral ligament to determine the score of 3.75 or 4.0 or more.



Sacral Barely Visible
Tailhead Not Visible

- Neither ligament easily seen
- BCS = 3.75



Sacral Not Visible
Tailhead Not Visible

- Neither ligament visible
- Will score 4.0 or more



All bony prominences rounded and covered in fat

- Tailhead buried in fat
- Fat deposits readily seen on rump and legs
- BCS = 5

# Appendix 2: Cleanliness Scoring System

Cleanliness scoring was performed using the scoring system shown below, which was taken from AHDB, 2014b.

## Scoring cleanliness

| Score | Clean | Flank (including tail) | Lower hind leg | Udder |
|---|---|---|---|---|
| 0 | • No dirt or only minor fresh or dried splashing present |  |  |  |
| Score | Dirty | | | |
| 1 | • An area of dirtiness (ie layer or plaques of fresh or dried dirt) at least palm size (10 x 15cm) |  |  |  |
| Score | Very dirty | | | |
| 2 | • An area of dirtiness (ie layer or plaques of dried dirt) amounting to at least forearm length (40cm) in any dimension |  |  |  |
| | **Factors that may affect cleanliness** | • Cubicle cleanliness<br>• Cubicle comfort<br>• Amount and type of bedding<br>• Manure consistency and health of the herd | • Frequency of:<br>– alley cleaning<br>– running of automatic scrapers<br>– cleaning of crossover alleys<br>– cleaning of collecting yard<br>• Space allowance per cow | • Cubicle cleanliness<br>• Cubicle comfort<br>• Amount and type of bedding<br>• Cleanliness of alleys<br>• Amount of hair on udder<br>• Manure consistency and health of the herd |

## Appendix 3: PCR results for the cows tested

The table below reports the individual faecal sample results for the cows which were present in the herd in Study 1 of Chapter 3, for different sampling time points. It represents whether the gene *sub0888* was identified within the DNA extracted from the faecal sample (P = positive, N = negative, ** = not present).

| Cow ID | Oct 0888 | Dec 0888 | Feb 0888 | Apr 0888 | June 0888 |
|---|---|---|---|---|---|
| **1** | N | N | ** | N | N |
| **2** | N | N | N | N | N |
| **7** | N | P | P | N | N |
| **11** | N | N | N | N | P |
| **12** | N | N | N | N | N |
| **13** | N | N | P | N | N |
| **15** | P | N | P | ** | N |
| **17** | N | N | ** | P | N |
| **18** | N | N | P | N | N |
| **20** | N | N | N | P | N |
| **22** | N | N | P | N | N |
| **23** | N | N | N | ** | N |
| **24** | N | N | P | N | P |
| **25** | N | N | ** | N | ** |
| **26** | N | ** | ** | N | ** |
| **27** | N | N | N | N | P |
| **28** | N | N | P | N | N |
| **29** | N | N | P | ** | ** |
| **30** | N | N | P | ** | ** |
| **31** | N | P | N | N | N |
| **32** | N | N | N | N | N |
| **33** | N | N | N | ** | N |
| **34** | N | N | N | N | N |
| **35** | N | N | ** | N | N |
| **36** | N | N | P | ** | ** |
| **37** | N | N | N | ** | ** |
| **38** | N | P | P | P | P |

| Cow ID | Oct 0888 | Dec 0888 | Feb 0888 | Apr 0888 | June 0888 |
|---|---|---|---|---|---|
| **39** | N | N | P | N | N |
| **40** | N | N | P | N | N |
| **41** | P | N | N | N | N |
| **42** | N | N | N | P | N |
| **43** | P | P | N | N | N |
| **44** | N | N | N | N | N |
| **45** | N | N | P | ** | N |
| **46** | N | N | P | N | N |
| **47** | N | N | P | N | N |
| **49** | ** | ** | ** | N | P |
| **50** | ** | ** | ** | N | N |
| **51** | N | N | ** | P | N |
| **53** | N | N | P | N | N |
| **55** | N | ** | N | P | P |
| **56** | N | N | ** | N | ** |
| **58** | P | N | N | N | N |
| **59** | N | N | P | N | P |
| **60** | N | P | P | N | N |
| **61** | N | N | ** | N | N |
| **62** | N | N | P | P | N |
| **63** | N | N | P | N | N |
| **64** | N | N | P | ** | N |
| **65** | N | N | N | N | N |
| **66** | P | ** | ** | ** | ** |
| **67** | N | N | N | N | N |
| **68** | P | N | N | N | ** |
| **69** | N | N | ** | N | N |
| **70** | N | N | N | N | N |
| **71** | N | N | N | N | ** |
| **72** | N | ** | P | ** | ** |
| **73** | ** | P | N | N | N |
| **74** | N | N | P | N | N |
| **75** | N | N | P | N | N |
| **76** | N | N | P | ** | P |
| **77** | ** | N | P | N | N |
| **78** | ** | N | P | N | N |
| **79** | P | P | ** | N | N |
| **80** | N | P | N | N | N |

| Cow ID | Oct 0888 | Dec 0888 | Feb 0888 | Apr 0888 | June 0888 |
|---|---|---|---|---|---|
| **81** | N | ** | P | P | P |
| **82** | ** | N | P | N | N |
| **83** | N | ** | P | ** | ** |
| **85** | N | N | N | N | N |
| **86** | ** | ** | ** | N | N |
| **87** | N | N | N | N | N |
| **88** | ** | ** | ** | N | N |
| **89** | ** | ** | ** | P | N |
| **90** | P | N | N | P | P |
| **92** | ** | ** | ** | N | N |
| **93** | N | N | N | N | N |
| **94** | N | N | N | N | P |
| **95** | N | N | N | P | P |
| **96** | N | N | N | ** | ** |
| **98** | N | P | N | N | N |
| **99** | ** | ** | P | N | P |
| **100** | ** | ** | ** | N | N |
| **101** | N | N | N | N | ** |
| **102** | N | ** | N | N | P |
| **106** | ** | ** | N | ** | ** |
| **107** | N | P | P | N | N |
| **108** | N | N | P | ** | ** |
| **111** | N | N | P | N | N |
| **114** | N | N | N | ** | ** |
| **115** | N | ** | N | N | N |
| **116** | N | N | ** | ** | ** |
| **117** | N | P | N | N | N |
| **120** | N | ** | N | ** | ** |
| **121** | N | N | ** | N | N |
| **122** | N | P | N | N | ** |
| **123** | N | N | N | N | N |
| **124** | N | N | N | N | P |
| **125** | N | P | N | N | N |
| **126** | N | N | ** | ** | ** |
| **129** | N | N | N | N | N |
| **131** | N | N | N | N | N |
| **133** | N | ** | P | N | N |
| **138** | N | ** | N | N | N |

| Cow ID | Oct 0888 | Dec 0888 | Feb 0888 | Apr 0888 | June 0888 |
|---|---|---|---|---|---|
| 141 | N | N | ** | P | N |
| 142 | N | N | N | N | N |
| 143 | N | N | N | N | ** |
| 145 | N | N | P | ** | ** |
| 146 | N | ** | N | N | N |
| 147 | P | ** | P | N | N |
| 148 | N | P | N | ** | ** |
| 149 | P | N | N | N | N |
| 150 | P | N | N | N | N |
| 153 | N | N | N | P | N |
| 155 | N | N | ** | N | N |
| 156 | N | N | P | N | N |
| 157 | N | N | N | P | N |
| 158 | N | N | N | N | N |
| 159 | N | ** | ** | ** | ** |
| 161 | N | N | P | N | N |
| 162 | N | N | P | P | N |
| 163 | N | N | N | N | ** |
| 164 | N | P | N | N | N |
| 165 | N | P | N | N | ** |
| 166 | N | N | N | N | N |
| 169 | N | N | N | N | N |
| 171 | N | ** | ** | ** | N |
| 172 | N | N | P | N | N |
| 173 | N | N | N | N | N |
| 174 | N | N | N | N | N |
| 176 | N | N | N | N | N |
| 177 | N | N | N | N | ** |
| 180 | N | ** | ** | ** | ** |
| 181 | N | N | N | N | N |
| 183 | N | N | P | N | N |
| 185 | N | N | N | N | ** |
| 186 | ** | ** | N | P | N |
| 191 | N | N | N | N | P |
| 192 | N | P | N | N | N |
| 193 | N | N | N | N | N |
| 195 | N | N | N | N | P |
| 196 | N | P | P | N | P |

| Cow ID | Oct 0888 | Dec 0888 | Feb 0888 | Apr 0888 | June 0888 |
|---|---|---|---|---|---|
| 197 | P | ** | P | N | ** |
| 198 | N | P | N | N | N |
| 200 | N | ** | ** | N | N |
| 201 | N | p | N | N | N |
| 202 | N | p | N | N | N |
| 204 | N | N | N | N | N |
| 205 | N | N | N | N | N |
| 206 | N | N | P | P | N |
| 208 | N | P | N | N | N |
| 213 | N | P | N | P | N |
| 215 | ** | N | ** | ** | ** |
| 217 | N | P | N | N | N |
| 218 | N | N | N | N | N |
| 219 | N | N | N | N | N |
| 221 | N | N | N | N | N |
| 222 | N | N | N | N | N |
| 223 | N | N | N | ** | ** |
| 225 | N | P | N | ** | P |
| 226 | N | N | P | P | N |
| 227 | N | N | ** | N | N |
| 228 | N | N | N | ** | N |
| 229 | N | N | N | N | N |
| 230 | N | N | P | N | N |
| 231 | N | ** | N | N | N |
| 232 | N | N | ** | N | ** |
| 233 | N | P | N | N | N |
| 234 | N | N | N | N | N |
| 235 | N | P | P | N | ** |
| 236 | N | N | N | N | N |
| 237 | P | N | N | N | N |
| 238 | P | N | ** | N | N |
| 239 | N | P | N | N | N |
| 240 | N | N | N | N | N |
| 241 | N | N | N | N | N |
| 242 | N | N | N | N | N |
| 243 | N | ** | N | N | N |
| 244 | N | N | ** | N | N |
| 245 | N | ** | ** | ** | ** |

| Cow ID | Oct 0888 | Dec 0888 | Feb 0888 | Apr 0888 | June 0888 |
|---|---|---|---|---|---|
| 246 | ** | N | N | N | N |
| 247 | N | P | P | N | N |
| 248 | N | N | N | N | N |
| 249 | N | P | N | N | N |
| 250 | N | p | N | ** | ** |
| 251 | N | N | N | P | N |
| 252 | N | P | P | N | N |
| 253 | N | N | ** | N | P |
| 254 | N | N | N | N | N |
| 256 | N | P | ** | ** | ** |
| 257 | N | N | N | N | N |
| 258 | N | N | ** | N | N |
| 260 | N | ** | ** | ** | ** |
| 261 | N | N | N | N | N |
| 262 | N | N | N | N | N |
| 263 | N | N | N | N | N |
| 264 | P | N | N | ** | N |
| 265 | N | P | N | N | N |
| 266 | N | N | N | N | P |
| 267 | N | P | N | N | N |
| 271 | N | N | P | P | N |
| 272 | N | N | N | N | N |
| 273 | N | P | P | N | N |
| 274 | N | N | P | P | P |
| 275 | P | N | P | N | N |
| 276 | N | P | N | N | N |
| 277 | N | P | N | ** | N |
| 278 | N | N | N | N | N |
| 280 | P | N | N | N | N |
| 281 | P | N | P | ** | N |
| 282 | N | N | N | N | ** |
| 283 | ** | ** | P | N | N |
| 284 | ** | N | N | N | ** |
| 285 | N | ** | ** | N | N |
| 286 | N | P | N | N | N |
| 287 | P | N | P | N | N |
| 288 | N | N | N | N | N |
| 289 | N | P | P | N | N |

| Cow ID | Oct 0888 | Dec 0888 | Feb 0888 | Apr 0888 | June 0888 |
|---|---|---|---|---|---|
| 290 | N | N | N | N | N |
| 292 | ** | ** | N | N | ** |
| 293 | ** | ** | ** | P | N |
| 294 | ** | ** | ** | N | N |
| 295 | ** | ** | ** | P | N |
| 298 | N | N | P | P | ** |
| 299 | ** | ** | ** | N | N |
| 300 | P | P | N | P | ** |
| 302 | ** | ** | ** | ** | N |
| 305 | ** | ** | ** | N | ** |
| 307 | N | N | N | N | ** |
| 308 | ** | ** | ** | ** | N |
| 309 | N | P | P | ** | ** |
| 310 | ** | ** | ** | N | N |
| 311 | ** | ** | ** | ** | N |
| 312 | ** | ** | ** | ** | N |
| 313 | ** | ** | ** | ** | N |
| 314 | ** | ** | ** | ** | N |
| 315 | ** | ** | ** | ** | N |
| 316 | ** | ** | ** | N | N |
| 317 | ** | ** | ** | ** | N |
| 318 | ** | ** | ** | ** | N |
| 319 | ** | ** | ** | N | N |
| 320 | ** | ** | ** | N | N |
| 321 | ** | ** | ** | N | N |
| 322 | ** | ** | ** | ** | N |
| 323 | ** | ** | ** | ** | N |
| 324 | N | N | N | N | ** |
| 325 | ** | ** | ** | N | ** |
| 326 | ** | ** | ** | ** | N |
| 327 | ** | ** | ** | ** | ** |
| 328 | ** | ** | ** | N | N |
| 329 | ** | ** | ** | ** | N |
| 330 | ** | ** | ** | ** | N |
| 331 | ** | ** | P | ** | N |
| 332 | ** | ** | ** | ** | N |
| 334 | ** | ** | ** | ** | N |
| 335 | ** | ** | ** | ** | N |

| Cow ID | Oct 0888 | Dec 0888 | Feb 0888 | Apr 0888 | June 0888 |
|---|---|---|---|---|---|
| **338** | ** | ** | ** | N | ** |
| **339** | N | P | ** | N | N |
| **349** | N | p | N | ** | ** |
| **350** | N | P | N | ** | N |
| **353** | N | N | P | P | N |
| **360** | N | N | N | N | P |
| **361** | n | ** | ** | ** | ** |
| **364** | N | N | ** | ** | ** |
| **369** | N | p | N | N | N |
| **374** | P | P | N | N | N |
| **376** | N | P | N | N | N |
| **379** | N | ** | P | ** | N |
| **381** | N | p | N | N | ** |
| **382** | P | P | N | N | N |
| **392** | P | N | ** | ** | ** |
| **394** | ** | ** | ** | ** | ** |
| **395** | N | N | ** | N | N |
| **396** | N | P | P | N | N |
| **666** | N | ** | ** | ** | ** |
| **301450** | ** | N | ** | ** | ** |
| **2J** | ** | N | ** | N | N |
| **500113** | ** | ** | ** | N | ** |
| **602351** | ** | ** | ** | N | ** |
| **Total Number tested** | 226 | 209 | 202 | 219 | 224 |
| **Total Number Positives** | 23 | 53 | 67 | 27 | 25 |
| **Prevalence** | 10.2% | 25.4% | 33.2% | 12.3% | 11.2% |

# Appendix 4: Clinical Mastitis Bacteriology

| Cow ID | Date of Clinical Mastitis | Days in milk | Environment | Result |
|---|---|---|---|---|
| 309 | 12/09/2014 | 74 | Pasture | Heavy, pure growth of *S. uberis* |
| 101 | 27/10/2014 | | Pasture | No significant bacterial growth after 72 hour's incubation |
| 197 | 07/11/2014 | 109 | Cubicles | No significant bacterial growth after 72 hour's incubation |
| 382 | 20/11/2014 | 187 | Cubicles | Contaminated |
| 31 | 28/11/2014 | 214 | Cubicles | Contaminated |
| 120 | 09/12/2014 | 95 | Cubicles | Heavy pure growth of *Candida albicans* |
| 114 | 10/12/2014 | 109 | Cubicles | No significant bacterial growth after 72 hour's incubation |
| 191 | 10/12/2014 | 7 | Straw yard | Heavy pure growth of *E.coli* |
| 108 | 19/12/2014 | 230 | Straw yard | Heavy pure growth of *S. uberis* |
| 226 | 19/12/2014 | 85 | Cubicles | No significant bacterial growth after 72 hour's incubation |
| 116 | 05/01/2015 | 49 | Straw yard | Scant, mixed growth of *Staphylococcus sciuri* and *Staphylococcus hominis* |
| 140 | 05/01/2015 | | | Scant, pure growth of *E. coli* |
| 181 | 06/01/2015 | 226 | Cubicles | Heavy, pure growth of *S. uberis* |
| 116 | 08/01/2015 | 77 | Straw yard | Heavy growth of *E. coli* |
| 87 | 12/01/2015 | 165 | Cubicles | Mixed heavy growth of *S. uberis* and *Staphylococcus aureus* |
| 197 | 15/01/2015 | 178 | Cubicles | Scant growth of *E. coli* |
| 202 | 26/01/2015 | 66 | Cubicles | Heavy, pure growth of *E. coli* |
| 197 | 29/01/2015 | 192 | Cubicles | Mixed, heavy growth of *S. uberis* and Bacillus sp |
| 149 | 03/02/2015 | 78 | Straw yard | Heavy, pure growth of *Staphylococcus sciuri* |
| 373 | 09/02/2015 | 310 | Cubicles | Mixed growth of *Staphylococcus haemolyticus* and *Staphylococcus epidermidis*. Scant growth of Bacillus sp. |
| 125 | 12/02/2015 | 72 | Cubicles | Scant, mixed growth of *E. coli* and *Staphylococcus aureus* |
| 208 | 23/02/2015 | 22 | Straw yard | Scant, mixed growth of *E. coli* and *S. uberis*. Heavy growth of *Staphylococcus chromogenes* |
| 148 | 24/02/2015 | 107 | Cubicles | Heavy, pure growth of *Staphylococcus aureus* |

| Cow ID | Date of Clinical Mastitis | Days in milk | Environment | Result |
|---|---|---|---|---|
| 309 | 24/02/2015 | 236 | Straw yard | Scant, mixed growth of *E.coli* and *Staphylococcus hominis* |
| 70 | 25/02/2015 | 34 | Straw yard | Scant, pure growth of *S. uberis* |
| 238 | 25/02/2015 | 145 | Cubicles | Heavy pure growth of *Staphylcoccus aureus* |
| 107 | 27/03/2015 | 108 | Straw yard | Heavy growth of *S. uberis* |
| 238 | 03/04/2015 | 151 | Cubicles | Heavy, pure growth of *Staphylococcus aureus* |
| 373 | 08/04/2015 | 1 | Straw yard | Mixed and heavy growth of *E. coli* and *S. uberis* and scant growth of *Staphylococcus warneri* |
| 95 | 16/04/2015 | 1 | Straw yard | Scant pure growth of *Staphylococcus xylosus* |
| 373 | 8/4/15 | 1 | Straw yard | Mixed, scant growth of *S. uberis* and Bacillus sp. |
| 373 | 19/04/2015 | 12 | Straw yard | Heavy growth of *Streptococcus epidermidis* |
| 298 | 20/04/2015 | 53 | Pasture | Mixed heavy growth of Bacillus sp and *Streptococcus lutetiensis* |
| 146 | 28/05/2015 | 3 | Straw yard | Heavy and pure growth of *E. coli* |
| 146 | 09/02/2015 | 248 | Pasture | Heavy pure growth of *Streptococcus dysgalactiae* |
| 373 | 19/06/2015 | 72 | Pasture | Heavy growth of *S. uberis* |
| 360 | 02/09/2015 | 118 | Pasture | Heavy growth of E. coli and scant mixed Bacillus sp and *Enterococcus casseliflavus* |
| 82 | | | | Scant, pure growth of *E. coli* |
| 98 | | | | Contaminated |
| 120 | | | | Heavy pure growth of Corynebacterium sp |
| 145 | | | | Contaminated |
| 163 | | | | Scant growth of Streptococcus lutetiensis |
| 213 | | | | Heavy pure growth of *S. uberis* |
| 221 | | | | Moderate, mixed growth of *Staphylococcus equorum* and *Staphylococcus chromogenes*. Scant growth of *Aerococcus viridans* |
| 233 | | | | Moderate, mixed growth of *Enterococcus avium* and *Aerococcus viridans* |
| 251 | | | | Contaminated |
| 262 | | | | Scant, pure growth of *Staphylococcus equorum* |