Report to the Stapledon Memorial Trust

The effect of a sub-acute ruminal acidosis challenge induced by either grain or alfalfa-pellet based diets on the pathogenicity of rumen *Escherichia coli* populations in dairy cows

Dr Patricia Aikman

Animal Science Research Group, Department of Agriculture, University of Reading, Earley Gate, Reading RG6 6AR, UK

p.c.aikman@reading.ac.uk

Details of the Fellowship

Canadian Contacts: Dr D. O. Krause,[1,2] Dr J. C. Plaizier[1] and Dr E. Khafipour[1]

[1]Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada

[2]Department of Medical Microbiology, University of Manitoba, Winnipeg, MB, Canada

The fellowship took place between 17th February and 8th April 2009 with a follow-up visit between 11th and 17th July 2009.

Overall Objectives of the Fellowship

Researchers at the University of Manitoba have previously demonstrated that rumen microbial populations differ in animals with sub-acute ruminal acidosis (SARA), depending on whether the SARA was caused by high starch, cereal-based diets or by forage-based diets with low physically-effective fibre concentrations. The primary objective of this Fellowship was to investigate the effect of method of inducing SARA on the disease-causing capabilities, or pathogenicity, of rumen microbes, in particular *Escherichia coli*. In addition to improving understanding of the etiology of SARA, this work may also indicate whether shedding of pathogens into the environment by animals with SARA is a cause for concern. The secondary objective of the Fellowship was to develop expertise in microbiological and molecular techniques. To achieve these objectives, *E. coli* isolated from rumen samples taken in the course of two previous studies conducted at the University of Manitoba (Khafipour *et al.*, 2009a, Khafipour *et al.*, 2009b) were used.

Background and Objectives

Sub-acute ruminal acidosis is a metabolic disorder of ruminants which arises when the rate of production of volatile fatty acids from microbial fermentation of dietary starch and sugars exceeds the rate at which they are absorbed, leading to prolonged periods of sub-optimal rumen pH (<pH 5.6). In dairy cows SARA is associated with reduced milk yield and milk fat concentration, fluctuating feed intake, internal abscesses and laminitis. Although most commonly associated with intensively managed herds consuming diets containing high proportions of cereals, SARA is also found in more extensively managed pasture-based herds (Bramley *et al.*, 2008) and is thus a major welfare and economic issue across the dairy industry. Brent (1976) described how microlesions develop in the rumen epithelium during periods of low pH, due to the acidic nature of the rumen contents, changes in osmotic pressure and the presence of lipopolysaccharides (LPS), endotoxins produced by the Gram-negative bacteria which predominate under these conditions. Current thinking is that damage to the epithelium of the rumen or further down the digestive tract allows LPS to pass into the bloodstream, inducing an inflammatory response from the immune system, and playing a role in the development of the characteristic internal abscesses associated with SARA and possibly contributing to the development of laminitis.

Researchers at the University of Manitoba detected LPS and acute phase proteins indicative of an inflammatory response in the peripheral bloodstream of dairy cows fed a high starch, cereal-based diet to induce SARA, demonstrating that LPS had translocated from the gastrointestinal tract into the bloodstream (Khafipour *et al.*, 2009a). In contrast, when SARA was induced by feeding a diet which was low in effective fibre, achieved by providing alfalfa pellets as the main forage source, rumen LPS concentrations were higher than those seen in the cereal-induced SARA, but blood concentrations of LPS and acute phase proteins were negligible, thus there was no evidence of LPS translocation into the bloodstream or of an inflammatory response (Khafipour *et al.*, 2009b). This suggests that high rumen LPS concentrations are not solely responsible for damage to the rumen epithelium and its associated inflammatory response, and that other contributory factors must be involved. Detailed analysis of the rumen microbial populations showed substantial differences in the types of microorganisms which proliferated when SARA was induced by cereal or alfalfa-based diets (Khafipour *et al.*, 2009c). Of particular note were shifts in the type of *Escherichia coli* present – not only were *E. coli* substantially more abundant in animals with cereal-induced SARA compared to control and alfalfa-induced SARA, but the strains present could be classified differently according to the ABD typing system developed by Clermont *et al.* (2000) to separate virulent and non-virulent *E. coli* (E. Khafipour, unpublished data). This clear evidence that there is a shift in the type and abundance of ruminal *E. coli* suggests a link between the presence of *E. coli* and the inflammatory response observed in cows with cereal-induced SARA. These strains of *E. coli* may possess virulence factors, in addition to LPS, which are responsible for the immune response observed in these animals.

The objective of this Stapledon Fellowship project was to determine if the specific rumen conditions elicited by cereal-induced SARA selected for strains of *E. coli* with enhanced virulence properties. To achieve this, the polymerase chain reaction (PCR) technique (Appendix 1) was used to screen *E. coli* isolated from rumen samples in which SARA had been induced by different methods for a range of genes, or groups of genes, known to be responsible for specific factors involved in the virulence of *E. coli*.

Materials and Methods

Two previous studies each using four ruminally cannulated dairy cows examined induction of SARA using a high starch, cereal-based diet (Khafipour *et al.*, 2009a) or a diet low in effective fibre, containing alfalfa pellets as the main forage source (Khafipour *et al.*, 2009b). Based on previous work in the same laboratory (Gozho *et al.*, 2005), SARA was defined as rumen pH < 5.6 for more than 180 minutes per 24 hour period. In each study, individual animals acted as their own control. Rumen samples were collected at the beginning of the study before SARA was induced over a number of days by gradual introduction of the cereal or pelleted forage as appropriate, when further rumen samples were collected. For the purposes of the current report, these sample periods will be defined as cereal-control (CC), cereal-SARA (CS), forage-control (FC) and forage-SARA (FS). Enumeration and isolation of *E. coli* from samples obtained during these studies was conducted by Dr Ehsan Khafipour before the start of the current Stapledon Fellowship study. In brief, *E. coli* isolates were obtained by culturing rumen fluid on *E. coli*-specific chromogenic medium, then growing up individual colonies to obtain DNA for further genetic analysis. From the initial 129 isolates, a total of 92 unique (nonclonal) isolates were identified by Rep-PCR[1] DNA fingerprinting, distributed between sampling periods as follows: CC – 31; CS – 32; FC – 17; FS – 12. The increased abundance of total *E. coli* in animals fed the cereal-based diets is reflected in the greater number of nonclonal isolates from these animals.

In the Stapledon Fellowship study, the *E. coli* isolates were screened for a total of 15 known *E. coli* virulence genes (Table 1). The PCR for each virulence gene was run separately using the appropriate forward and reverse primers and optimum annealing temperature (Table 1) for that gene. Strains of *E. coli* of known genotype were included in each run to act as positive and negative controls, along with a water blank. The products of the PCR, or amplicons, were separated on agarose gels stained with ethidium bromide then visualised by photographing them under a UV light (Figure 1).

[1] Repetitive extragenic palindromic PCR

Table 1. Sequences of the primer pairs and optimum annealing temperature for detection of 15 virulence genes in *E. coli* isolates.

Type of virulence factor	Primer name	Primer sequence (5′→ 3′)	Annealing temperature, °C	Description of gene(s)	Ref
Toxin	Cnff	AGTACTGACACTCACTCAAGCCGC	62	Cytotoxic necrotising factors (Cnf1 and Cnf2)	1
	Cnfr	GCAGAACGACGTTCTTCATAAGTATCACC			
Toxin	Vt1f	CGCATAGTGGAACCTCACTGACGC	64	Verocytotoxin 1	1
	Vt1r	CATCCCCGTACGACTGATCCC			
Toxin	Vt2f	CGGAATGCAAATCAGTCGTCACTCAC	65	Verocytotoxin 2	1
	Vt2r	TCCCCGATACTCCGGAAGCAC			
Toxin	HlyAf	TGCAGCCTCCAGTGCATCCCTC	63.5	The *hlyA* gene encoding α haemolysin	1
	HlyAr	CTTACCACTCTGACTGCGATCAGC			
Toxin	Eaef	CCAGGCTTCGTCACAGTTGCAGGC	66.5	The *eae* gene coding for intimin present in AEEC strains	1
	Eaer	CGCCAGTATTCGCCACCAATACC			
Adhesin	Papf	CCGGCGTTCAGGCTGTAGCTG	65	The genes coding for pathogenicity islands (*PAI I*, *PAI II*), which carries a *pap* operon encoding P-fimbria, and *sfp* gene cluster	1
	Papr	GCTACAGTGGCAGTATGAGTAATGACCGTTA			
Adhesin	PAI1	TAGCTCAGACGCCAGGATTTTCCCTG	61		
	pai2	CCTGGCGCCTGCGGGCTGACTATCAGGG			
Adhesin	Sfaf	CGGAGGAGTAATTACAAACCTGGCA	64	S-fimbrial adhesins encoded by *sfaD* to *sfaE*	2
	Sfar	CTCCGGAGAACTGGGTGCATCTTAC			
Adhesin	Afaf	TATGGTGAGTTGGCGGGGATGTACAGTTACA	58	*AfaE-3* gene cluster	1
	Afar	CCGGGAAAGTTGTCGGATCCAGTGT			
Adhesin	AIDA1	TATGCCACCTGGTATGCCGATGAC	69	The *aidA* gene coding *E. coli* AIDA-I adhesin in DAEC strains	1
	AIDA2	ACGCCCACATTCCCCCAGAC			
Adhesin	Ag43f	TGACACAGGCAATGGACTATGACCG	67	The *agn43* gene coding for antigen involved in *E. coli* autoaggregation	1
	Ag43r	GGCATCATCCCGGACCGTGC			
Adhesin	AggRf	GAGTTAGGTCACTCTAACGCAGAGTTG	61	The *aggR* gene for adhesin of aggregative adherence fimbria I	1
	AggRr	GACCAATTCGGACAACTGCAAGCATCTAC			
Adhesin	BmaEf	CTAACTTGCCATGCTGTGACAGTA	59	The *bmaE* gene for M-agglutinin subunit; *Afa-8* gene cluster	2
	BmaEr	TTATCCCCTGCGTAGTTGTGAATC			
Fimbriae	FimHf	CTGGTCATTCGCCTGTAAAACCGCCCA		The FimH gene encoding FimH subunit of type 1 pili	1
	FimHr	GTCACGCCAATAATCGATTGCACATTCCCT			
Autotransporters	SPATE1	GAGGTCAACAACCTGAACAAACGTATGGG	57	The genes encoding SPATE (serine protease autotransporter)	1
	SPATE2	CCGGCACGGGCTGTCACTTTCCAG			

[1]Kotlowski, R., C. N. Bernstein, S. Sepehri, and D. O. Krause. 2007. Gut 56:669-75.

[2]Martin, H. M., B. J. Campbell, C. A. Hart, C. Mpofu, M. Nayar, R. Singh, H. Englyst, H. F. Williams, and J. M. Rhodes. 2004. Gastroenterology 127:80-93.

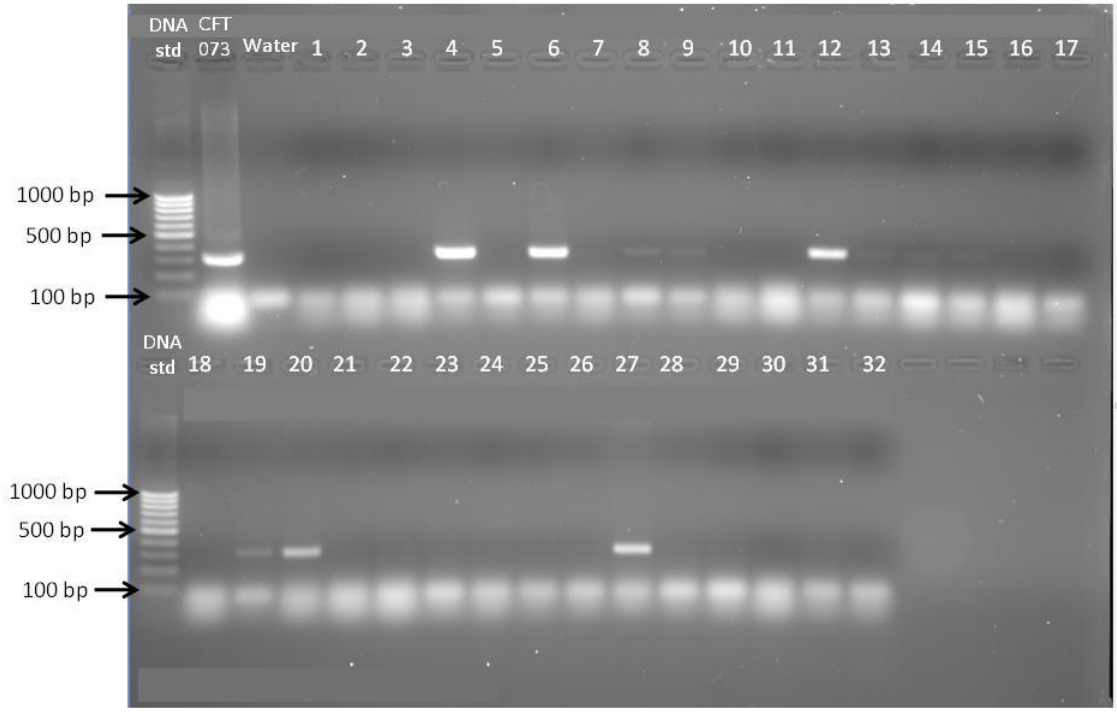

Figure 1. PCR amplicons visualised on ethidium bromide-stained agarose gel. This example shows the products of PCR to detect the presence of the *Agn43* gene in *E. coli* isolates of unknown genotype. A DNA standard, which provides a scale which the PCR amplicons can be measured against, was placed in the first well in each row. The amplicon of interest was 317 base pairs (bp) in size, and can be seen clearly as a white band in the column containing the PCR product of *E. coli* CFT073, which was used as a positive control. The water blank was placed next to the positive control and is followed by *E. coli* isolates 1-32. Numbers 4, 6 8, 9, 12, 13, 14, 15, 19, 20 and 27 were positive for *Agn43*.

Results and Discussion

Subacute ruminal acidosis did not result in significant increases in any of the virulence factors that were screened for, irrespective of the method used to induce SARA (Table 2). The *FimH* gene which encodes for the fimbria virulence factor is known to be widespread in *E. coli,* including commensal strains (Chapman *et al.*, 2006), and so its presence in nearly all of the *E. coli* samples was expected. Fimbriae are hair-like protein appendages with which *E. coli* attach themselves to the epithelium of the digestive tract, thus preventing them from being flushed out. Of the eight adhesin virulence factors that were screened for, only three were detected in any of the samples, but there were no significant effects of treatment. It is interesting to note the relatively high frequency of the *aidA* and *Agn43* genes, which encode for the AIDA and Ag43 virulence factors respectively, in samples from all treatments. Like fimbriae, these proteins help *E. coli* adhere to the intestinal epithelium and they also have a major role in aggregation, or clumping together, of the *E. coli* (Le Bouguenec, 2005, Sherlock *et al.*, 2004), an ability which increases bacterial resistance to host defence mechanisms. Genes encoding for the serine protease autotransporter (SPATE) family of virulence factors were detected in two samples from each of the CC and CS treatments, and in 3 samples from the FC

treatment. SPATE proteins have been detected in *E. coli* responsible for diarrhoeal diseases and urinary tract infections in humans, and although the mode of action has not been fully determined, it is thought that the SPATE proteins induce degeneration of host tissue cells by disrupting barrier function and ion secretion (Nataro *et al.*, 2003). The samples were screened for a total of five different toxin-producing genes, but only *cnf*, encoding for cytotoxic necrotising factors 1 and 2 (CNF1 and CNF2) was detected, in two samples from animals on the FC treatment. These toxins induce cell necrosis in the host tissues (DeRycke *et al.*, 1990).

Table 2. Distribution of the phylotypes and virulence factors present in *E. coli* isolated from controls, cereal and alfalfa pellet-induced SARA groups. Values indicate percentage (number) of *E. coli* isolates in each group.

	Cereal model			Alfalfa-pellet model		
	Control	SARA	*P*-value	Control	SARA	*P*-value
Phylotype[1]						
A	57.5 (23)	32.5 (13)	0.04	50.0 (15)	47.4 (9)	1.00
B1	37.5 (15)	57.5 (23)	0.07	43.3 (13)	36.8 (7)	0.77
B2+D	5.0 (2)	10.0 (4)	0.67	6.7 (2)	15.8 (3)	0.36
Virulence factors[2]						
Total number of isolates screened	31	32		17	12	
Toxins						
Cnf	0	0	-	11.8 (2)	0	0.23
Vt1	0	0	-	0	0	-
Vt2	0	0	-	0	0	-
Hly	0	0	-	0	0	-
Eae	0	0	-	0	0	-
Adhesins						
Pap	3.2 (1)	0	0.31	0	0	-
PAI	0	0	-	0	0	-
Sfa	0	0	-	0	0	-
Afa	0	0	-	0	0	-
AIDA	51.6 (16)	43.8 (14)	0.53	52.9 (9)	25 (3)	0.14
Ag43	35.5 (11)	15.6 (5)	0.07	58.8 (10)	83.3 (10)	0.17
AggR	0	0	-	0	0	-
BmaE	0	0	-	0	0	-
Fimbrae						
FimH	96.8 (30)	100(32)	0.31	100 (17)	100(12)	-
Autotransporter						
SPATE	6.5 (2)	6.3 (2)	0.97	17.6 (3)	0	0.13

[1]Phylotypic classification according to Clermont *et al* (2000). Phylotypic grouping data provided by E. Khafipour (unpublished).

[2]See Table 1 for definition and description of each virulence factor.

The work conducted for the Stapledon Fellowship detected the presence of genes encoding for a number of virulence factors in *E. coli,* but did not prove that these genes were being expressed – i.e. that the virulence factors were actually being produced. It is possible that environmental conditions within the rumen were not challenging enough, under either normal or acidotic conditions, to necessitate the "switching on" of these genes in defence of the *E. coli*. Alternatively, the genes may have been fully expressed in the *E. coli* carrying them, but competition from other bacteria within the rumen meant that the pathogenic *E. coli* did not predominate.

The set of virulence genes that were screened for during the Stapledon Fellowship are associated with highly virulent phylotypes of *E. coli* such as enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), and uropathogenic *E. coli* (UPEC). These strains are the causative agents of severe intestinal or extraintestinal infections and the results presented here indicate that *E. coli* isolated during SARA does not fit in these clusters. Insufficient time during the Fellowship prevented screening of the *E. coli* isolates for the full range of virulence genes which can be studied at the University of Manitoba. Consequently, Dr Khafipour plans to conduct PCR screening of the samples for additional virulence genes. These will include genes associated with capsule formation, a bacterial defence mechanism, as well as *E. coli* adherence to and colonisation of the rumen epithelial surface. Although some of these genes might be present in commensal strains of *E. coli*, any small difference in the genes structures and sequences can vastly affect the adhesion and invasion properties of these strains. This is important as *E. coli* colonisation can create an imbalance in the normal microflora of the rumen epithelium, which can result in immune activation and an increase in gut permeability during SARA. Microbial fermentation in the hindgut of animals with cereal-induced SARA is also being explored by the group at the University of Manitoba. It is thought the epithelium of the large intestine may be more vulnerable to low pH and LPS than that of the rumen, so this may be another site where LPS passes into the bloodstream, thereby inducing an immune response in the animal.

Perhaps the most notable finding from this study is that *E. coli* carrying genes for a range of pathogenic abilities are present in the rumens of dairy cows under both normal and acidotic conditions, where they could presumably act as a reservoir of pathogenicity should conditions become favourable for their proliferation.

Outcomes of the Fellowship

It is anticipated that the results from this Stapledon Fellowship will be included, along with those of the further analysis detailed above, in a paper for submission to a peer-reviewed journal. In addition to working on the specific project described here, I had the opportunity to observe and assist with a number of other microbiological and molecular techniques during the Fellowship. This has greatly increased my knowledge and understanding of molecular biology, which has already proved useful in the writing of proposals for funding for further research in the field of ruminant microbiology.

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Appendix 1. Key concepts of the polymerase chain reaction

The polymerase chain reaction (PCR) is a technique whereby a specific section of a single DNA molecule is amplified (copied). The amplification of the DNA sequence of interest allows its presence to be detected and so provide information about the genotype of the organism from which the DNA was derived. In this Stapledon Memorial Fellowship project, PCR was carried out on DNA from *E. coli* isolated from dairy cows in which SARA had been induced by different means in order to detect the presence or absence of genes encoding for specific features which increase the virulence, or pathogenicity, of the microorganism.

The principal components required for PCR are:

• Primers – oligonucleotides, usually 17-30 nucleotides in length, which are complementary to specific nucleotide sequences flanking the area of DNA that is to be amplified. Each PCR requires a pair of primers, one for each strand of the double-stranded DNA molecule to be copied. These are often referred to as the *forward* and *reverse* primers. They initiate copying of the target section of DNA by annealing (binding) to the complementary section of the DNA molecule.

• Deoxynucleotide diphosphates –"free" nucleotides (adenine, thymine, cytosine and guanine) used in the assembly of the new strands of DNA.

• DNA polymerase – a heat stable enzyme which binds to the free end of the primer and assembles a new strand of DNA by adding deoxynucleotide diphosphates complementary to the original strand of DNA.

The three principal steps in a PCR cycle are:

1. Denaturation – the two strands of the target DNA molecule are subjected to a short period of high temperature, causing them to peel apart and separate.
2. Annealing – the primers bind to the appropriate sections of the separated DNA strands. The optimum temperature for this stage of the reaction is dependent upon the length and nucleotide sequence of the primer.
3. Extension – assembly of the new DNA strand by the DNA polymerase enzyme. The temperature of the reaction has to be adjusted again to ensure optimal activity of the DNA polymerase.

This series of steps is repeated 25-30 times in one PCR run. The new DNA strands created in each cycle are used as templates in the next cycle, thereby doubling the number of strands of DNA copied each time.

The temperature steps used in the PCR in this study were as follows:

	First cycle	Cycles 2-29	Cycle 30
Denaturation	94°C, 300 seconds	94°C, 60 seconds	94°C, 60 seconds
Annealing	Primer-specific annealing temperature, 60 seconds	Primer-specific annealing temperature, 60 seconds	Primer-specific annealing temperature, 60 seconds
Extension	72°C, 60 seconds	72°C, 60 seconds	72°C, 300 seconds