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Developing better methods for culturing rumen bacteria

A Confidential Report prepared for The Ministry of Agriculture and Forestry

Dr Peter H. Janssen, Ms Nikki Kenters and Dr Gemma Henderson

30 June 2008

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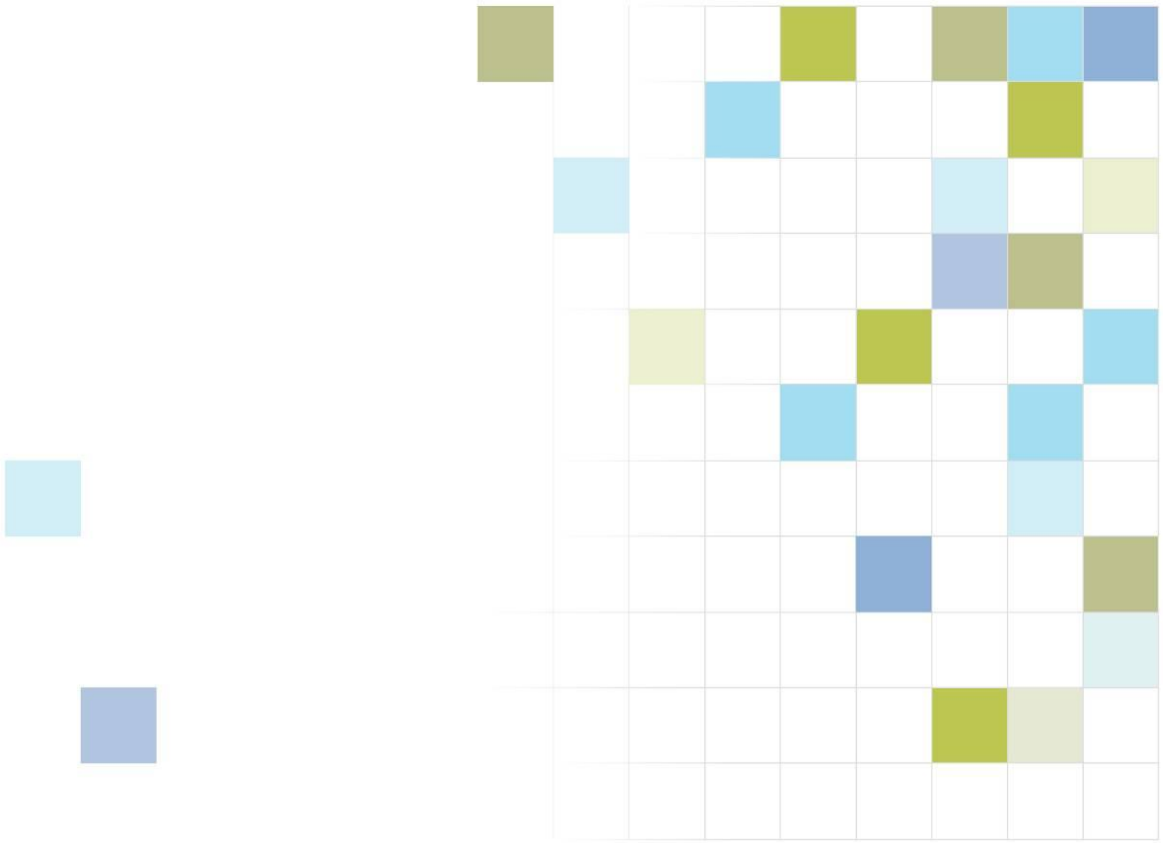

Developing better methods for culturing rumen bacteria

Agreement Number: CC MAF POL_2008-27 (138-4)

Confidential Final Report

Prepared for: **The Ministry of Agriculture and Forestry**

30 June 2008

Dr Peter H. Janssen (Programme Leader),

Ms Nikki Kenters and **Dr Gemma Henderson**

Food, Metabolism & Microbiology Section
Food & Textiles Group
AgResearch Limited
Private Bag 11008
Palmerston North

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TABLE OF CONTENTS

Summary **4**

Context of the project **4**

Approach **4**

Outcomes **5**

Summary **5**

Publications **5**

Science Report **6**

1. Introduction **6**

1.1 General introduction **6**

1.2 Developing new growth media **6**

1.3 Optimising a high-throughput culturing method **7**

1.4 Testing success in culturing approach **10**

2. Materials and Methods **11**

2.1 Sheep and sampling **11**

2.2 Medium preparation **11**

2.3 Preparation of rumen fluid and substrates **11**

2.4 Vitamin 10 concentrate **12**

2.5 Viable counts **13**

2.6 Isolation experiments **13**

2.7 DNA extraction by heating cultures **14**

2.8 DNA extraction using a Chelex resin **14**

2.9 PCR amplification of 16S rRNA genes **15**

2.10 Microscopy **16**

2.11 Determination of fermentation products **16**

2.12 Storage of cultures **16**

3. Results **17**

3.1 Development of growth medium **17**

3.2 Development of dilution approach **17**

3.3 Estimation of MPN of rumen heterotrophs **18**

3.4 Isolation of rumen bacteria **20**

4. References **22**

SUMMARY

Agreement Number and Title: CC MAF POL_2008-27 (138-4)

Business/Institution: AgResearch Limited

Programme Leader: Dr Peter H. Janssen

Goal:
This project aims to develop better methods for culturing rumen bacteria, to make them available for more detailed investigation.

Context of the project:
The major precursor of the methane formed in the rumen is hydrogen. This hydrogen is produced during the fermentation of feed by the rumen microbial community, and is used by methanogens to produce methane. Some of the fermentation pathways operating in the rumen produce little hydrogen and hence contribute little to methane formation. Other, parallel, fermentation pathways produce large amounts of hydrogen, and lead to significant amounts of methane. Preferentially stimulating the activity of the bacteria that use the pathways that produce little hydrogen will result in less methane formation. This approach can be termed “hydrogen management”. Much of the research into methane mitigation has focussed on inhibition of methanogens, the microorganisms that convert hydrogen to methane. Research into “hydrogen management” by manipulating the hydrogen producing bacteria is, in contrast, not being pursued, because of the lack of knowledge of the bacteria involved. This project begins a new line of research by making the first steps towards overcoming the lack of understanding of hydrogen-producing bacteria in the rumen.

Approach:
The composition of microbiological media is nearly always a compromise between mimicking the environment and ease of preparation. For media used to culture rumen microbes, ease of preparation is favoured over mimicking the rumen conditions. Using previous experience in developing media for anaerobic sediment bacteria and aerobic soil bacteria, new medium formulations were trialled to produce growth media that chemically mimic the rumen. Culturing anaerobic microorganisms is generally a tedious process that limits the number of isolates, severely limiting the chances of isolating so-called uncultured species. Statistical treatment of dilution theory was applied to anaerobic culture techniques to produce the maximal number of pure cultures of rumen bacteria within any given large-scale isolation experiment. Bacteria isolated using the new media and high-throughput culturing approach were identified by sequencing their 16S rRNA genes, to confirm that the approach could isolate members of the uncultured majority of rumen bacteria

Outcomes:

A new medium formulation that chemically mimics the rumen was devised, and a simple way of preparing this medium was developed. Statistical treatment of dilution theory was applied to anaerobic culture techniques to produce the maximal number of pure cultures of rumen bacteria within any given large-scale isolation experiment. The calculated expected number of pure cultures was compared with the actual number obtained, and this confirmed the applicability of the approach. Bacteria were isolated using the new media and high-throughput culturing approach, and were identified by sequencing their 16S rRNA genes. Bacteria belonging to lineages known to exist in the rumen but not previously grown in pure culture, were identified among the collection of bacteria isolated in the high-throughput culturing step. Approximately half of the 78 new isolates appeared to be members of 10 new genera.

This project has generated a new approach to rapid isolation of large numbers of rumen bacteria, including representatives that form part of the so-called “uncultured majority”. This approach has provided isolates for detailed study to better understand why some rumen bacteria produce large amounts of hydrogen and so lead to large amounts of methane being formed. The methods are widely and easily applicable, and allow isolation of large number of new species at a fraction of the effort previously required.

Summary:

This project has developed better methods for culturing rumen bacteria, to make them available for more detailed investigation. This research underpins the development of a good fundamental understanding of rumen bacteria, which is a critical step towards manipulation and successful “hydrogen management” as a tool to mitigate rumen methane. The advance was achieved by developing better growth media, and using a statistical approach to rapidly isolate pure cultures of fermentative bacteria. Isolates of new genera of rumen bacteria were obtained that will allow a better understanding of the rumen to be achieved.

Publications:

No publications have been generated at this time. It is expected that one publication in a peer-reviewed international microbial ecology journal will be a direct outcome of this project, and that isolates obtained will be characterised and named in subsequent publications. These subsequent publications will be generated from further research stemming from isolates obtained and methods developed within this project.

SCIENCE REPORT

1. Introduction

1.1. General introduction

Ruminants themselves do not produce fibre-degrading enzymes, but they harbour bacteria, fungi and protozoa that can. The host provides the microorganisms with a suitable habitat for growth, and the microbes supply protein, vitamins, and short-chain organic acids for the animal.

Cellulose, other sugar polymers, free sugars and proteins are fermented by anaerobic microbes in the rumen, including bacteria. Recent surveys based on the presence of 16S rRNA genes indicate that there are many unstudied species of bacteria in the rumen. Krause and Russell (1996) surmised that the species diversity of rumen bacteria was much higher than the 22 species described as being dominant when they reviewed the literature. Since then, it has been shown that the rumen contains a wide diversity of bacterial species (An *et al*., 2005; Edwards *et al*., 2004; Firkins and Yu, 2006; Kobayashi, 2006; Kocherginskaya *et al*., 2001; Koike *et al*., 2003; Larue *et al*., 2005; Nelson *et al*., 2003; Ramsak *et al*., 2000; Sundset *et al*., 2007; Tajima *et al*., 1999; Tajima *et al*., 2000; Tajima *et al*., 2001). A large part of this bacterial diversity is from genus-level groups with no cultured relatives. The physiologies and properties of these uncultured bacteria are not known. The availability of cultures would greatly simplify attempts to understand their roles in the rumen.

1.2. Developing new growth media

To culture representatives of this wide diversity of rumen bacteria, it is important to mimic their natural environment in the laboratory growth medium. Any cultivation approach has to be a compromise between providing conditions that approach those the bacteria grow under in their natural environment, and requirements for the human-scale microbiologist attempting to culture them. Developing such a medium is the aim of objective 1.

Objective 1

Objective Title:	Develop growth media
Objective Leader:	Peter H. Janssen
Description:	To develop growth media that better mimic the rumen environment.
Methodology:	The composition of microbiological media is nearly always a compromise between mimicking the environment and ease of preparation. For media used to culture rumen microbes, ease of preparation is favoured over mimicking the rumen conditions. Using previous experience in developing media for anaerobic sediment bacteria and aerobic soil bacteria, new

medium formulations will be trialled to produce growth media that chemically mimic the rumen.

1.3. Optimising a high-throughput culturing method

Dilution culture can be used to isolate bacteria from mixed communities. By diluting the inoculum to introduce small numbers of culturable cells into a tube (or other culture vessel), a significant number of cultures can be obtained that are derived from single culturable cells. The inoculum may contain unculturable cells, but after growth, these will essentially be of no consequence. Subsequent subculture by further dilution culture will eliminate these. It should be borne in mind that mixed cultures may also be obtained, where one microorganism will grow because of the activity of another (or both may be mutually dependent on each other).

The proportion of all inoculated tubes that develop cultures derived from single cells is greatest when the expected inoculum size is 1, but at this inoculum size, only ~58% of all positive cultures will be derived from single cells. If the expected inoculum size is decreased, the number of positive cultures (i.e. receiving ≥1 cells) and the number of pure cultures (i.e. receiving exactly 1 cell) decreases, but the proportion of positives that are potentially pure cultures increases (Fig. 1).

The theory behind this is described by Button *et al*. (1993), and is presented here in a different form (and with some changes in terminology). The terminology used in the formulas is explained in Table 1.

Table 1. Definition of used terms in dilution culture theory.

C	The proportion of culturable cells in the inoculum
d	Reduction of inoculum size from the original sample to the inoculum introduced into each tube for any value of X
n	Total number of tubes receiving inoculum X
p	Proportion of growth-positive tubes; $p = z/n$
P_1	Proportion of growth-positive tubes derived from exactly one culturable cell.
p_T	Proportion of growth-positive tubes under the assumption that all cells in the inoculum are viable, i.e., $X = X_T$
r_z	Proportion of growth-positive tubes that will be derived from a single cell.
u	Number of pure cultures among n tubes with p growth positive
X_0	Number of cells in sample before dilution to prepare actual inoculum for tubes
X_T	Expected number of culturable cells introduced into a tube; $X_T = X_0Cd$
z	Number of growth-positive tubes at any value X

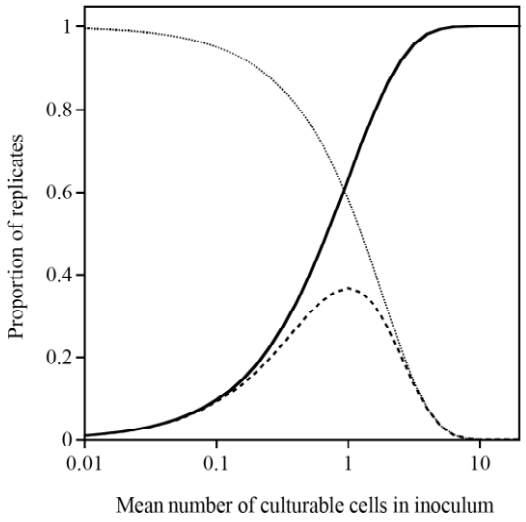

Fig. 1. Proportion of growth positive cultures as a function of the mean number of culturable cells in the inoculum of a culture (solid line), and the calculated proportion of cultures that will be inoculated with a single culturable cell (dashed line). The proportion of positive cultures that will be derived from a single culture cell decreases with increasing inoculum size (dotted line).

The proportion of growth positive tubes, p, at any value of X, is related to X and the actual culturability, C, as

$$1 - p = \exp(-CX)$$

Assuming all cells are culturable, or alternatively considering only the viable cells since the others are essentially "invisible" in the experiment (i.e., $X = X_T$), C can be set to 1, and the number of tubes that will be growth positive under that assumption, p_T, can be calculated

$$p_T = -\exp(-X_T) + 1$$

This gives the proportion of tubes that will be growth positive at any value of X_T.

The culturability, C, is the ratio of the number of culturable cells to the total number of cells. This is the term viability (V) used by Button *et al*. (1993).

We know the number of cells in the original sample, X_0, and the expected number of cells in the inoculum for any tube, which is X. This can be calculated from the proportion of the sample, d, that is used to inoculate the tube. Button *et al*. (1993) call this the dilution, d. If the sample is

diluted 1 in 100, then d = 0.01. For example, if the sample contained 10^9 cells, and 1/1000th of this was used to inoculate a tube, then d = 0.001, and $X = X_0 d = 10^9 \times 0.001 = 10^6$.

The proportion of growth-positive cultures derived from 1 cell, p_1, can be calculated as

$$p_1 = -(1 - p)\ln(1 - p)$$

where p is the proportion of growth-positive tubes. At $X = X_T$, $p = p_T$, since only the viable cells in the inoculum are considered.

The number of pure cultures, u, among the p positive tubes can be calculated as

$$u = -n(1 - p)\ln(1 - p)$$

The proportion of growth-positive tubes that contain a pure culture at any value of X_T will be

$$r_z = p_1/p_T$$

To achieve about a 90% incidence of growth-positive cultures that are derived from single cells, the inoculum should contain an expected 0.2 cells. The number of tubes that will be growth positive will be 18% of the total, and the number of tubes containing cultures derived from a single viable cell will be about 16% of the total. Using this approach is the aim of objective 2.

Objective 2

Objective Title:	Optimise high-throughput culturing methods
Objective Leader:	Peter H. Janssen
Description:	To develop new strategies for high-throughput culturing.
Methodology:	Culturing anaerobic microorganisms is generally a tedious process that limits the number of isolates, severely limiting the chances of isolating so-called uncultured species. Statistical treatment of dilution theory will be applied to anaerobic culture techniques to produce the maximal number of pure cultures of rumen bacteria within any given large-scale isolation experiment. The calculated expected number of pure cultures will be compared with the actual number obtained, to confirm the applicability of the approach.

1.4. Testing success in culturing approach

The aim of this project was to use a simple-to-prepare growth medium for culturing rumen anaerobes. This needed to reflect the chemical and physicochemical conditions of the rumen and allow them to be studied in a research laboratory setting. Also, an efficient method is needed to isolate many strains easily. This medium had to be used in an approach to isolate a maximum number of pure cultures with minimum effort. Separating out mixed cultures to produce pure cultures is a time-consuming and often frustrating aspect of anaerobic microbiology, and the method developed should avoid this step. Success of the approach will be determined if allows isolation of phylogenetically – novel rumen bacteria. In most studies only a few strains are cultured. The approach should yield many new species without being complicated, or requiring specialised equipment, intensive training or high levels of experience. Testing the utility of the combination of medium and dilution culture is the aim of objective 3.

Objective 3

Objective Title:	Testing success in culturing approaches
Objective Leader:	Peter H. Janssen
Description:	To show that use of optimised high-throughput culturing methods with newly-developed media allow isolation of rumen bacteria hitherto thought to be intractable to standard microbiological techniques.
Methodology:	Bacteria isolated using the new media and high-throughput culturing approach will be identified by sequencing their 16S rRNA genes. Bacteria belonging to lineages known to exist in the rumen but not previously grown in pure culture, will be identified among the collection of bacteria isolated in the high-throughput culturing step.

2. Materials and Methods

2.1 Sheep and sampling

All samples were collected from the same ruminally-fistulated sheep (Romney X, 8 years, 70–80 kg). This animal was kept at the AgResearch Grasslands Campus, and allowed to feed *ad libitum* on a rye-grass clover pasture. Samples of total rumen contents were collected from the fistula on 4 different occasions. All sampling was conducted at 10 am, within 30 min of the animal being taken from the pasture. Samples were collected in 450 ml glass containers with screw-top sealable metal lids. The containers were gassed with CO_2 prior to use, and filled to the top with sample, before being capped and brought to the laboratory for use within 30 min of collection.

2.2 Medium preparation

A bicarbonate-buffered mineral medium supplemented with vitamins was used to culture rumen bacteria. The mineral salts solution contained (per litre of medium) 1.4 g of KH_2PO_4, 0.6 g of $(NH_4)_2SO_4$, 1.5 g of KCl, 1 ml trace element solution SL10 (Widdel *et al*., 1983), 1 ml of selenite/ tungstate solution (Tschech and Pfennig, 1984) and 4 drops of 0.1% (w/v) resazurin solution. This solution was mixed and then boiled under O_2-free 100% CO_2, before being cooled in an ice bath while it was bubbled with 100% CO_2. Once the medium was cool, 4.2 g of $NaHCO_3$ and 0.5 g of L-cysteine·HCl·H_2O was added per litre. The medium was dispensed into 16 ml Hungate tubes while gassed with 100% CO_2, at 9.5 ml of medium per tube, and the tubes capped with butyl rubber seals and perforated plastic caps, with a headspace of 100% CO_2. These tubes were sterilised by autoclaving for 20 min at 121ºC. Before use, media were stored in the dark for at least 24 h. Substrates and other supplements were added to sterile media before inoculation.

2.3 Preparation of rumen fluid and substrates

Rumen contents were collected from a ruminally-fistulated cow that had been fed hay for 48 h after being on a rye-grass clover pasture. Feed was withheld from the animal after 4 pm and rumen contents collected the next day at 9.30 am. The material was filtered through a single layer of cheesecloth and then fine particulate material was removed by centrifugation at 10,000 *g* for 20 min. The supernatant (rumen fluid) was stored frozen at -20ºC. The rumen fluid was thawed before use, and any precipitates removed by centrifugation at 12,000 *g* for 15 min. The supernatant was bubbled for 10 min with 100% N_2 gas, before being autoclaved under 100% nitrogen for 15 min to remove viruses. The anoxic conditions are to limit the formation of unwanted oxidation products during autoclaving. The following was then added per 100 ml of rumen fluid while stirring under air: 1.63 g of $MgCl_2·6H_2O$ and 1.18 g of $CaCl_2·2H_2O$. This forms a heavy precipitate, which is removed by centrifuging at 30,000 *g* and 4ºC for 60 min. The supernatant is the clarified rumen fluid. Substrates and vitamins were added to this to obtain the final desired concentrations after the addition of 0.5 ml of supplemented rumen fluid to 9.5 ml of

medium. Five supplemented rumen fluid preparations were made. After preparation (see below), each was well mixed and then bubbled with N_2 gas for 15 min, before being transferred to a N_2-flushed sterile serum vial through a 0.2 µm pore size sterile filter using a syringe and needle. Two ml of Vitamin 10 concentrate (see below) was then added per 100 ml of preparation by syringe and needle.

Preparation GenRFV contained 100 ml of clarified rumen fluid, 0.34 g of D-glucose, 0.34 g of D-cellobiose, 0.30 g of D-xylose, 0.30 g of L-arabinose, 0.88 ml of Na L-lactate syrup (50%), 2 g of casamino acids, 2 g of Bacto-peptone and 2 g of yeast extract. Addition of 0.5 ml of this preparation to 9.5 ml of medium gave final concentrations of 5% (vol/vol) rumen fluid, 0.5 mM of cellobiose, 1 mM each of glucose, xylose and arabinose, 5 mM of lactate, and 1 g each of casamino acids, Bacto-Peptone and yeast extract per litre.

Preparation NoSubRFV contained 100 ml of clarified rumen fluid and 2 g of yeast extract. Addition of 0.5 ml of this preparation to 9.5 ml of medium gave final concentrations of 5% (vol/vol) rumen fluid and 1 g of yeast extract per litre.

Preparation 5GluRFV contained 100 ml of clarified rumen, 1.80 g of D-glucose, and 2 g of yeast extract. Addition of 0.5 ml of this preparation to 9.5 ml of medium gave final concentrations of 5% (vol/vol) rumen fluid, 5 mM glucose, and 1 g of yeast extract per litre.

Preparation 20LacRFV contained 100 ml of clarified rumen, 3.52 ml of Na L-lactate syrup (50%), and 2 g of yeast extract. Addition of 0.5 ml of this preparation to 9.5 ml of medium gave final concentrations of 5% (vol/vol) rumen fluid, 20 mM lactate, and 1 g of yeast extract per litre.

Preparation 5CpyRFV contained 100 ml of clarified rumen fluid, 10 g of casamino acids, 10 g of Bacto-peptone, and 10 g of yeast extract (10% (wt/vol). Addition of 0.5 ml of this preparation to 9.5 ml of medium gave final concentrations of 5% (vol/vol) rumen fluid, and 5 g each of casamino acids, Bacto-peptone, and yeast extract per litre.

2.4 Vitamin 10 concentrate

Preparation Vitamin 10 concentrate contained 1000 ml of distilled water, 40 mg of 4-aminobenzoate, 10 mg of d-(+)-biotin, 100 mg of nicotinic acid, 50 mg of hemicalcium D-(+)-pantothenate, 150 mg of pyridoxamine hydrochloride, 100 mg of thiamine chloride hydrochloride, 50 mg of cyanocobalamin, 30 mg of D,L-6,8-thioctic acid, 30 mg of riboflavin and 10 mg of folic acid. After preparation, the solution was well mixed and then bubbled with N_2 gas for 15 min, before being transferred to a N_2-flushed sterile serum vial through a 0.2 µm pore size sterile filter using a syringe and needle.

2.5 Viable counts

Total rumen content from the sheep were blended under anaerobic conditions for different time intervals (15, 20, 40, 60, 80, 100, 120 s) and different speeds (low or high settings) using a Waring blender (Waring, USA) with a glass chamber and tight-fitting rubber lid. Samples: A, 15s, low; B, 20 s, high; C, 40 s, high; D, 60 s, high; E, 80 s, high; F, 100 s, high; G, 120 s, high. The glass vessel was gassed with CO_2 prior to use, and during and subsequent manipulations. Samples treated in these ways were serially diluted in thirteen 10-fold dilutions, each in 5 replicates for the initial dilutions and 10 replicates for the more diluted steps, to give a Most Probable Number (MPN) estimate of culturable bacteria. The dilutions were made by syringe and needle after flushing them with CO_2, transferring from each dilution to a 10-fold more dilute step. All manipulations were made in Hungate tubes containing 9.5 ml of medium supplemented with 0.5 ml of GenRFV. These MPN tubes were incubated at 39ºC. Gram-staining (Gram, 1884) was carried out on the growth-positive MPN tubes after 1 week, to assess the diversity in the highest dilutions and determine the purity of the cultures. MPN counts were calculated from the tubes with positive growth, the dilution factor and tables for 5 parallel dilution series based on the statistical treatment of the counting methods (Cochran, 1950).

2.6 Isolation experiments

Forty grams of freshly collected rumen contents from the sheep were added to 360 ml of unsupplemented medium in a CO_2-gassed glass vessel capped with a rubber seal, and blended 4 times using a Waring blender (Waring, USA), each time for 20 s burst on high, with 1 min pauses between each burst. Using a large bore needle, 1 ml was transferred from the blender into a 150 ml serum vial containing 94 ml of medium supplemented with 5 ml of clarified rumen fluid, to produce a suspension containing an estimated 4×10^7 viable cells/ml. One millilitre of this suspension was then added to another serum vial containing 94 ml of medium supplemented with 5 ml of clarified rumen fluid, and this was repeated, to produce suspensions of 4×10^5, 4×10^3 and 40 viable cells/ml. Three different experiments were carried out with the final suspension. These experiments were performed on different dates, using freshly prepared suspensions from the same sheep.

First experiment: From the serum vial with an estimated 40 cells/ml, 20 ml was added to 76 ml medium supplemented with 4 ml of GenRFV, to give an 8 cells/ml dilution, then 20 ml of that suspension was added to 57 ml of medium supplemented with 3 ml of GenRFV to give a 2 cells/ml dilution, before 20 ml of that suspension was added to 57 ml of medium supplemented with 3 ml of GenRFV to give a 0.5 cells/ml dilution. The last three dilutions were used to inoculate 50 cultures each with 0.1 ml of suspension in Hungate tubes containing 9.5 ml of medium supplemented with 0.5 ml of GenRFV. These tubes were incubated at 39ºC degrees in the dark.

Second experiment: From the serum vial with an estimated 40 cells/ml, 20 ml was added to 57 ml medium supplemented with 3 ml of GenRFV, to give a 10 cells/ml dilution, then 20 ml of that suspension was added to 57 ml medium supplemented with 3 ml of GenRFV, to given a 2.5 cells/ml dilution, before 20 ml of that suspension was added to 57 ml of medium supplemented with 3 ml of GenRFV to give a 0.625 cells/ml dilution. The last 4 dilutions were used to inoculate 125 cultures each with 0.1 ml of suspension in Hungate tubes containing 9.5 ml of medium supplemented with 0.5 ml of GenRFV. These tubes were incubated at 39ºC in the dark.

Third experiment: From the serum vial with an estimated 40 cells/ml, 20 ml was added to 57 ml medium supplemented with 3 ml of GenRFV, to give a 10 cells/ml dilution, before 20 ml of that suspension was added to 57 ml of medium supplemented with 3 ml of GenRFV to give a 2.5 cells/ml dilution. The last 4 dilutions were used to inoculate 250 (1^{st} dilution), 125 (2^{nd} dilution) and 125 (3^{rd} dilution) cultures each with 0.1 ml of suspension in Hungate tubes containing 9.5 ml of medium supplemented with 0.5 ml of GenRFV. These tubes were incubated at 39ºC in the dark.

2.7 DNA extraction by heating cultures

Cells from 1 ml of growing culture were harvested by centrifuged at 14,100 *g* for 5 min. The supernatant was removed and 500 µl of DNA free water was added. The pellet was resuspended, and then harvested again by centrifugation at 14,100 *g* for 5 min. The supernatant was removed and a small portion of the pellet added to a PCR tube containing 5 µl of Taq buffer (Roche, Germany), 5 µl (25 mM) of $MgCl_2$ (Roche, Germany) and 34.5 µl of DNA free water. This mixture heated to 95 degrees for 10 min in a thermocycler, before primers, dNTPs (see below) and Taq polymerase (Roche, Germany) were added and 16S rRNA genes amplified by PCR (see below).

2.8 DNA extraction using a Chelex resin

Cells were harvested from 2 ml of growing culture by centrifugation at 14,100 *g* for 5 min. The supernatant was removed and 500 µl of DNA free water was added. The pellet was resuspended, and then harvested again by centrifugation at 14,100 *g* for 5 min. DNA was extracted from a suspension of the pellet in 200 µl 6% Chelex 100 (InstaGene Matrix; BioRad, USA) by heating at 56ºC for 30 min, then mixing by vortexing for 10 s, followed by boiling at 100ºC for 8 min and again mixing by vortexing for 10 s. Cell debris was removed from the suspension by centrifugation at 12,000 *g* for 3 min. The supernatant that was obtained was then used for PCR.

2.9 PCR amplification of 16S rRNA genes

Each reaction contained 5 µl DNA polymerase Taq buffer (Roche, Germany), 5 µl of 25 mM $MgCl_2$ (Roche, Germany), 1 µl of 27f primer (5´-AGAGTTTGATCCTGGCTCAG-3´; Lane, 1991), 1 µl 1492r Primer (5´-GGTTACCTTGTTACGACTT-3´; Lane, 1991), 1 µl dNTPs (Roche, Germany), 0.5 µl of Taq DNA polymerase (Roche, Germany), template DNA as described above and 34.5 µl DNA free water.

DNA-free water was prepared by autoclaving distilled water, followed by 0.2 µm pore size sterile filter into a falcon tube, before the distilled water was exposed to UV light for 4 h.

After an initial denaturation step at 94ºC for 4 min, amplification occurred during 35 cycles consisting of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min, extension at 72ºC for 1.5 min. Following these 35 cycles, there was a final extension step at 72ºC for 10 min. A P x 2 thermal cycler was used with a gradient PCR block (Thermo Electron Corporation, USA). PCR products were purified using the Wizard® Gel and PCR Clean-up System (Promega, USA), according to the manufacturer's instructions. The quantity of recovered amount of template DNA was measured using a ND–1000 Spectrophotometer (Nanodrop®, USA). ND-1000 v3.2.1. software (Nanodrop®, USA) was used to measure template DNA (ng/µl).

An amount of 10–30 ng/15 µl template DNA was used for sequence reactions. Partial sequencing of the 16S r RNA genes of new isolates was carried out using the sequencing primer 514r (5´-TCCGTCCATTGCCGAAGATTC-3´) (Lane, 1991). All DNA sequencing reactions were conducted by the Allan Wilson Centre Genome sequencing service. This service included fluorescent labelling of products using the BigDye^{TM} Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit, a Ready Reaction Cycle Sequencing kit cycle by sequencing PCR, subsequent removal of unincorporated fluorescent dideoxy NTPs (ddNTPs) by cleanup and precipitation of products and capillary separation on a AB13730 Genetic Analyzer (Applied Biosystems Inc., USA). Results were returned as ABI tracefiles. Sequencing data was analysed using the Staden package and associated software (Staden *et al*., 1998). Sequencing traces were base-called using *Phred* (Ewing and Green, 1998) and quality clipped using *qclip* (Staden *et al*., 1998).

The 16S rRNA gene sequences were compared with those in the GenBank database (Benson *et al*., 2007) using the BLASTN program (Altschul *et al*., 1990) to obtain an approximate phylogenetic placement by comparison to genes from classified organisms.

2.10 Microscopy

Agarose slides were prepared and a small amount of culture added as described by Pfennig and Wagener (1986). Phase contrast photomicrographs were made using a DM2500 microscope (Leica, Germany) with a 100× oil phase contrast objective, and the images captured digitally using Leica Application Suite software (Leica, Germany).

2.11 Determination of fermentation products

Isolates were grown for 7 days on medium containing only background yeast extract rumen fluid and vitamins (i.e. supplemented with NoSubsRFV), 20 mM lactate (20LacRFV), 5 mM glucose (5GluRFV) and a mixture of casamino acids, Bacto-peptone, and yeast extract (5CpyRFV). Growth was monitored in duplicate cultures, and growth on lactate, glucose or the amino acids and extract mixture assessed by comparing the culture density with the medium containing the background supplement. Samples for high performance liquid chromatography were centrifuged at 17,000 *g* for 5 min, and 1 ml of the supernatant was transferred to 1.5 ml tubes and frozen at –20°C. Prior to analysis, samples were thawed at room temperature, then 50 µl of 1 M H_2SO_4 was added and mixed. The samples were allowed to stand at room temperature for 10 min, then centrifuged at 17,000 *g* for 5 min.

Fermentation end products were separated using high performance liquid chromatograph with a Rezex 8 µm 8% H 300 x 7.80 mm organic acid column (Phenomenex, USA), in an Isocratic UV Analyzer (BioRad, USA) fitted with a Model AS-48 Automatic Sampler (BioRad, USA) and a Model 1770 Differential Refractometer (BioRad, USA). Data were collected using Shimadzu Class – VP version 5.0 software. The column was operated at 50°C with 5 mM H_2SO_4 as the mobile phase, at 0.8 ml/min.

2.12 Storage of cultures

The cultures were grown in medium supplemented with GenRFV, before 9.2% (final concentration) of glycerol was added, and the cultures then frozen at –80ºC.

Preparation anaerobic glycerol solution contained 85 ml of Salt solution A, 85 ml of Salt solution 2B, 130 ml of distilled water, 200 ml of glycerol, 2.5 g of $NaHCO_3$ and 2 drops of Resazurin (0.1% solution). This solution was mixed and then boiled under O_2-free 100% CO_2, before being cooled in an ice bath while it was bubbled with 100% CO_2. Once the medium was cool 0.25 g of L-cysteine.HCl.H_2O was added per litre. The anaerobic glycerol solution was dispensed into 125 ml serum vials while gassed with 100% CO_2, 30 ml of anaerobic glycerol solution was added per serum vial, and the serum vials capped with butyl rubber seals and metal caps, with a headspace of 100% CO_2. These serum vials were sterilised by autoclaving for 20 min at 121ºC.

3. Results

3.1 Development of growth medium

Many rumen bacteria have a requirement for components in the rumen fluid, and it is possible that some of the uncultured bacteria in the rumen have similar requirements. However, rumen fluid also contains large amounts of suspended and colloidal material that results in a cloudy medium. Since growth of bacteria can be most easily assessed by an increase in the turbidity of the medium as cell number increase, background turbidity due to the medium itself will make detection more difficult. This will be complicated even more if the concentrations of energy sources in the medium is to be kept low to mimic the steady state concentrations in the rumen, with low expected culture densities as a result.

The new medium was devised to reflect the composition of elements in rumen fluid. The composition of rumen fluid reported in 5 different studies (Durand and Kawashima, 1980; Emanuele and Staples, 1994; von Engelhardt and Hauffe, 1975; Geishauser and Gitzel, 1996; Johnson and Aubrey Jones, 1989) was used as a guide to develop a balanced salt solution that resulted in elemental concentrations within the reported ranges. Substrates and other additions were made to the sterilised salts solution from separately sterilised stock solutions. This was done to minimise the formation of products in the Maillard reaction. These products can inhibit the growth of rumen bacteria (Marounek *et al*., 1995).

Rumen fluid was clarified by precipitation of colloidal and other materials with calcium and magnesium salts. The clarified rumen fluid was then added to growth medium. The final concentrations of calcium and magnesium were analysed by plasma emission spectrometry to be 2.99 mM and 4.02 mM, respectively.

3.2. Development of dilution approach

In an attempt to isolate large numbers of pure culture isolates, a modified dilution approach was developed. In combination with an estimate of the number of viable cells (see below), it should then be possible to select an inoculum that should lead to a 18% of inoculated tubes being a growth positive, and some 90% of these being pure cultures. The method used is depicted in Fig. 2.

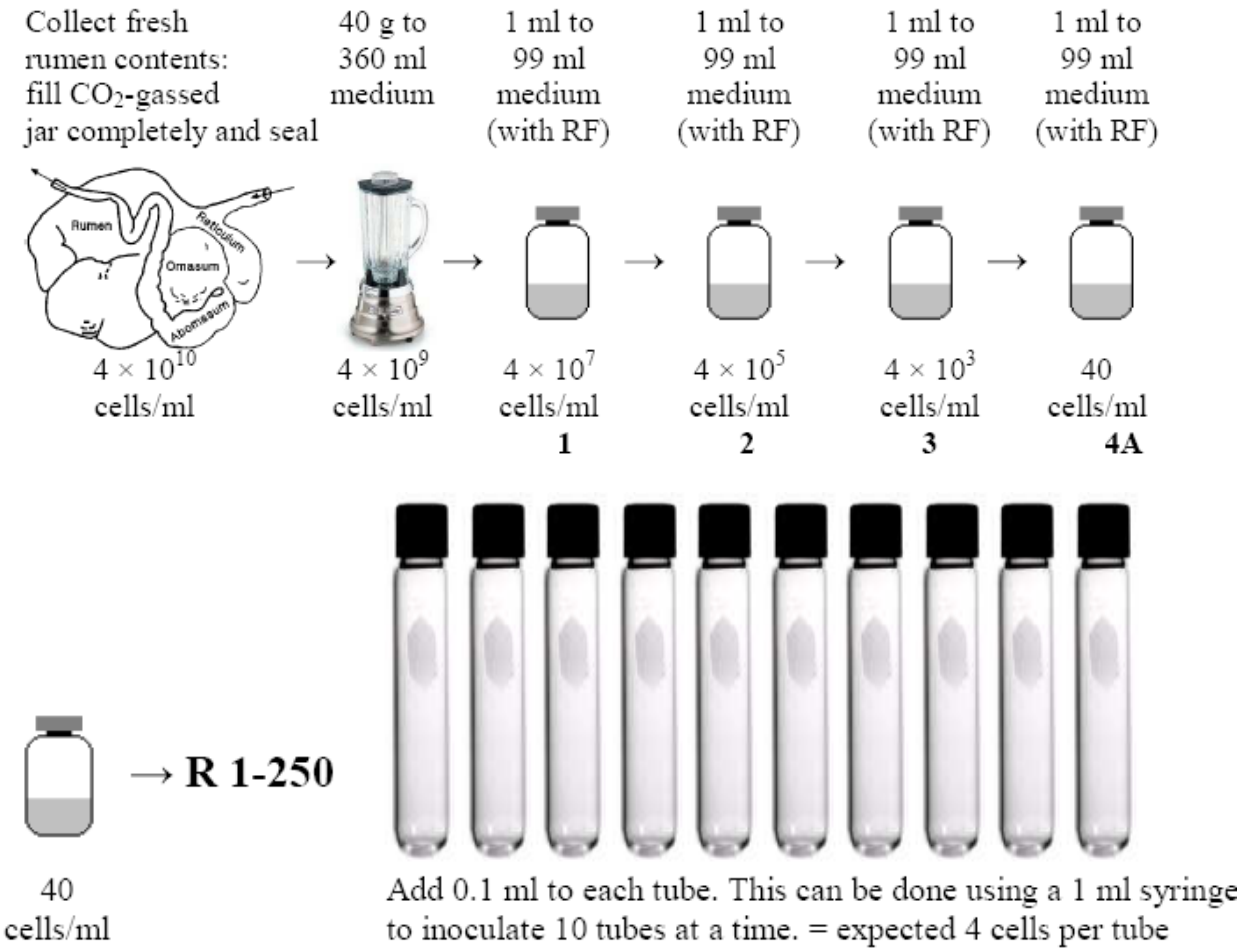

Fig. 2. Schematic of dilution approach. R 1-250 represents up to 250 replicate cultures inoculated from obe vial containing an estimate 40 cells per ml. RF, rumen fluid.

3.3. Estimation of MPN of rumen heterotrophs

Heterotrophic bacteria present in rumen samples were quantified by most probable number (MPN) counts. Using a variety of isolation procedures (A–G; detailed in materials and methods) culturable populations of heterotrophic rumen bacteria were estimated to be present at approximately 4 x 10^{10} cells per g of rumen content (wet weight). The viable counts obtained for each procedure were not noticeably different (Table 2). The MPN tubes were assessed each day, for 11 days and the MPN counts did not increase any further after 7 days incubation (Fig. 3). The greatest bacterial diversity, as determined by Gram stain microscopy, was found in MPNs prepared isolation procedure E. The greatest number of culturable cells per g (wet weight) were found in extraction procedures B, D, E and F. Isolation procedure G had the lowest culturable cells per g (wet weight). Isolation procedure E was selected for all further experimentation.

Table 2. MPN counts with lower (95%) and upper (95%) confidence limits from the different treatments A to G after 7 days of incubation.

Treatment	Blender time in seconds at high speed	Cell count per g rumen content	Lower 95% confidence limit	Upper 95% confidence limit
A	15 (low speed)	1.7×10^{10} /g	4×10^{9} /g	5×10^{10} /g
B	20	3.5×10^{10} /g	2×10^{9} /g	2×10^{11} /g
C	40	1.0×10^{10}/g	3×10^{9} /g	2.4×10^{10} /g
D	60	3.5×10^{10} /g	1.2×10^{10} /g	20×10^{10} /g
E	80	3.5×10^{10} /g	1.2×10^{10} /g	20×10^{10}/g
F	100	3.5×10^{10} /g	1.2×10^{10} /g	20×10^{10} /g
G	120	1.6×10^{10} /g	4×10^{9} /g	4.1×10^{10} /g

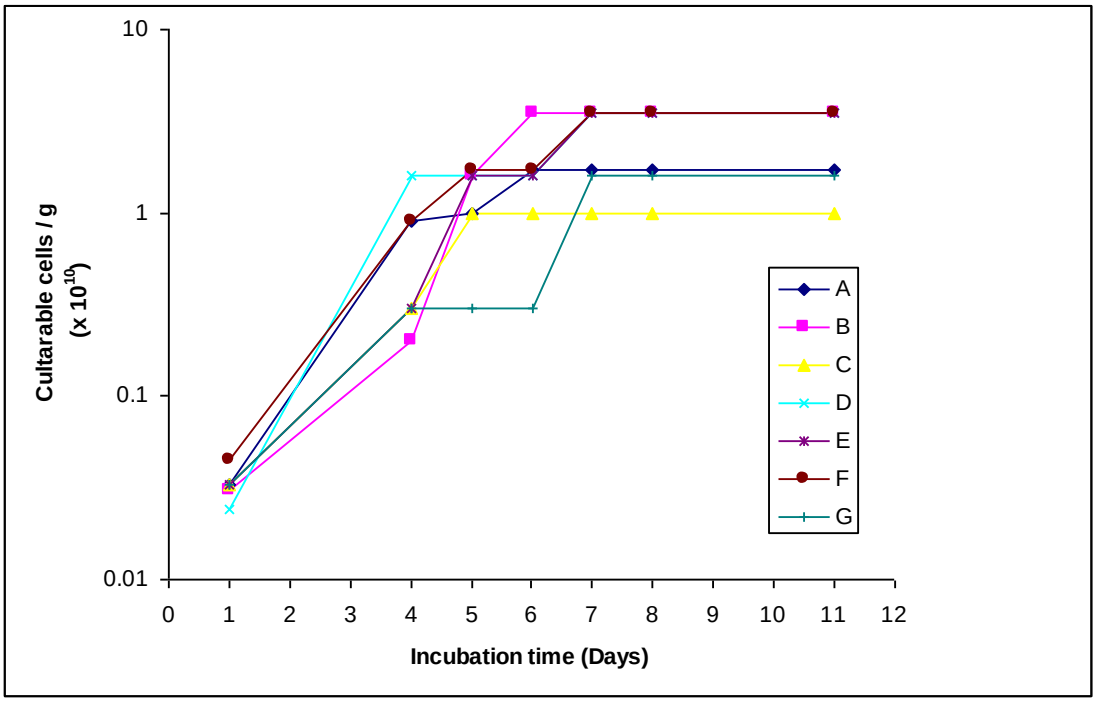

Fig. 3. The increase in MPN over time in experiments prepared using different treatment of the rumen contents.

3.4 Isolation of rumen bacteria

A total of 147 cultures were obtained from three further isolation experiments from the rumen contents of a ruminally-fistulated sheep set up using isolation procedure E. Cultures were grown in supplemented media at 39°C in the dark until DNA was extracted. The actual number of pure cultures were compared to the expected number of pure cultures which was calculated, as described by Button *et al.* (1993). There were in total an actual number of 78 pure cultures; a total of 93 pure cultures were expected (Table 3). Forty-five (30.1%) of the 147 cultures did not grow following subculture. Twenty-four of the remaining 102 cultures were found to be co cultures, giving a total of 78 pure isolates, slightly less than the number estimated above. When only the final isolation experiment was analysed, 61 pure cultures were obtained, compared to an expected 65. The 78 isolates were identified by comparative analysis of partial 16S rRNA sequences (Table 5). The sequence, spanning approximately 400 to 480 base pairs of the 5´ region of the 16S rRNA gene, was determined and compared with sequences in the GenBank database (Benson *et al*., 2007) using BLASTN (Altschul *et al*., 1990). All sequences were compared in the GenBank database (Benson *et al*., 2007). Members of 4 different bacterial phyla were cultured, *Firmicutes*, *Spirochetes*, *Bacteroidetes* and *Fusobacteria*. *Firmicutes* were the most abundant isolates, followed, in order, by *Bacteriodetes*, *Spirochetes* and *Fusobacteria*. Of the 78 isolates, 40 (51%) were from 10 known genera, while 38 (49%) grouped into 14 clusters that may represent new genera (Fig. 2).

Table 3. Expected and actual pure cultures out of the three experiments that were done with samples the rumen content from a ruminally-fistulated sheep.

Experiment	Dilution	Expected cells in inoculum	Total tubes inoculated	Growth positive tubes	Proportion growth of positive tubes	Expected pure cultures	Cultures not able to be subcultured	Adjusted total inoculated tubes[a]	Adjusted growth positive tubes[a]	Adjusted proportion growth positive tubes[a]	Adjusted expected pure cultures[a]	Actual pure cultures	Actual mixed cultures
		X	n	z	p	u		n'	z'	p'	u'		
2	A	8	50	4	0.08	4	0	50	4	0.08	4	3	1
	B	2	50	3	0.06	3	0	50	3	0.06	3	2	1
	C	0.5	50	1	0.02	1	0	50	1	0.02	1	1	0
3	A	40	125	33	0.264	28	21	104	12	0.115385	11	7	5
	B	10	125	13	0.104	12	5	120	8	0.066667	8	3	5
	C	2.5	125	2	0.016	2	2	123	0	0	0	0	0
	D	0.625	125	1	0.008	1	0	125	1	0.008	1	1	0
4	A	40	250	70	0.28	59	13	237	57	0.240506	50	47	10
	B	10	125	16	0.128	15	4	121	12	0.099174	11	11	1
	C	2.5	125	4	0.032	4	0	125	4	0.032	4	3	1
Total			1150	147			45	1105	102		93	78	24
Total experiment 4			500	90			17	483	73		65	61	12

[a] The total number of inoculated tubes and the total number of tubes positive for growth were adjusted by subtracting the number of tubes that failed to subculture.

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