FINAL REPORT on FSA project G03031

a) *Title*

Project Code	G03031
Project Title	**Factors influencing transgene survival and transfer in the rumen**
Lead Contractor (Organisation)	Rowett Institute of Nutrition and Health, University of Aberdeen
Sub- Contractor (Organisation)	Aberystwyth University
Contractor Project Leader	Karen Scott (Senior Research Fellow)
Sub-Contractor Project Leader	Prof. Jamie Newbold (Professor of Animal Science
Project start and end-dates	15/03/08 to 31/07/11
Report Date	31 July 2011
Agency Project Officer	David Jefferies

Other staff involved

Dr Kostas Minas	Post doctoral Researcher, carrying out research and organising procedures at RINH and U Aberystwyth. From Oct 08 to date.
Prof. Harry Flint (Band 3 Group Leader RINH)	Head of research group, RINH. Supervisory and trouble shooting as required, from Oct 08.
Dr Neil McEwan (Lecturer in Animal and Equine Sciences, Aberystwyth University)	Supervising post-doc whilst at U Aberystwyth, Liaising with RINH. October 08 to date

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PRIFYSGOL ABERYSTWYTH UNIVERSITY

b) Executive Summary

Background

This proposal was designed to address possible risks associated with transgenic food/feed that arise from gene transfer to the gut microbiota in the gut of farm animals. In the European Union genetically modified plants are regulated by the GM Food and Feed Regulation 1829/2003.. The authorisation of GM plants includes a safety assessment, one aspect of which is the risk of gene transfer to other organisms, including bacteria.

In ruminant animals, minimally processed feed material is exposed to a very high density of micro-organisms within the reticulo-rumen without previously passing through an acidic stomach. Release of DNA through digestion of plant material by the resident microbial community may lead to DNA acquisition via transformation of naturally competent microrganisms. Gene transfer to rumen bacteria does not constitute an entirely new risk, nor is it unique to consumption of GM feed, since if such transfer events can occur, then animals (and humans) have been ingesting potentially transforming DNA via their normal diets throughout evolutionary history.

The research undertaken in this project attempted to clarify two particular questions. First, what are the potential consequences of transfer events involving genes and gene combinations from GM material that are not normally present at significant levels in our food supply? Second, do any features of the genetic constructs found in GM food plants increase the likelihood of onward transgene transfer? Previous work has indicated that homology between GM constructs and potential target DNA in gut bacteria appears to be a crucial factor determining transgene acquisition and stable incorporation by the resident gut community. Because most early GM plants have had bacterial sequences used in their construction, it can be argued that they are far more likely to share regions of homology with gut bacteria than DNA arising from non-GM food. Marker genes designed to aid the selection of GM plants by discriminating them from parental strains have included bacterial antibiotic resistance genes, which could adversely alter the phenotype of any gut bacterium acquiring them.

This project set out to develop a body of evidence indicating the ‘risk of gene transfer’ as a result of animal consumption of GM feed.

Objectives and Approach

The work was divided into three key objectives:

1. Examine the factors affecting GM plant DNA survival in rumen fluid
2. Investigate the influence of regions of DNA homology upon acquisition of DNA by rumen bacteria.
3. Assess the release and survival of transgene DNA from GM plant material under conditions prevailing in the rumen

The release and survival of DNA during the degradation of three types of GM plant material, that would be used in animal diets – namely GM soya, maize and rapeseed was assessed following exposure to rumen fluid. Realtime quantitative PCR detection of specific gene sequences (200-250bp) was used to examine the relative rates of degradation of specific transgenes present in the individual GM plants compared to normal (housekeeping) plant genes. In the first set of experiments plant material was incubated with fresh rumen fluid in test-tubes, providing the opportunity

to assess DNA survival/degradation under a large number of different experimental conditions, all of which could be encountered in the rumen. Overall, twelve different variable were compared, making this the most comprehensive study of DNA survival under physiological rumen conditions performed to date. These experiments were used to narrow down the parameters to include in subsequent survival experiments where plant DNA survival was assessed in an *in vitro* model of the rumen. This model system was also used to analyse the survival of model transgene constructs carrying multiple selectable marker genes. These model transgenes provided a 'worst case scenario' for DNA acquisition and were used to study factors that influence acquisition of external DNA by the gut microbial community, including antibiotic treatment and the effect of sequence homology with resident genes. Specific marker genes were included to allow selection of transformed gut bacteria, and also their recovery from metagenomic libraries.

Key Results

Most of the variables tested had no significant effect on the survival time of DNA in rumen fluid.

The observed half-life for detection of DNA sequences from maize and soya plant material following exposure to rumen fluid was 4-6h, while rapeseed was degraded a bit more rapidly and had a half-life of about 3h. The only variable that significantly affected DNA survival was the presence or absence of protozoa in the rumen fluid. These eukaryotic microbes engulf plant material, and prolonged the time the GM sequences could be detected.

When the plant material was incubated under simulated rumen conditions, transgenes were detected in the solid fraction – residual plant material – 24h after incubation, in those vessels where GM soya and maize had been added. However transgenes were never detected in the bacterial fractions extracted from either the liquid or solid-associated bacterial phase, indicating that there was no detectable uptake of GM DNA from plant material by rumen bacteria.

In the final experiment specific DNA constructs, designed to contain detectable marker genes and regions of bacterial DNA, were added to simulated rumen conditions and the persistence of the DNA followed. The model transgenes were taken up by a sub-population of the rumen bacteria, at a low level. The results indicated that linear GM DNA could be taken up by naturally transformable commensal rumen bacteria – if bacterial DNA sequences are associated with the transgene. A range of bacterial species acquired, incorporated and expressed the plasmid DNA. The number of DNA uptake events was increased when antibiotic selection pressure was applied, but were still so rare that they could not be analysed statistically, using standard methods.

Conclusions

The main findings from this work were that GM DNA sequences can be detected within residual GM plant material that has been exposed to rumen fluid *in vitro*, even after 24h. However these sequences were never detected in any of the rumen bacteria using rt-PCR. Rumen bacteria were only able to acquire GM DNA if there was a region of bacterial DNA associated with the transgene, to facilitate homologous recombination events. Even then, the uptake of DNA by rumen bacteria was an extremely rare event.

b) Glossary

A list of all abbreviations used in the main text and definitions of key terms

Cm^R – chloramphenicol resistance
Em^R – erythromycin resistance
gfp – green fluorescent protein
GIT - gastrointestinal tract
GM – genetically modified
qPCR – quantitative realtime PCR
RUSITEC - **Ru**men **Si**mulation **Tec**hnique (a model fermentation system designed to simulate ruminant digestion)
SCFA - short chain fatty acids
tRFLP – terminal Restriction Fragment Length Polymorphism

c) Aims and Objectives of the Investigation

Background/Introduction

Farmers are under increasing pressure to produce sufficient food to feed the ever-increasing world population. One method to increase crop production is the use of genetically modified crops, resistant to pesticide treatments. Further modifications to GM crops are under investigation to improve agronomic or nutritional qualities (eg. the incorporation of drought tolerance, salt tolerance etc). Legislation governing the growth and use of GM crops in human and animal food vary in different countries, with commercial GM crops widely grown throughout the world and also in five EU Member States: Spain, Portugal, France, Germany and the Czech Republic. Transgenic plant material, particularly GM soya and maize, is already used widely in animal feedstuffs in many parts of the world, including the EU. Despite this, the potential risks associated with gene transfer to the microbiota inhabiting the gastrointestinal (GI) tracts of humans and farm animals have not been fully assessed.

Ruminants harbour a huge and diverse microbial population, and consume vast amounts of minimally treated plant material which directly enters the rumen without prior passage through an acidic stomach, that would otherwise contribute to DNA degradation. Plant material remains in the rumen, being digested by fermentation by rumen microbial inhabitants for 24 - 48hr. This combination means that the possibilities of DNA survival and acquisition by rumen micro-organisms are theoretically higher than in humans, who consume less, more highly processed plant material, which passes through the acidic stomach before reaching the colon where most bacterial fermentation occurs. Release of DNA through digestion of plant material by the microbial community resident in the GIT may lead to DNA acquisition via transformation of naturally competent microrganisms. Previous work by ourselves and others has demonstrated natural transformation in members of the rumen microbiota in pure culture (Mercer et al., 1999; Duggan et al., 2000).

Gene transfer to gut bacteria does not constitute an entirely new risk, nor is it unique to consumption of GM feed, since if such transfer events can occur, then animals (and humans) have been ingesting potentially transforming DNA via their normal diets throughout evolutionary history. This research project has attempted to address

two particular questions.

1. What are the potential consequences of transfer events involving genes and gene combinations from GM material that are not normally present at significant levels in our food supply?
2. Do any features of the genetic constructs found in GM food plants increase the likelihood of onward transgene transfer?

Bacterial transformation using transgenic plant DNA was originally demonstrated to occur in the soil under optimised laboratory conditions (Gebhard & Smalla 1998). Subsequent work with soil bacteria demonstrated that transformation can also happen in a natural setting, but the efficiency depended on the type of soil (Nielsen et al 1997). Previous work in our laboratory implied that homology between GM constructs and potential target DNA in bacteria could be crucial in determining transgene acquisition and stable incorporation by the resident gut community (Mercer et al 2001). In addition, following a single event of bacterial transformation, further lateral gene transfer also needs to be considered (Nielsen & Townsend 2004) Because many earlier GM plant constructs included bacterial sequences , it can be argued that they are far more likely to share regions of homology with gut bacteria than DNA arising from non-GM food. New molecular tools – specifically qPCR - now widely available enable the survival of GM DNA to be quantified with much greater sensitivity than in the past, when competitive PCR was used (Mercer et al 1999; Martin-Orue et al 2002; Duggan et al 2003, Nielsen & Townsend 2004).

Objectives list and tasks

<u>Objective 1</u> GM plant DNA survival in rumen fluid

Assess the relative rates of survival/degradation of relevant GM plant DNA under conditions simulating those found in the rumen.

Task 1.1 DNA extraction and amplification methods.

Task 1.2 Assessment of DNA release and survival by quantitative realtime PCR (qPCR).

Task 1.3 Statistical analysis to compare DNA degradation rates

<u>Objective 2</u> Investigate the influence of regions of DNA homology upon acquisition of DNA by rumen bacteria.

The presence of homologous DNA squences may increase the likelihood of DNA acquisition by rumen bacteria. The persistence of model constructs, specifically designed to contain regions of bacterial DNA, under rumen conditions was assessed.

Task 2.1 Preparation of GM DNA constructs

Task 2.2 Detection of DNA transfer under antibiotic selection

Task 2.3 Detection of DNA transfer with two regions of homology

Task 2.4 Statistical analysis to compare DNA transfer rates

<u>Objective 3</u> Assess the release and transfer of transgene DNA under conditions prevailing in the rumen using a metagenomic approach

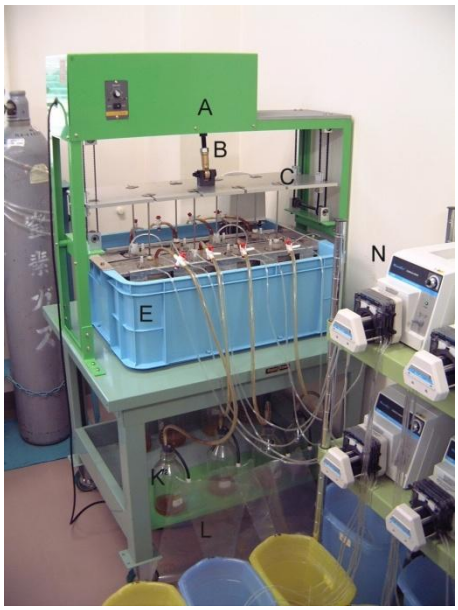
Since some of the bacteria acquiring GM DNA may be unculturable, preparation and screening of a total rumen DNA library will enable the identification of clones containing selectable marker genes, and the bacteria carrying them identified.

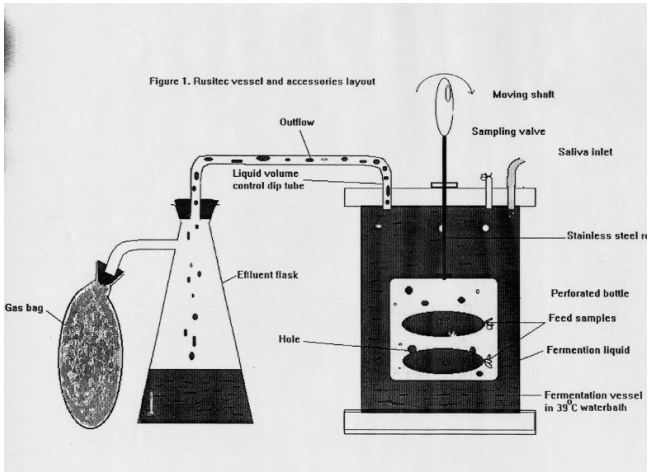
Task 3.1 Preparation of metagenomic libraries

d) Experimental Procedures

1. An essential component of the project was determining a DNA extraction procedure that was appropriate for the large scale extraction of DNA suitable for qPCR quantification. Various methods were compared and finally a modification of the CTAB method was adopted (appendix 1). This led to the preparation of the manuscript *Optimisation of a high-throughput CTAB-Based protocol for the extraction of qPCR-grade DNA from rumen fluid, plant and bacterial pure cultures.*
2. The second essential procedure was the design and validation of primers suitable for amplification of transgenes and housekeeping genes in the GM plant material. Where possible, previously validated primers described in the literature were used. Primers were selected depending on the amplification efficiency and limit of detection (LOD), and tested experimentally in qPCR before entering routine use. Primers used for the detection of cruciferin (CruF, CruR), Cry1F (Cry1F, Cry1R) and lectin (lecF, LecR) were originally designed by Delano *et al.,* 2003. Primers for the amplification of Bialaphos Resistance (BarF, BarR) and invertase (IvrF, IvrR) were originally described by Ehlers *et al.,* 1997, whereas the primers used for the amplification of 5-enolpyruvylshikimate-3-phosphate synthase (EpspsF, EpspsR) were designed in this project. The protocol for qPCR amplification and the sequences of all primers are described in appendix 2.
3. In order to investigate DNA survival/degradation under rumen conditions we were dependent on being able to simulate ruminant digestion – which can be done using an *in vitro* fermentation system known as the RUSITEC (Rumen Simulation Technique). Performing the experiments in the RUSITEC vessels enables more variables to be compared than would be possible *in vivo* using cannulated animals, aswell as permitting interventions such as antibiotic addition.

Figure 1 Photograph of the RUSITEC, containing 8 vessels, and a diagrammatic representation of a single RUSITEC vessel.





4. The final objective, investigating the influence of DNA homology on DNA uptake utilised modified DNA constructs that had been prepared in a previous FSA-funded project. In these experiments constructs with single and double regions of homology were used, in order to see if there was any effect on DNA acquisition.

e) Results and Discussion

Objective 1 GM plant DNA survival in rumen fluid

Assess the relative rates of survival/degradation of relevant GM plant DNA under conditions simulating those found in the rumen.

This objective set out to investigate the survival/degradation of DNA within GM plant material under rumen conditions in as robust a way as possible. Thus many different variables, that would normally be encountered *in vivo* in the rumen of different animals were compared in a large *in vitro* test-tube experiment, within Tasks 1.2 and 1.3. The results obtained were used to decide which parameters were the most critical in afffecting DNA survival, and these were then compared in a survival experiment under simulated rumen conditions, designed to mimic conditions *in vivo*, by using the RUSITEC (rumen simulaiton technology).

Task 1.1 DNA extraction and amplification methods.

Extraction of quality DNA, suitable for qPCR amplification, from both GM plant material and microbial cells in the same sample required comparison and modification of various DNA extraction methods. The method chosen had also to be suitable for high throughput extractions, as the DNA survival experiment would involve extracting DNA from >6000 tubes. The protocol developed (Appendix 1) relied on a CTAB-based buffer for cell lysis, and further purification of DNA with phenol : chloroform : isoamyl alcohol. The additional phenol extraction step improved the yield from rapeseed and maize, and improved the DNA quality from soya (Table 1).

Table 1 – Comparison of DNA yield from different amounts of starting material, with/without including a phenol extraction step

	Soya				
	Amount of Starting Material			DNA extraction reagent	
	20 mg	50 mg	100 mg	C:IAA (24:1)	P:C:IAA (25:24:1
Average (ng/µl)	211.54	354.68	669.78	522.18	301.81
p value	>0.01			0.04	
Average A_{260}/A_{280}	1.97	1.97	1.99	1.52	1.96
p value	0.83			>0.01	
	Rapeseed				
	Amount of Starting Material			DNA extraction reagent	
	20 mg	50 mg	100 mg	C:IAA (24:1)	P:C:IAA (25:24:1
Average (ng/µl)	281.63	296.86	420.27	245.09	420.75
p value	0.10			>0.01	
Average A_{260}/A_{280}	2.21	2.01	1.95	2.17	2.05
p value					
	Maize				
	Amount of Starting Material			DNA extraction reagent	
	20 mg	50 mg	100 mg	C:IAA (24:1)	P:C:IAA (25:24:1
Average (ng/µl)	208.39	484.72	239.27	215.15	406.44
p value	0.01			0.03	
Average A_{260}/A_{280}	2.22	1.99	1.81	2.04	1.98
p value	0.26			0.79	
Abbreviations: **P =** Phenol, **C =** Chloroform, **IAA =** Isoamyl Alcohol					

The DNA yield using the CTAB method exceeded that from the same samples using commercial kits (Table 2), and the quality was confirmed by successful qPCR applications. The protocol was used successfully for DNA purification from rumen fluid and plant cells, and could also be altered for large-scale extraction of DNA from pure cultures of Gram +ve and Gram –ve bacteria. The protocol was also successfully adapted to a high-throughput, small-scale 96-well plate format.

Table 2 – Comparison of DNA yield from plant material using different methods

Original Material	Yield (Conc. - ng/µl)	A_{260}	A_{280}	A_{260}/A_{280}	Method
20 µl of R.F. and 20 µg of ground maize	1.60	0.03	0.01	2.46	Wizard genomic DNA purification Kit (Promega)
40 mg of ground maize seeds	**31.50**	**0.63**	**0.31**	**2.04**	Wizard genomic DNA purification Kit (Promega)
10 µl of R.F. and 10 mg of ground maize	10.00	0.20	0.09	2.15	Dneasy Plant Mini Kit (QIAGEN)
20 mg of ground maize seeds	5.70	0.11	0.07	1.76	Dneasy Plant Mini Kit (QIAGEN)
100 µl of R.F. and 100 mg of ground maize	**226.00**	**4.52**	**2.15**	**2.10**	QIAamp DNA Stool Mini Kit
100 µl of R.F. and 100 mg of ground maize	Undetectable	Undetectable	Undetectable	1.64	QIAamp DNA Stool Mini Kit
2 ml R.F.	153.30	3.07	1.97	1.56	Chloroform-Isoamyl Alcohol
400 mg of ground maize seeds	Undetectable	Undetectable	Undetectable	1.56	Chloroform-Isoamyl
200 mg of ground maize seeds	**332.00**	**6.64**	**3.16**	**2.10**	CTAB Extraction method
100 mg of ground maize seeds	**460.30**	**9.21**	**4.49**	**2.05**	CTAB Extraction method
500 µl of R.F.	**326.00**	**6.52**	**3.20**	**2.04**	Phenol-Chloroform Extraction
250 µl of R.F. and 250 µg of ground maize	**807.70**	**16.15**	**8.12**	**1.99**	Phenol-Chloroform Extraction
100 µl of R.F. and 100 mg of ground maize	**571.10**	**11.42**	**5.77**	**1.98**	Phenol-Chloroform Extraction
200 mg of ground maize seeds	**770.20**	**15.40**	**7.74**	**1.99**	Phenol-Chloroform Extraction
50 mg of lyophilised, bead-beaten Soya and rumen fluid	**332.13**	**6.64**	**3.42**	**1.94**	This Study / 2 ml format
50 mg of lyophilised, bead-beaten maize and rumen fluid	**495.88**	**9.92**	**4.88**	**2.03**	This Study / 2 ml format
50 mg of lyophilised, bead-beaten oilseed rape and rumen fluid	**459.75**	**9.20**	**4.44**	**2.07**	This Study / 2 ml format
5 mg of lyophilised, bead-beaten Soya and rumen fluid	**266.55**	**5.33**	**2.80**	**1.94**	This Study / 96-well format

5 mg of lyophilised, bead-beaten maize and rumen fluid	**400.04**	**8.00**	**4.03**	**1.97**	This Study / 96-well format
5 mg of lyophilised, bead-beaten oilseed rape and rumen fluid	**174.92**	**3.50**	**1.80**	**1.95**	This Study / 96-well format
50 ml of E. faecalis culture	**429.25**	**8.59**	**4.25**	**2.01**	This Study / 50 ml format
50 ml of E. coli culture	**280.50**	**5.61**	**2.88**	**1.96**	This Study / 50 ml format

qPCR detection methods were optimised for both the transgenes and specific endogenous plant genes for each of the three plant materials (soya, oilseed rape and maize). These plants were chosen as they can all be components of the ruminant diet and are three of the commonest GM crop plants worldwide. Various different primer pairs, either published in the literature or developed in this study were tested for efficient amplification of the DNA purified from the GM plant material. Each of the primer pairs was initially checked in a standard PCR amplification, to confirm formation of the appropriately sized band (Appendix 3). Information on the primers ultimately chosen (sequences and additional parameters) for the qPCR amplification of transgenic and housekeeping DNA is shown in Table 3. Melt-curve analysis of qPCR products confirmed the amplification of single bands from total plant DNA.

Table 3: Sequence of the Primers and Annealing Temperatures used in qPCR amplification

RoundupReady Soya							
	Primer Name	**Sequence 5'-3'**	**Orientation**	**Annealing Temperature (°C)**	**Number of Cycles**	**Amplicon Size (bp)**	Reference
Transgene (EPSPS)	EPSPS F5	CCGTCTTCCCGTTACCTTG	Forward	63.7 (for 30 s)	43	215	This Study
	EPSPS R5	AAGCCCTGCAGCATCTTTT	Reverse				
Housekeeping (Lectin)	Lec F	GGGTGAGGATAGGGTTCTCTG	Forward	59 (for 30 sec)	43	210	Delano, J. *et al.,* 2003
	Lec R	GCGATCGAGTAGTGAGAGTCG	Reverse				
Herculex I maize							
	Primer Name	**Sequence 5'-3'**	**Orientation**	**Annealing Temperature (°C)**	**Number of Cycles**	**Amplicon Size (bp)**	**Reference**
Transgene (Cry1F)	Cry1F F	CTGCCTTCATACGCTATTTATTTGC	Forward	60 (for 20 sec)	40	179	Delano, J. *et al.,* 2003
	Cry1F R	GGAACAAACTCAGACAACAGGAAAC	Reverse				
Housekeeping (Invertase)	Ivr F	CCGCTGTATCACAAGGGCTGGTACC	Forward	63.2 (for 15 Sec)	50	226	Ehlers, Von B., *et al.,* 1997
	Ivr R	GGAGCCCGTTGTAGAGCATGACGATC	Reverse				
InVigor 5020 oilseed rape							
	Primer Name	**Sequence 5'-3'**	**Orientation**	**Annealing Temperature (°C)**	**Number of Cycles**	**Amplicon Size (bp)**	**Reference**
Transgene (Bialaphos resistance)	Bar F1	AAGCACGGTCAACTTCCGTA	Forward	60 (for 60 sec)	50	221	Ehlers, Von B., *et al.,* 1997
	Bar R1	CTTCAGCAGGTGGGTGTAGAG	Reverse				
Housekeeping (Cruciferin)	Cru F	TGGCTAAAGGTACGTTGAATCTG	Forward	60 (for 15 sec)	40	258	Delano, J. *et al.,* 2003
	Cru R	CTCTCCCCATAAGACCTTCTCC	Reverse				
16S rDNA							
	Primer Name	**Sequence 5'-3'**	**Orientation**	**Annealing Temperature (°C)**	**Number of Cycles**	**Amplicon Size (bp)**	**Reference**
Housekeeping (16S)	FD2	AGAGTTTGATCATGGCTCAG	Forward	55 (for 30 sec)	35	1,500	Weisburg, W.G., *et al.,* 1991
	RP1	ACGGTTACCTTGTTACGACTT	Reverse				
Housekeeping (16S)	27f	AGAGTTTGATCMTGGCTCAG	Forward	50 (for 60 sec)	30	1,362	Hongoh, Y *et al.,* 2000
	1389R	ACGGGCGGTGTGTACAAG	Reverse				

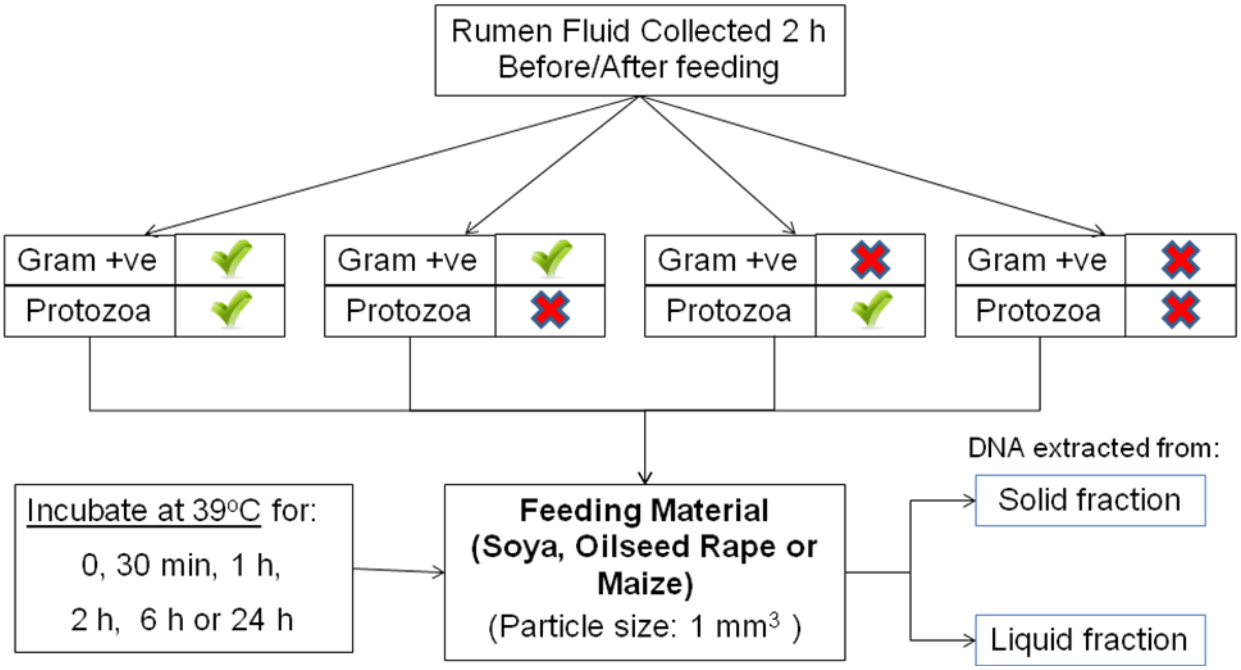
The development of the DNA extraction methods and qPCR amplification of the GM DNA and housekeeping genes were crucial for the remainder of the project. Details of each of the methods can be found in Appendices 1 and 2.

Task 1.2. Assessment of DNA release and survival by realtime PCR (qPCR).

Initial experiments investigating the survival/degradation of GM plant DNA were carried out in vitro, in test-tubes using fresh rumen fluid. In order to compare the

effects of diet, fresh rumen fluid was collected from cows fed on either a high forage (70% forage: 30% concentrate), or a high concentrate (30% forage:70% concentrate) diet. Full analysis of the diets is provided in Appendix 4. Feeding such different diets affects the pH of the rumen, as well as probably having an effect on the composition of the rumen microbiota. During preliminary discussions on this project it was suggested that this piece of work be expanded to include as many different variables encountered in the rumen as possible. The experimental outline is shown in figure 3 and included: sampling the rumen fluid before or 2h after feeding; the presence or absence of protozoa; the presence or absence of Gram positive bacteria (by monensin treatment); and incubating with the GM plant material for various times. Finally the pH of the whole rumen fluid (plus protozoa and Gram-positive bacteria) was adjusted to pH5.5, 6, 6.5 or pH7. Following incubation of the GM plant material in each rumen fluid fraction (in two biological replicates) for the specified time, DNA was extracted using the CTAB method described above, and the survival of the specific GM DNA from each plant compared to that of the housekeeping genes by qPCR amplification (performed in duplicate) using the primers shown in Table 3.

Figure 3 Schematic representation of the different incubations carried out to test the survival of GM plant DNA in rumen fluid.



Task 1.3 Statistical analysis to compare DNA degradation rates

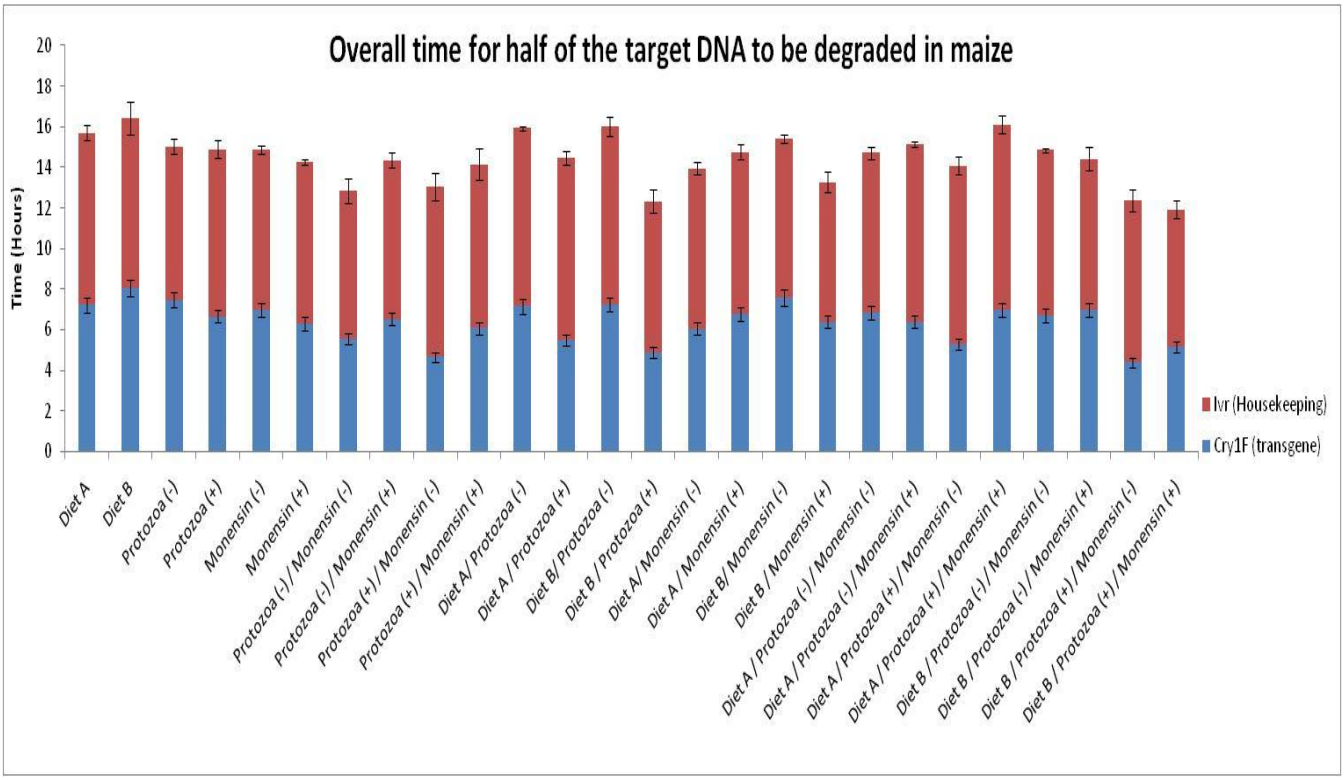
This robust analysis of DNA survival under rumen conditions resulted in qPCR data for >6000 DNA samples (2 biological replicates, amplified in duplicate), which required careful statistical analysis to obtain meaningful results. Dr Grietje Holtrop (BioSS at RINH) assisted in this analysis, as a sub-contractor. The experimental data were analysed by repeating measures ANOVA.

DNA survival/degradation rates were assessed for the GM DNA within each plant material, at each timepoint, and compared to the survival of the housekeeping

genes. The data is illustrated as the half-life of the DNA – the time taken for half of the DNA detected at t0 to disappear. For each plant material the housekeeping genes could consistently be detected for slightly longer than the GM DNA (t½ = ~7h compared to t½ = <6h). Oilseed rape DNA seemed to be degraded (t½ less) faster than GM maize or soya.

The survival of the Maize housekeeping and GM DNA in rumen fluid extracted before feeding is shown in Figure 4. Detection of the housekeeping genes was not affected by any of the experimental parameters, so in all other cases only the survival of the GM DNA is shown.

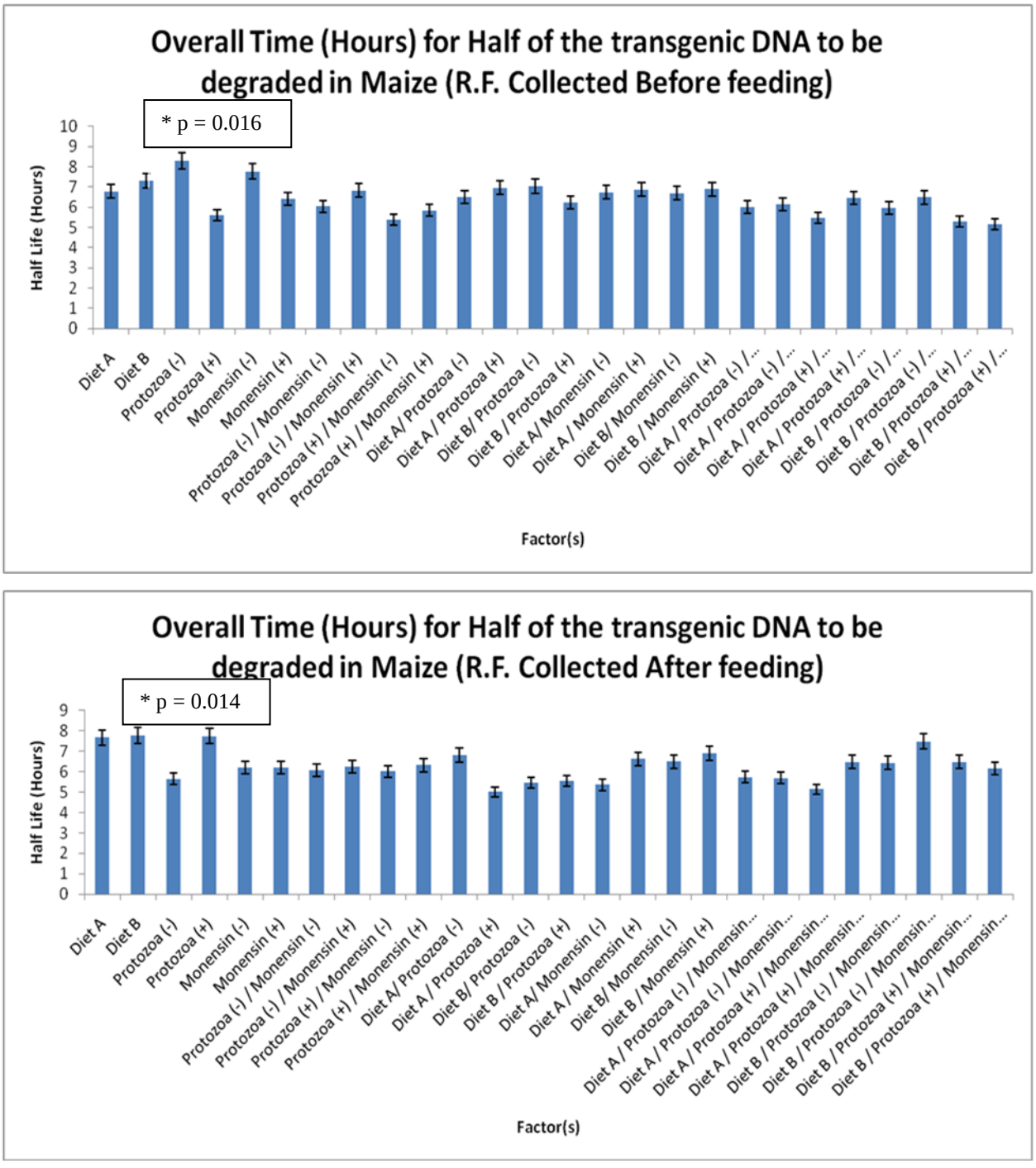
Figure 4 – Survival time of maize DNA in rumen fluid extracted before feeding



The main factor affecting DNA survival, for all types of GM plant material, was the presence or absence of protozoa. In ruminants the large protozoa, which are involved in plant fibre digestion, replicate slowly and spend much of the time attached to the rumen wall to prevent them being washed out of the rumen. Shortly after the animals are fed these protozoa detach from the wall and attach to plant material which they engulf, and degrade. This phenomenon means that the protozoal population in the rumen fluid removed from the rumen before and after feeding could be different. In these experiments protozoa had a significant effect on GM DNA survival, but had different effects depending on whether the rumen fluid had been collected before or after feeding (Figure 5). For the maize GM DNA, if the rumen fluid was collected before feeding the DNA was more rapidly degraded when protozoa were present compared to the incubation where the protozoa had been removed ($p=0.016$). In the incubation with rumen fluid collected after feeding the DNA survived for significantly longer if the protozoa were present ($p=0.014$). The biological

explanation for this is that when these feeding protozoa engulf the plant particles, the DNA within the plant material is protected from degradation by other factors in the rumen fluid.

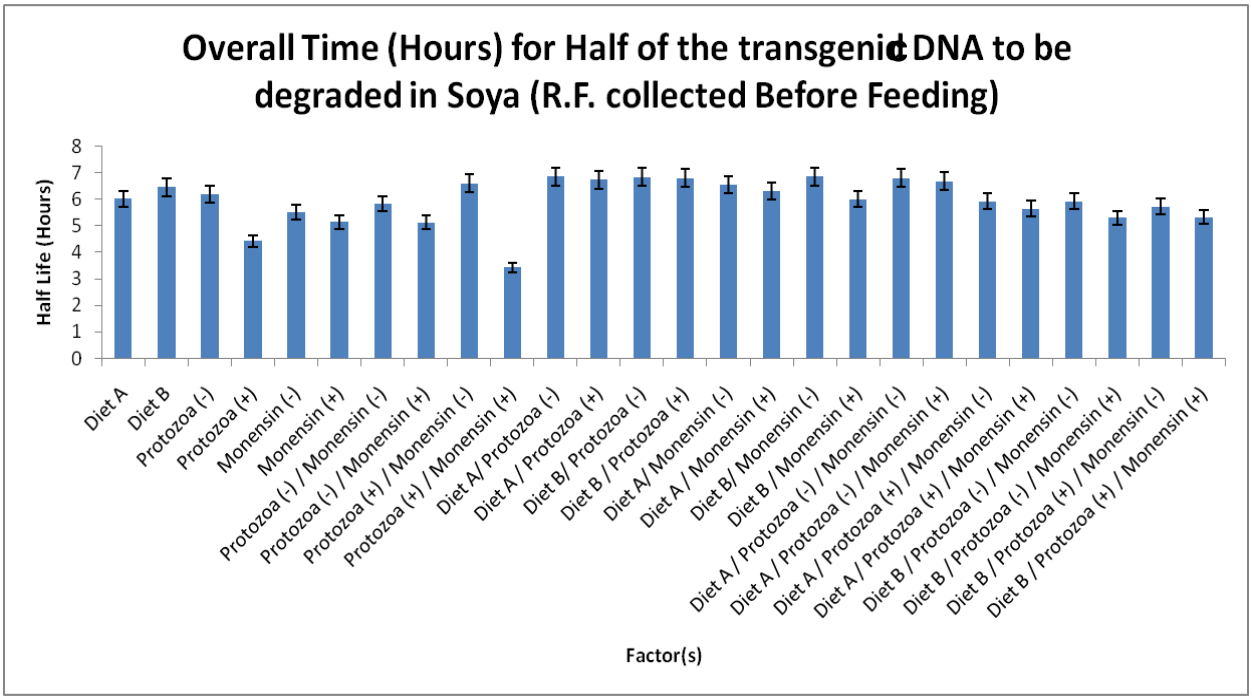
Figure 5 – Comparison of maize GM DNA survival in rumen fluid collected before or after feeding



Similar results were obtained for the survival of GM DNA in rapeseed (not shown) and soya (Figure 6). In the latter case, the presence of protozoa in rumen fluid collected before feeding reduce DNA survival time by about 2h ($p<0.001$) and a combined effect of reduced survival time was observed when protozoa were present

and Gram-positive bacteria were removed. The reasons for this are not known. A 3D representation, comparing the relative half-lives of the house-keeping and transgenes for each of the three transgenic crops is shown in Appendix 6.

Figure 6 Survival time of DNA in GM soya plant material in rumen fluid



In summary, the first experiments within objective 1, assessing the *in vitro* survival of DNA within GM plant material, can be summarised as follows, and as indicated in Table 4:

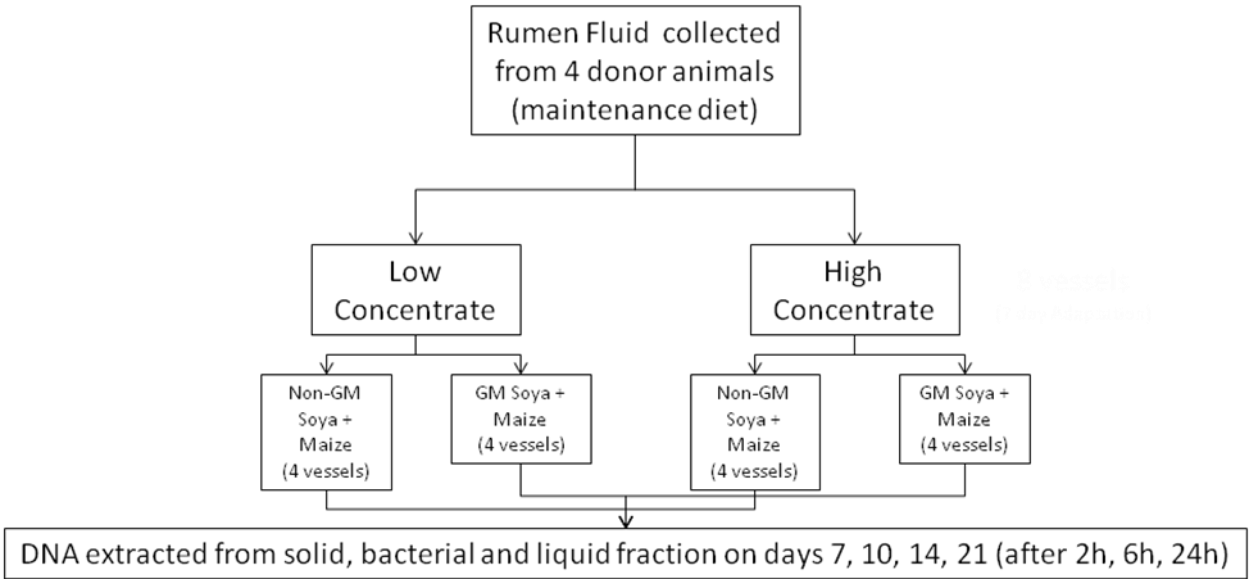
- Forage/concentrate diets do not have different effects on DNA survival
- Plant housekeeping genes can be detected for longer than GM DNA genes
- Incubation pH has no effect on DNA survival times
- Treatment with monensin does not affect survival of GM DNA
  (although GM soya DNA is degraded more quickly if protozoa are present and G+ve bacteria absent)
- The presence/absence of protozoa has a significant effect on DNA survival
  The protozoa present before feeding contribute to more rapid DNA degradation
  The protozoa present after feeding significantly increase the survival time of plant DNA
- Transgenic DNA can be detected up to 24hr after exposure of plant particles to rumen fluid in vitro

Table 4 Statistical significance of factors influencing DNA degradation

	Rapeseed		Soya		Maize	
Factor	Before Feeding	After Feeding	Before Feeding	After Feeding	Before Feeding	After Feeding
Protozoa	0.353	0.033	<.001	0.373	0.016	0.013
Monensin	0.834	0.124	0.209	0.979	0.129	0.388
Diet.Protozoa	0.258	0.392	0.3	0.115	0.107	0.027
Diet.Monensin	0.975	0.047	0.53	0.554	0.042	0.09
Protozoa.Monensin	0.495	0.676	0.037	0.952	0.586	0.289
Diet.Prsotozoa.Monensin	0.301	0.037	0.684	0.836	0.343	0.441
pH	---	0.658	---	0.217	---	0.562
Diet.pH	---	0.76	---	0.658	---	0.181

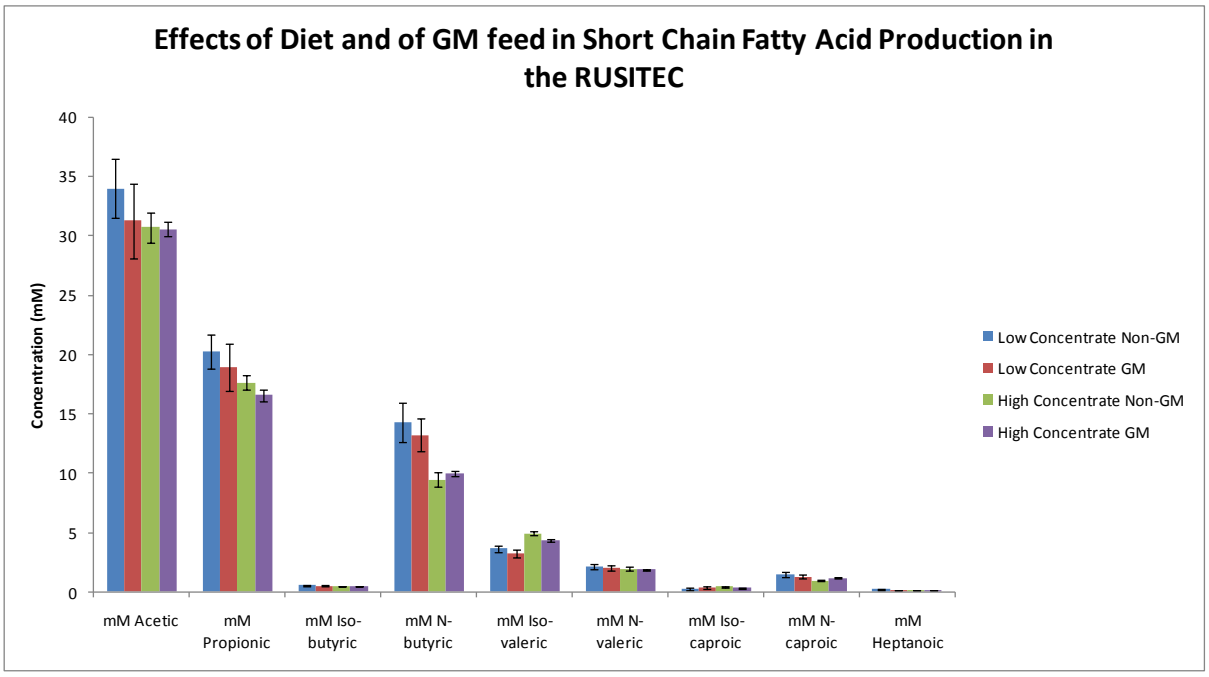
These results were used to design the experiment to address the second part of this objective, and the first part of Objective 3; specifically the **survival of DNA in GM plant material in the rumen**. These experiments were carried out using the RUSITEC model system to simulate conditions in the rumen (Figure 1), and the experimental outline is shown in Figure 7. The degradation of only GM Maize and soya DNA were assessed as they had been found to survive for longer than oilseed rape in the preliminary experiments. Both types of DNA could be added to the same RUSITEC vessels as the detection primers for both the GM DNA and housekeeping genes were different, and would distinguish the DNA during amplification (Table 3). All vessels were maintained at the same pH in order to maintain a consistent fermentation profile and microbial composition. Varying the pH had not affected DNA survival in the first experiments, and monensin treatment was not used. Following DNA extraction, qPCR was again used to assess the relative survival/degradation of the GM DNA within the plant material.

Figure 7 – Schematic of RUSITEC experiment to assess the release and survival of transgene DNA from plant material in the rumen



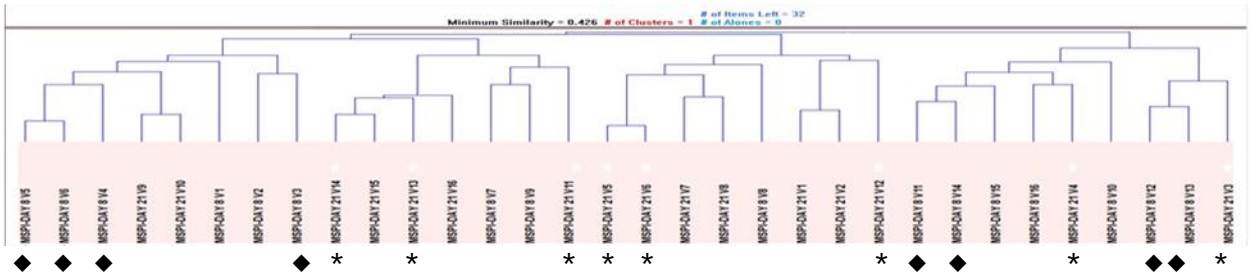
The integrity of the RUSITEC system throughout the experiment was additionally monitored by confirming that both the bacterial profile and the production of short chain fatty acids (SCFA) (Figure 8) was not different between those vessels to which transgenic plant material was added and the control vessels containing normal plant material.

Figure 8 Measurements of SCFA concentrations in samples removed from RUSITEC vessels on days 20 and 21. Figures quoted are averages of all vessels over the two days.



The microbial composition in each individual RUSITEC vessel was monitored by tRFLP analysis, as described in Appendix 5. Total DNA extracted from the vessels was amplified using the generic primers 27f and 1389R which amplify 16S rDNA from all eubacteria (Table 3). For use in tRFLP analysis the forward primer (27F) was purchased with the fluorescent Cy5 label on the 5'-end. The resulting PCR amplicons were digested individually with one of three different restriction enzymes (MspI, HaeII and HhaI). The resultant bands were then separated using the Beckman CEQ8800 DNA sequencer and the banding profiles analysed using the integral fragment analysis program. The dendrograms generated for each of the separate restriction enzymes used indicated that addition of GM or non-GM plant material did not have different effects on the bacterial composition in the RUSITEC vessels (Figure 9). It is clear that the profiles from the different vessels, on the 2 days analysed, are randomly distributed.

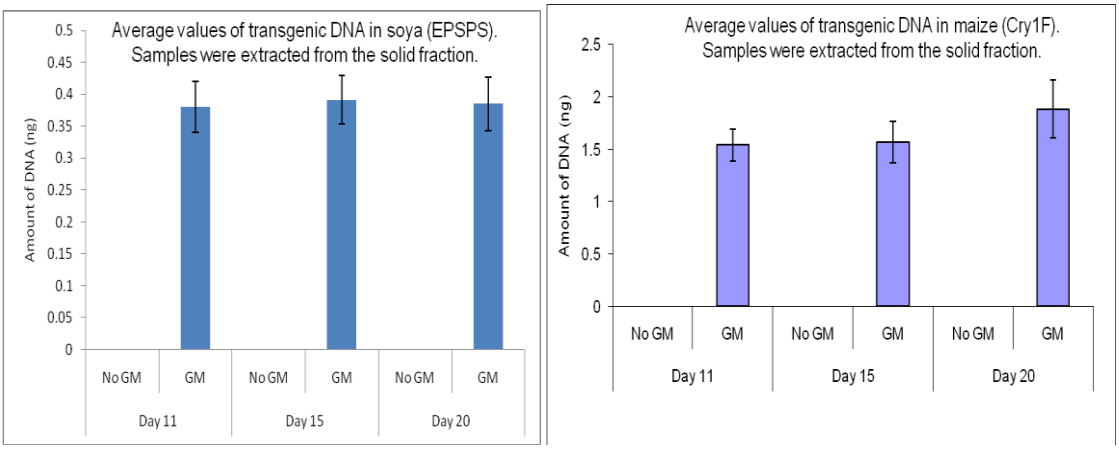
Figure 9 Dendrogram generated using data from restriction enzyme MspI



The position of vessels 3, 4, 5, 6, 11, 12, 13, 14 which received GM plant material on day 8 (◆) and day 21(*) is shown.

The DNA extracted for the solid (plant material), solid associated bacteria and liquid fraction was amplified by qPCR, in triplicate, using the 4 primer sets specific for the housekeeping and GM genes in the transgenic soya and maize (Table 3). Transgenic DNA was readily detected in the solid fraction (plant material) even after 24h but never in the bacterial fractions – either solid-associated or planktonic (Figure 10). As expected, transgenic DNA was not detected in control vessels (containing non-GM material). However there was a lot of variability in the ability to detect the GM DNA in the different vessels.

Figure 10 Detection of transgenic DNA sequences (ng DNA remaining) in residual plant material in the RUSITEC vessels



GM/NoGM- RUSITEC vessels to which transgenic soya or maize were added respectively. Values given are averages for four vessels.

The overall conclusion from this work is that uptake of GM DNA from plant material by rumen bacteria could not be detected using qPCR.

<u>Objective 2</u> Investigate the influence of regions of DNA homology upon acquisition of DNA by rumen bacteria.

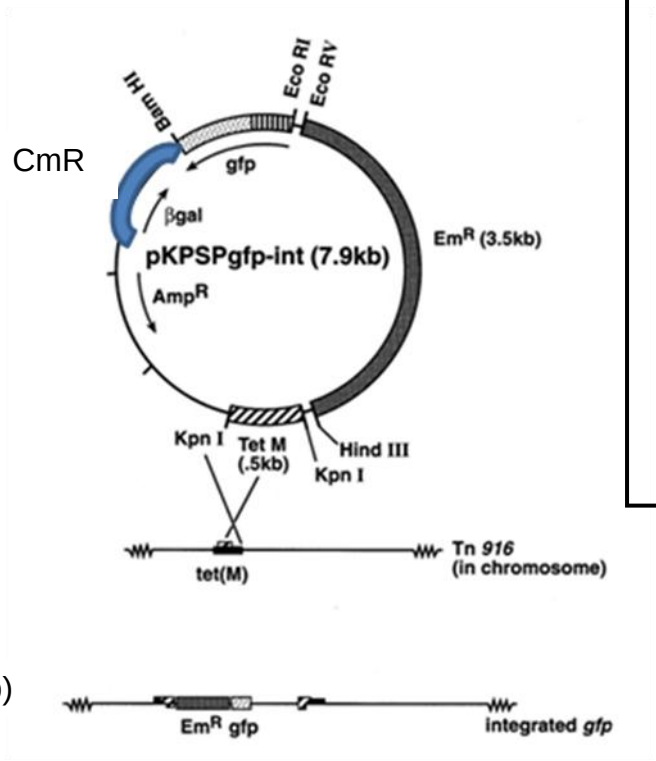
The presence of homologous DNA sequences may increase the likelihood of DNA acquisition by rumen bacteria. Thus any GM crops that retain any of the bacterial sequences used in their construction – for example selective antibiotic resistance markers – may be more likely to be acquired by rumen bacteria. In order to test this hypothesis, the persistence and acquisition of model constructs, specifically designed to contain regions of bacterial DNA, under rumen conditions was assessed.

Task 2.1. Preparation of GM DNA constructs

Two distinct types of DNA were prepared. The **circular plasmid DNA** was a construct containing the backbone of the *E. coli* plasmid pBluescript, modified to contain the green fluorescent protein (gfp) and an erythromycin resistance (Em^R) marker gene. In addition this construct contains a short 500bp sequence of the tetracycline resistance gene *tet*(M). This plasmid was prepared in a previous FSA-funded project (FSG01007). However many rumen bacteria are erythromycin resistant, so the presence of the Em^R marker gene does not provide a strong positive selection. This plasmid was modified to include a chloramphenicol resistance gene (Cm^R) (Figure 11a). The very low background Cm^R in the rumen was confirmed by plating out a rumen fluid sample on anaerobic plates containing 10µg/ml chloramphenicol. The gfp gene originates from a salt water jelly fish, and there are no homologues in any rumen bacteria. This means that there is no background of this marker in rumen contents, making it a very strong selective marker gene. The green fluorescence can be visualised by exposing cells or bacterial colonies grown aerobically to long wave UV light.

Figure 11 Diagrams of plasmid and linear model GM DNA constructs

a). Plasmid pKPSPgfp-int-Cm



This plasmid cannot replicate in Gram +ve bacteria but can recombine into the genome of any bacteria containing the *tet(*M) gene.

The plasmid confers Em^R, Cm^R and gfp fluorescence

The linear DNA contains the Tn916 backbone and confers Em^R and gfp fluorescence

b)

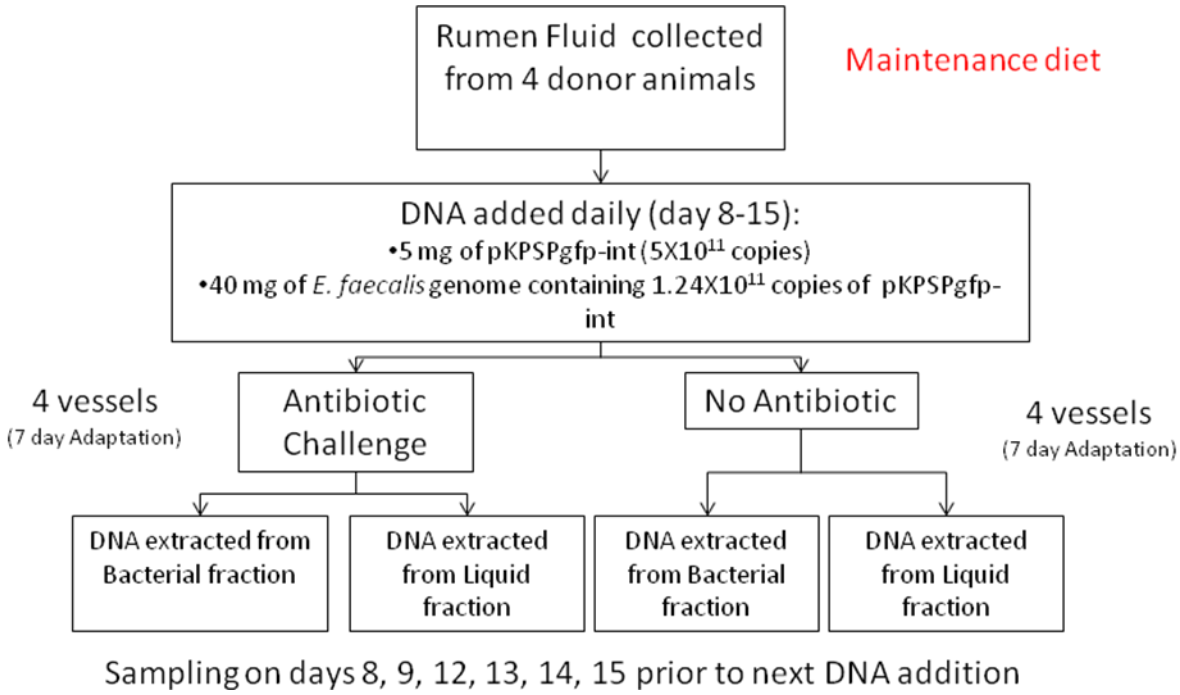
Prior to insertion of the Cm^R gene, the plasmid, had been integrated into the conjugative transposon Tn*916* as a consequence of homologous recombination with the *tet*(M) gene on this transposon (Figure 11b; Mercer et al 2001; Scott et al 2001). DNA purified from these transconjugants formed the **linear DNA** construct used in the DNA survival experiments (Figure 11b). This DNA can recombine into the genome of any bacteria containing Tn*916,* and has regions of homology on both sides of the gfp transgene. The integrated form of Tn*916*::pKPSgfp is able to transfer between bacteria by conjugation based on the mobility of Tn*916*. The modified transposon confers Em^R and gfp fluorescence.

Task 2.2 Detection of DNA transfer under antibiotic selection
Task 2.3 Detection of DNA transfer with two regions of homology
Task 2.4 Statistical analysis to compare DNA transfer rates

The persistence and acquisition of both forms of transgenes (with one or two potential regions of homology with prospective host bacterial DNA) was tested under simulated rumen conditions in the RUSITEC. The experimental outline is shown in Figure 12. The free DNA was added to the RUSITEC vessels each day, in the F57 nylon bags also containing 1g of RUSITEC diet, after a 7 day stabilisation period. Samples were subsequently removed daily, and the persistence of the marker genes assessed using qPCR. At the same time potential transformants were identified by plating out the total bacterial fraction on selective anaerobic agar plates. Four vessels were additionally infused with chloramphenicol from day 8. The

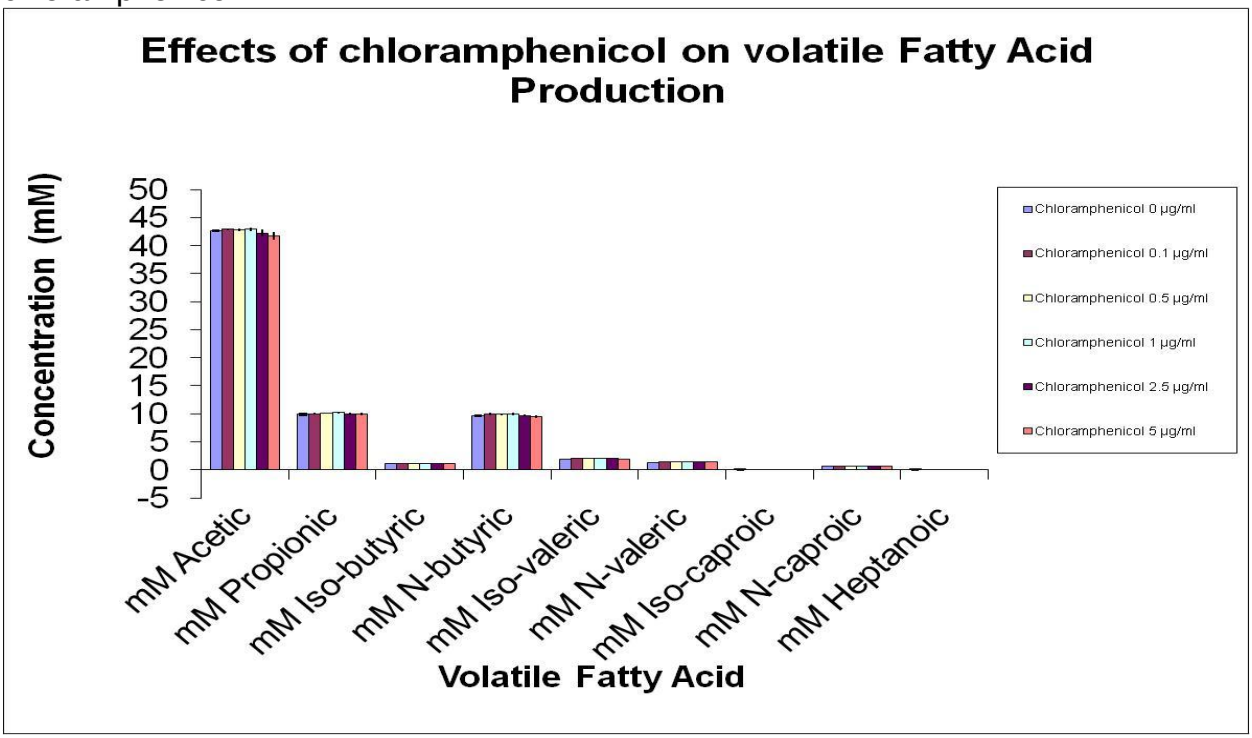
chloramphenicol was added to the feed vessels, initially at a concentration of 1μg/ml, meaning that with a turnover time of approximately 30hr it would take 1.5 days for the concentration in the RUSITEC vessels to reach this level. Infusion of the higher concentration of 5μg/ml Cm started on day 13, so that the final concentration in the RUSITEC vessels reached 5 μg/ml only on day 15.

Figure 12 Protocol to assess survival and uptake of model transgenes under rumen conditions



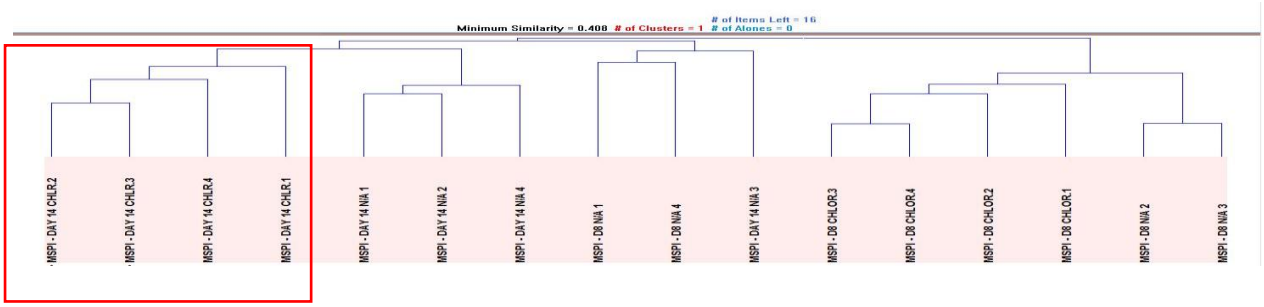
As before the integrity of the RUSITEC system was confirmed by checking the SCFA production, and also investigating the microbial profile by tRFLP analysis. Vessels with and without the addition of chloramphenicol were compared, in order to see whether this had any effect. Addition of low concentrations of chloramphenicol did not affect SCFA production, although concentrations of 2.5μg/ml or 5μg/ml had a small effect, particularly on acetate concentrations (Figure 13).

Figure 13 SCFA production in RUSITEC vessels containing increasing amounts of chloramphenicol



The infusion of chloramphenicol to the RUSITEC vessels did affect the composition of the microbial community. tRFLP analysis carried out as described previously (Appendix 5) indicated clustering of the bacterial profiles from vessels containing chloramphenicol, with all three enzymes even on day 8, after only 1 day of antibiotic addition (Figure 14).

Figure 14 Dendrogram indicating clustering of the microbial profile in vessels containing chloramphenicol following tRFLP analysis



Day 14, plus chloramphenicol

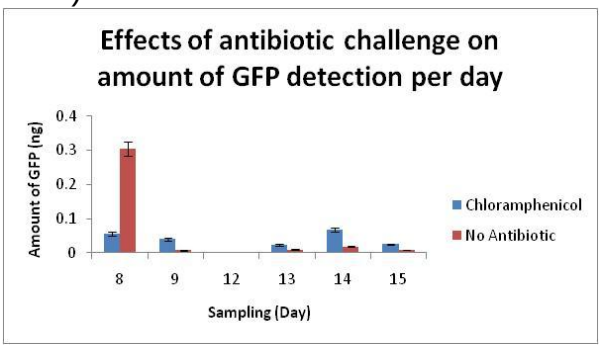
Detection of transgenes by qPCR

The amount of DNA sequences encoding the gfp and Cm^R genes detectable in the solid-associated and liquid bacterial fractions on different days were quantified by qPCR. Detection of the gfp marker generally seemed to be higher in vessels with Cm pressure, but there was considerable variability in the detection of the transgenes, even between vessels which had the same treatments, on different days, (Figure 15). The fact that the difference in the amount of GFP in each experimental group

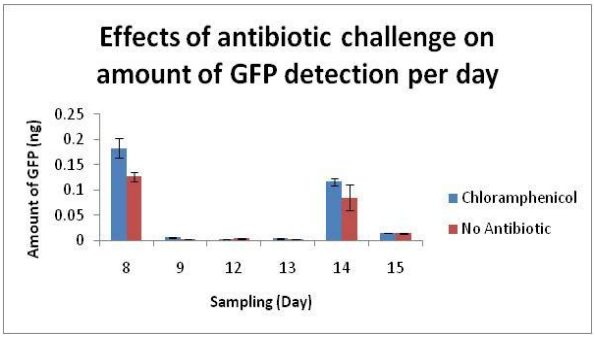
was larger than the difference between different groups, combined with the low detection frequencies, meant that following ANOVA analysis, no statistical significance could be assigned to the data ($p>0.05$ in all cases). The comparatively high detection of gfp on day 8, compared to the subsequent days, may have been due to initial DNA acquisition by competent bacteria, at a low frequency. However since antibiotic infusion had only just started, the selective pressure to maintain the integrated DNA would have been limited, and these insertion events would not have been maintained, and the bacteria may even have been washed out of the RUSITEC, which is a continuous flow culture. The free DNA itself would have survived for less than 2hr, as determined by the previous experiment, and consistent with other work (Duggan et al 2000, Mercer et al 2001).

Figure 15 Detection of the gfp marker gene in RUSITEC vessels in the solid associated (A) and liquid (B) bacterial fractions.

A)

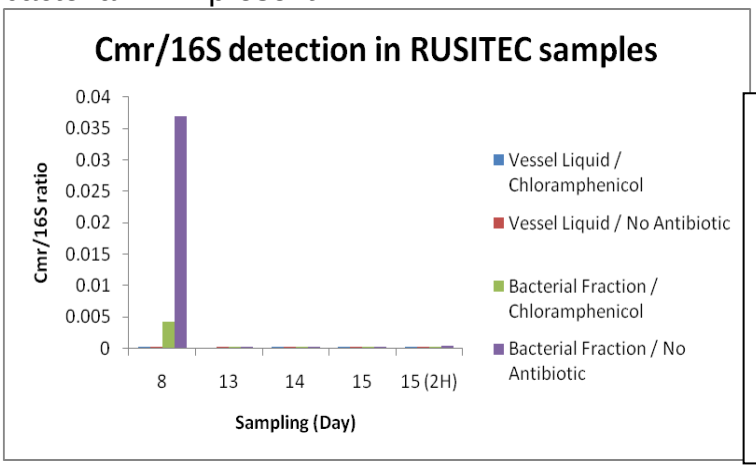


B)

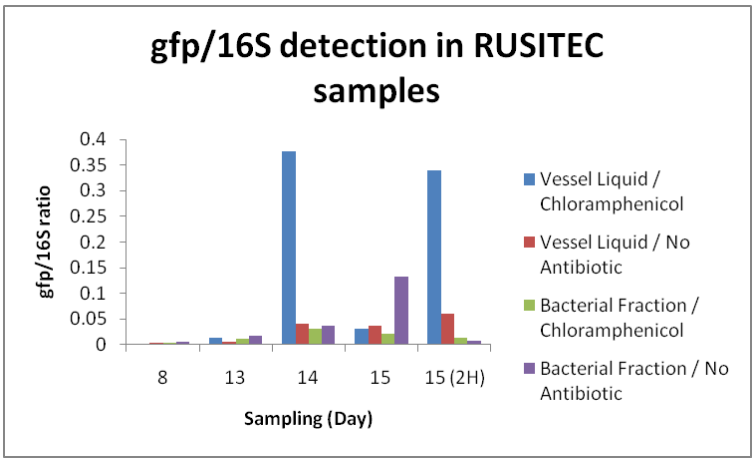


In order to try and assess the relative persistence of the two transgenes, the amount of transgenic DNA remaining was compared with the amount of total bacterial DNA present. Thus in addition to the qPCR on the gfp transgenes, specific qPCR amplification of the bacterial 16S rDNA was performed. This enabled us to calculate the relative ratios of gfp and Cm^R DNA remaining at the different time points, in the solid-associated and liquid bacterial fractions (Figure 16).

Figure 16 Relative detection of the Cm^R and gfp transgenes compared to the total bacterial DNA present



Detection of Cm^R gene only indicates level of uptake of plasmid DNA – very low.
Detection of added gfp gene in vessel liquid AND solid associated bacterial fractions = uptake of linear and plasmid DNA



The Cm^R gene was detected in the solid associated bacteria in all vessels on day 8. This is probably the result of initial transformation of naturally competent bacteria, but at this time in the experiment the total concentration of chloramphenicol (even in the vessels to which it was infused) would be negligible and thus exert no selective pressure on the bacteria to retain the plasmid which may have subsequently been lost. In contrast the detectable amount of gfp increases with time. This can either be the result of an increasing number of bacteria acquiring the DNA, replication of those bacteria which have acquired GM DNA (resulting in amplification of a single acquisition event) or horizontal transfer of the conjugative transposon between related bacteria in the RUSITEC vessels. The graphs clearly indicate that the detection of the gfp gene is highest in the liquid bacterial fractions extracted from vessels containing the higher concentrations of chloramphenicol selective pressure. The final concentration of 5μg/ml chloramphenicol built up over time, but until day 13 was much lower than this. However the considerable variation observed between vessels on the same treatments meant that there was no statistical difference between vessels containing or lacking Cm pressure. Statistical analysis was done using the non-parametric Mann-Whitney U test, but the Z scores fell within the critical range ($-1.96 < x < 1.96$), meaning that there was no difference between the treatment vessels.
Overall approximately 10 X more gfp DNA was detected compared to Cm^R gene sequences, indicating higher uptake of the linear form of DNA than the plasmid form. This implies that the presence of flanking regions of homology would improve the likelihood of transgene acquisition.

Identification of putative transformants

On each sampling day colonies were plated on selective anaerobic M2GSC agar containing Cm and Em. Many colonies grew on these plates (>100/day), but it proved impossible to identify fluorescent colonies due to the background colour of the medium. Therefore, about 300 colonies were picked from plates on day 14 that were both Cm^R and Em^R. The presence of gfp DNA was confirmed in 21 of these colonies by PCR amplification of a 500bp fragment of the gfp gene. The full-length16S rDNA genes were amplified and sequenced from these 21 colonies in order to try and identify the bacteria that had acquired the gfp gene. The longest possible assembled 16S sequence was used to search the ribosomal database to try and assign identities to the bacteria. In general an identity of >97% enables a species to be assigned to a bacterium with some certainty. Following this analysis

only 4 bacteria could be fully identified. The closest matches of all the isolates are shown in Table 5. In summary 6 were related to Enterococcus sp.; 5 were Streptococcus; 1a possible Staphylococcus while the remaining 9 were most closely related to uncultured bacteria (Table 5). Of particular potential interest are isolates 9 and 11 which are potentially new bacterial species, previously uncultured.

These bacterial isolates all expressed Cm^R and Em^R, and also contained the gfp gene making it likely that they arose due to acquisition of the plasmid DNA. Again whether there have been 21 independent DNA acquisition events, or a smaller number followed by onward transfer of the integrated plasmid DNA between related bacteria is unclear. Uptake of the linear DNA, which appeared to have occurred at 10X greater frequencies based on the results of the qPCR analysis, would not have been detected with this selective plating due to the absence of the Cm^R marker gene in the GM DNA. Due to the high level of background resistance to erythromycin in rumen bacteria, it was not possible to detect acquisition of the linear fragment by selective plating.

Table 5 Identification of transconjugant bacteria following BLAST searching of the ribosomal database with assembled 16S rDNA sequences.

Isolate	Contig Size (bp)	BLAST ID	% Identity
1	1,549	Streptococcus equinus gene for 16S rRNA, partial sequence, strain: W3	97
2	1,442	Uncultured bacterium clone 1103200830709 16S ribosomal RNA gene, partial sequence	97
3	950	Streptococcus bovis isolate Sb5 16S ribosomal RNA gene, partial sequence	94
4	896	Uncultured bacterium clone 4-5M9 16S ribosomal RNA gene, partial sequence	97
5	962	Uncultured Firmicutes bacterium clone L2j19UD 16S ribosomal RNA gene, partial sequence	97
6	1,590	Enterococcus faecium strain G4 16S ribosomal RNA gene, partial sequence	89
7	1,020	Enterococcus faecalis strain R4 16S ribosomal RNA gene, partial sequence	88
8	1,440	Uncultured bacterium clone ncd2817d04c1 16S ribosomal RNA gene, partial sequence	94
9	1,190	Staphylococcus lugdunensis N920143 complete genome	84
10	1,545	Streptococcus bovis strain NCFB 2476 16S ribosomal RNA gene, partial sequence	99
11	1,549	Uncultured Firmicutes bacterium clone L2j19UD 16S ribosomal RNA gene, partial sequence	85
12	968	Streptococcus bovis strain NCFB 2476 16S ribosomal RNA gene, partial sequence	93
13	945	Uncultured Firmicutes bacterium clone L2j19UD 16S ribosomal RNA gene, partial sequence	94
14	875	Enterococcus faecalis strain R4 16S ribosomal RNA gene, partial sequence	97
15	726	Enterococcus faecalis strain DahE1 16S ribosomal RNA gene, partial sequence	92
16	1,440	Uncultured Firmicutes bacterium clone L2j19UD 16S ribosomal RNA gene, partial sequence	98
17	1,542	Uncultured Streptococcus sp. clone T0018 16S ribosomal RNA gene, partial sequence	99
18	756	Enterococcus faecalis gene for 16S rRNA, partial sequence, strain: A19-2	87
19	894	Uncultured bacterium clone 1li10 16S ribosomal RNA gene, partial sequence	97
20	995	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: N98	98
21	667	Enterococcus sp. SI8(2011) 16S ribosomal RNA gene, partial sequence	94

<u>Objective 3</u> Assess the release and transfer of transgene DNA under conditions prevailing in the rumen using a metagenomic approach

In Objective 2, the identification of bacteria acquiring the GM DNA constructs was based on cultivation of Cm^R, Em^R isolates. This precluded the selection of any bacteria only acquiring the linear DNA (no Cm^R phenotype) and also any unculturable bacteria acquiring either DNA type. In addition it is possible that the rumen protozoa themselves (which were protective towards degradation of the DNA present in GM plant material), may acquire the GM transgenes. In order to detect any such transfer events a total rumen metagenomic DNA library was prepared to identify clones containing selectable marker genes. Subsequent sequencing of positive clones would enable identification of DNA sequences flanking the insertion site, and could facilitate identification of the microbial host.

qPCR analysis had indicated that there were most potential DNA acquisition events on days 14 and 15. DNA was extracted from the solid and liquid fractions of samples removed from the RUSITEC on days 14 and 15 using a specific procedure for the isolation of high molecular weight DNA (See Appendix 1). Following isolation the DNA was randomly sheared into pieces about 45 Kb in size and finally 5µg cloned into the pCC1FOS vector. Following packaging the clones were plated out on selective plates containing Cm (marker in fosmid) and Em (marker on potential inserts). A calculation had indicated that in order to have a 95% probability of detecting the gfp in the fosmid library due to acquisition of plasmid or linear DNA, a total of 160 or 461 clones would have to be screened. In total 2000 of 8000 possible colonies were picked from the plates containing the metagenomic library. Unfortunately, even after storing the culture plates aerobically in the fridge to optimise the intensity of the gfp fluorescence, it proved impossible to identify fluorescent colonies either visually or using the spectrophotometer. Thus the 2000 colonies were pooled in groups of 10, and each group amplified by PCR to detect the gfp gene. Eight positive samples were obtained and the colonies within these mixed samples were amplified individually to identify 8 individual clones that contained the gfp DNA sequence. Thus a maximum of 0.4% of metagenomic clones contained transformed DNA.

Attempts to sequence these fosmid clones using fosmid and outward pointing gfp and Tn*916* primers to try and identify the regions flanking the DNA insertion site were not successful. Problems are often experienced in sequencing fosmid clones due to the size of the DNA fragment and the concentration of purified DNA. Whilst these problems could possibly have been overcome in time with further optimisation, to save time it was decided to sequence the 8 clones in full using the 454 sequencing facility at Aberystwyth University. This type of sequencing generates short sequence reads that are then assembled into contigs. The sequences of these contigs were screened against the database of genomic DNA sequences using the BLASTn programme to try and identify both the location of the gfp sequence, and elucidate the surrounding host DNA, to identify which bacterium had been transformed. Unfortunately, despite assembling approximately 150 DNA sequences of 500-900bp from each fosmid clone, the majority of these sequences matched to either *E.coli* genomic DNA, or to phage vector DNA. Sequences corresponding to the gfp gene or Tn*916* were not identified. This could have been because the purified fosmid DNA was contaminated with *E. coli* DNA since the fosmids were maintained in *E. coli* hosts, or because the inserts had not been stably maintained in the fosmids. It is unlikely that all the transformed bacteria were actually Gram-negative bacteria related to *E. coli*, as very few such bacteria were identified in Objective 2, following isolation of transformed bacteria (Table 5). The sequencing procedure was repeated with a further purification of DNA, but the same results were obtained (most sequence matches to *E. coli* DNA). It may be that the inserts were lost at some point in the purification procedure, which would mean that a new metagenomic library would have to be constructed in order to try and obtain fosmid clones with stable inserts for sequencing. Unfortunately there was no time left within this project to do this, but the samples are under storage and this will form a short student project next summer.

It is likely that at least some of these transconjugants would have arisen due to onward transfer of either the original interrupted Tn*916* or of a modified Tn*916* which acquired the gfp following an initial integration event. It would be relatively easy to test each transconjugant for the ability to act as a donor of the Tn*916*/gfp construct in laboratory matings, to determine the proportion that can act as donors of mobile DNA sequences.

The overall conclusion from this part of the work is that model GM DNA can be taken up by different species of rumen bacteria, under simulated rumen conditions, but at low frequencies. Comparing relative detection frequencies of the gfp gene (present in both linear and plasmid constructs) and the Cm^R gene (only present in the plasmid) indicates that the presence of two regions of homology flanking the transgene, is important for GM DNA uptake.

Conclusions and Recommendations

This project was initiated to assess the survival and potential for transfer of GM DNA under rumen conditions. **GM DNA contained within GM plant material was detectable in rumen fluid for 24h**, but was only ever detectable in the solid fraction containing residual plant material. **There was no detectable uptake of transgenic DNA from plant material by rumen bacteria**. This supports earlier work (Duggan et al., 2000, 2002) describing rapid DNA degradation in rumen fluid. In a model of the worst case scenario, in which the GM DNA contained sequences of bacterial DNA, **GM DNA could be acquired by a range of different bacterial species if appropriate homologous DNA sequences were present**. However these acquisition events were rare and random, and completely dependent on the presence of homologous DNA sequences. **The frequency of uptake of transgenic DNA by rumen bacteria was increased by actively selecting for acquisition of the transgene**, but the rarity and random nature of the transfer events meant that the frequency of DNA uptake could not be assessed statistically. Linear DNA with flanking regions of DNA homology was detected in bacteria at higher frequencies than plasmid DNA. This could be due to both greater uptake of linear DNA and the greater potential for horizontal transfer of the acquired DNA following initial acquisition events. The conjugative Tn*916* transposon, interrupted with the marker genes (gfp and Em^R), was shown in previous work to still be capable of conjugative transfer (Scott et al., 2000). In order to enumerate only events pertaining to acquisition of the linear DNA, a non-mobile version of the transposon would have to be developed. The range of bacterial isolates that acquired the gfp gene that were able to be identified (Table 5) are both naturally transformable and are established hosts for Tn*916*. Thus they are likely to contain the sequences necessary for homologous recombination with the incoming DNA, and are able to act as both donors and recipients of Tn*916* sequences – modified or native Tn*916*.

Suggestions for extensions to the work and areas that deserve further investigation.

There are several pieces of work that could feasibly be done to add value to this project.

1. Further analysis of the 21 transconjgants. Test each transconjugant for the ability to act as a donor of the Tn*916*/gfp construct in laboratory matings, to determine the proportion able to act as donors of mobile DNA sequences.

   In addition those isolates that have closest similarity to uncloned bacteria, or which have very low matches to any known cultured bacteria could represent important new bacterial isolates, and their phenotypes should be investigated fully.

2. A further metagenomic library could be prepared, and positive clones sequenced to establish whether linear and plasmid DNA were acquired by different bacterial species. (Points 1 and 2 could be a summer project).

3. In the DNA survival experiments that were carried out in the RUSITEC using the model GM DNA, both types of DNA were added together to the same vessels. Whilst we were able to estimate the relative incidence of acquisition of the two DNA types, it would be useful to compare the rates of detection of the gfp in separate, parallel experiments. Similarly, it took 7 days for the concentration of antibiotic to reach 5μg/ml in this RUSITEC, and thus apply a strong selective pressure. In a future experiment it would be useful to see if the rate/frequency of GM-DNA uptake increased with greater selective pressure being applied from the start. Furthermore, the way in which the linear DNA with flanking regions of DNA homology was constructed meant that it was independently mobile, making it impossible to differentiate initial uptake events from subsequent horizontal gene transfer of mobile DNA. This would only be possible if the genes encoding the mobility functions were disabled.

4. The differences in DNA survival in the presence/absence of protozoa before and after feeding were attributed to the presence of different protozoal populations in the rumen fluid removed at the two times. This could be checked relatively easily by comparing the protozoal populations in the two fractions by ribosomal DNA analysis.

5. Finally the results of this project indicated that GM (plant) DNA could not be detected in rumen bacteria unless there were regions of sequence homology present – and that even then such uptake events were extremely rare. It would be extremely useful to investigate the presence of GM DNA sequences in rumen bacteria that have been exposed to GM plant material for a prolonged time period (eg feedlot cattle in North America just prior to slaughter where they have been fed GM plants for their entire lives). Only by performing such a study, on quite a large scale (sampling several different farms and comparing with organic farms in the UK, certified GM-free) would it be possible to state more definitely that GM plant material does not get acquired by rumen bacteria (at least in the lifetime of an animal raised for beef production), by facilitating the detection of rare events. This aspect of our original proposal would give important information on the ultimate safety of GM plants as a feedstuff.

Outputs from the project

Rowett-INRA conference (Aberdeen, June 2010) poster:

Minas, K., Scott, K.P., McEwan, N.R. and Newbold, J. Survival of genes from Genetically Modified (GM) plants in the rumen

International Symposium on the Anaerobic Microbiology (Smolenice, June 2011) poster:

Minas, K., Scott, K.P., McEwan, N.R. and Newbold, J. Examination of the factors influencing transgenic and endogenous DNA survival in rumen fluid.

Expected Publications:

Minas, K., McEwan, N.R., Newbold, J. and Scott, K.P. Optimisation of a High-throughput CTAB-Based protocol for the extraction of qPCR-grade DNA from rumen fluid, plant and bacterial pure cultures. FEMS Microbiology Letters *in press*.

Minas, K., Scott, K.P., McEwan, N.R., Newbold, J. A comprehensive study of factors influencing tgDNA survival in the bovine rumen and the effects of GM crops in bacterial fermentation. [Proposed journal - British Journal of Animal Nutrition or Journal of Dairy Science.] *in preparation*

Minas, K., Scott, K.P., McEwan, N.R., Newbold, J. Examination of *in vitro* DNA survival in a system that is physiologically close to the rumen [Target journal not finalised.] *in preparation*

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g) References

Delano, J., Schmidt, A.M., Wall, E., Green, M. & Masri, S. (2003) Reliable detection and identification of genetically modified maize, soybean and canola by multiplex PCR analysis. *Journal of Agriculture and Food Chemistry* **51:** 5839−5834.

Duggan, P.S., Chambers, P.A., Heritage, J. & Forbes, J.M. (2002) Fate of genetically modified maize DNA in the oral cavity and rumen of sheep. *British Journal of Nutrition* **89:** 159-166.

Duggan, P.S., Chambers, P.A., Heritage, J. & Forbes, J.M. (2000) Survival of free DNA encoding antibiotic resistance from transgenic maize and the transformation activity of DNA in ovine saliva, ovine rumen fluid and silage effluent. *FEMS Microbiology Letters* **191:** 71–77.

Ehlers Von B., Strauch, E., Goltz, M., Kubsch, D., Wagner, H., Bendiek, H., Appel, B. & Buhk, H.J. (1997) Nachweis gentechnischer Veranderungen in Mais mittels PCR. B*undesgesundheitsbl* **40:** 118-121.

Gebhard, F. & Smalla, K. (1998) Transformation of *Acinetobacter* sp. strain BD413 by Transgenic Sugar Beet DNA. *Applied and Environmental Microbiology* **64:** 1550-1554.

Hongoh, Y., Yuzava, H., Ohkuma, M. & Kudo, T. (2003) Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. FEMS

Microbiology Letters **221:** 299-304.

Martın-Orue, S.M., O'Donnell, A.G., Arino, J., Netherwood, T., Gilbert H.J. & Mathers, J.C. (2002) Degradation of transgenic DNA from genetically modified soya and maize in human intestinal simulations. *British Journal of Nutrition* **87:** 533–542.

Mercer D. K., Melville, C. M., Scott K. P. & Flint H. J. (1999) Natural genetic transformation in the rumen bacterium *Streptococcus bovis* JB1. *FEMS Microbiology Letters* **179:** 485–90.

Mercer, D.K., Scott, K.P., Melville, C.M., Glover, L.A. & Flint, H.J. (2001) Transformation of an oral bacterium via chromosomal integration of free DNA in the presence of human saliva *FEMS Microbiology Letters* **200:** 163-167.

Nielsen, K.M. & Townsend, J.P. (2004) Monitoring and modelling horizontal gene transfer. *Nature Biotechnology* **22:** 1110-1114.

Nielsen, K.M., Van Weerelt, M.D.M., Berg, T., Bones. A,M., Hagler, A.N. & Van Elsas, J.D. (1997) Natural transformation and availability of transforming DNA to *Acinetobacter calcoaceticus* in Soil Microcosms. *Applied and Environmental Microbiology* **63:** 1945-1952.

Scott, K.P., Mercer, D.K., Richardson, A.J., Melville, C.M., Glover, L.A. & Flint, H.J. (2000) Chromosomal integration of the green fluorescent protein in lactic acid bacteria and the survival of introduced strains in human gut simulations. *FEMS Microbiology Letters* **182:** 23-27.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. & Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173:** 697-703.

h) List of Appendices