

# The effect of fibre source on the numbers of some fibre-degrading bacteria of Arabian camel's (*Camelus dromedarius*) foregut origin

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**Abstract** The total bacterial community of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* in fibre-enriched culture of the foregut contents of 12 adult feral camels (*Camelus dromedaries*) fed on native vegetation in Australia was investigated using quantitative PCR. Foregut contents were collected postmortem, pooled and filtered before divided into two fractions. One fraction was used for extraction of DNA, while the other fraction was inoculated straight away into BM 10 contained filter paper (FP), cotton thread (CT) or neutral detergent fibre (NDF) as the sole carbohydrate sources in Hungate tubes. The tubes were incubated anaerobically at 39 °C for 1 week. After a near complete degradation of the FP and CT and extensive turbidity in the NDF, media subculturing was carried out into fresh media tubes. This was repeated twice before genomic DNA was extracted and used for quantification of bacteria. Using an absolute quantification method, the numbers of cells in 1 ml of each sample ranged from  $4.07 \times 10^6$  to  $2.73 \times 10^9$  for total bacteria,  $1.34 \times 10^3$  to  $2.17 \times 10^5$  for *F. succinogenes* and  $5.78 \times 10^1$  to  $3.53 \times 10^4$  for *R. flavefaciens*. The mean cell number of *F. succinogenes* was highest in the FP enrichment medium at approximately 107-fold, whereas for the

*R. flavefaciens* targeted primer, the NDF enrichment media had the highest mean cell number at approximately 4-fold when compared to the rumen content. The data presented here provide evidence of fibre type preference by the two main fibre-degrading bacteria and would help us understand the interaction between fibre type and fibre-degrading microorganisms, which has ramification on camel nutrition at different seasons and environments.

**Keywords** Fibre-degrading bacteria · Real-time PCR · Arabian camel · Native vegetation

## Introduction

The fermentation processes in the foregut of the camel occur in a similar fashion as in the ruminant forestomach. By-products of the fermentation processes, especially the short-chain fatty acids are absorbed across the foregut wall and used by the host animal as energy sources. On the other hand, the microbial protein synthesised in the foregut enters the small intestine where it is digested by body enzymes, and the released amino acids are absorbed and also utilised by the animal body for the synthesis of different proteins. In addition to these basic processes, the activities of the different microbes have resulted in the synthesis of essential molecules and detoxified some of the plant secondary toxic compounds. Camels are unique browsing herbivores adapted to environmental conditions ranging from the African savannah and the desert of Arabia to the mountainous regions of Mongolia. In Australia, majority of camels are feral that are spread over a large part of the arid and semiarid interior of the continent. They exhibit a selective feeding behaviour towards a range of vegetation including the shrubs of salty soils and trees such as mulga (*Acacia aneura*), broadleaved whitewood (*Atalaya hemiglauca*), capper bushes (*Capparis spinosa*) and emu bush

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(*Eremophila* sp.) growing along dry river beds (Philips et al. 2001). Their feeding behaviour is influenced by season and abundance of vegetation. In addition to being highly lignified, the presence of a range of anti-nutritional factors in these shrubs and trees make them unpalatable and less attractive to cattle and sheep.

Fibre digestion in ruminants has been widely investigated, and the key bacterial species involved in the fermentation processes have been identified. Three bacterial species (i.e. *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus*) are considered to be the main fibre degraders, possibly due to their adherent ability towards dietary fibre feed particles (Morris 1988; Miron et al. 2001). In contrast, only few studies have dealt with the bacterial community of the camel foregut. While the focus of Ghali et al. (2004, 2011) was on lactic acid-producing and lactic acid-utilising bacteria, Samsudin et al. (2011, 2012) have provided an insight into the bacterial diversity using 16S rDNA clone library approach. It is important to note here that the three fibre-degrading bacterial species were harvested from the rumen using a culture-dependent technique and therefore do not represent all rumen bacteria with the capability of degrading fibre. With this recognition of the predominant fibre-degrading bacteria in the rumen, it can be argued that any study based on culture-dependent methods alone to enumerate the population of the corresponding bacteria (Bryant and Burkey 1953; Bryant and Doetsch 1954) will underestimate the population. Thus, with the advancement of molecular techniques, enumeration of particular species of bacteria can be covered out accurately. Molecular techniques proved to be a powerful tool to end the discrepancy between direct microscopic counts and the numbers of culturable bacteria from environmental samples (Russell et al. 2009).

Of the different available molecular techniques, real-time PCR allows for the rapid quantification (Freeman et al. 1999) of microorganism populations in environmental samples. It is the relative quantification of DNA molecules, based on the recognition of DNA products, which results from the amplification of the molecules by PCR through the design of specific primer sets. The relative amounts of amplified PCR products can be demonstrated at each cycle of the PCR (Mackay 2004). The small part of the targeted gene in the bacterial community that is contributed by a particular taxon can be estimated by using a taxon-specific signature probe and suitable standards that correct the variations of species specific in the efficiencies of PCR (Stevenson and Weimer 2007). Several studies have shown that this technique can be successfully applied to rumen samples to quantify and monitor microbial population changes in the rumen of domesticated ruminant (Denman and McSweeney 2006; Mosoni et al. 2007; Cherdthong et al. 2010). However, the study on the microbial population densities in the foregut of dromedary camels has received less attention and not well documented.

To our knowledge, only one study using real-time PCR that focussed on lactate-utilising bacteria population in response to diet change has been reported (Ghali 2006). There is no available information of the total bacteria and predominant fibre-degrading bacteria densities in the foregut of the dromedary camel that fed on native shrubs and trees. Thus, the work described in the present paper was carried out to determine the densities of the total bacteria, *F. succinogenes* and *R. flavefaciens*, in the foregut of the dromedary camel using real-time PCR.

## Material and methods

### Sampling methods

Foregut content samples were collected immediately postmortem from 12 adult feral Arabian camels that were fed on native vegetation available abundantly in the central desert of Australia and in a paddock adjacent to the abattoir. The experiment was conducted according to the animal ethics guideline set by The University of Queensland Animal Ethics Committee (AEC Approval Number: SAS/069/08/UQ). Approximately 1 ml of the foregut fluid that was strained using four layers of sterile cheesecloth was used to inoculate fibre enrichment media containing cotton thread (CT), filter paper (FP) or neutral detergent fibre (NDF) in Hungate tubes prepared in triplicates. The cultures were incubated anaerobically at 39 °C for 1 week. After a near complete degradation of the FP and CT and extensive turbidity in the NDF, media subculturing was carried out into fresh media tubes. This was repeated twice before genomic DNA was extracted and used for quantification of bacteria. In total, the cultures were incubated for 21 days starting from the first until the third subculture.

### DNA extraction

Approximately 1.5 ml from each of the three enrichment media originated from the third subculture was collected, and the extraction of DNA was performed following a previously published protocol (Wright et al. 1997). Briefly, the samples were centrifuged (12,000g) for 5 min, and the supernatant was removed. Then, 200 mg of silica/zirconium beads was added, and the pellet was resuspend in 800 µl of hexadecyltrimethylammonium bromide (CTAB) isolation buffer (100 mM Tris-HCl, pH 8; 1.4 M NaCl; 20 mM EDTA (sodium salt); 2 % CTAB). The samples were homogenised in a bead beater for 2 min, cooled on ice and homogenised for another 2 min. The samples were then incubated at 70 °C for 20 min followed by centrifugation at 10,000g for 10 min. The aqueous phase was transferred to a new microfuge tube, and 500 µl phenol/chloroform/isoamyl alcohol (25:24:1) was

added, vortexed and centrifuged at 13,000g for 10 min. Again, 500 µl of the upper aqueous layer was removed and dispensed into another Eppendorf tube. The DNA was precipitated with 300 µl of isopropanol at room temperature for 5 min. DNA was collected by centrifugation at top speed (14,000g) for 15 min, and the nucleic acid pellet was washed with 1 ml of 70 % ice-cold ethanol. The pellet was incubated at 70 °C for 10 min before being centrifuged again at 14,000g for 10 min. The ethanol was removed, and the pellet was let to air-dry before being resuspended in 50 µl of DNA/RNA free water. Each sample of DNA was diluted 50-fold to remove PCR inhibitors as it was established laboratory practice (Ms. Korinne Northwood, Livestock Industries, CSIRO, personal communication).

### Real-time PCR

DNA quantification of each bacterial species in the respective samples was carried out using a Bio-Rad iCycler thermal cycler (Bio-Rad, Hercules, CA). The reaction was set up in a total volume of 25 µl which contained 1 µl of dilutions of DNA as mentioned above. The reaction contained 12.5 µl of SYBR® green mix fluorescent dye (QuantiTech™ SYBR® Green PCR, Qiagen) and 1.0 µl of each set primers (Table 1) with 10 µM concentration (Denman and McSweeney 2006). The DNA was amplified according to the cycling conditions described by Denman and McSweeney (2006). The same concentration of DNA was used in triplicate, and the external standard, five log dilutions series, was also included in the same assay. The external standards used in the present study for the real-time PCR amplifications were prepared according to a method reported by Denman and McSweeney (2006). Briefly, DNA was extracted from *F. succinogenes* strain S85 and *R. flavefaciens* strain Y1 fresh cultures that were grown overnight at 39 °C. The cultures were centrifuged, the supernatant was discarded and the cells were resuspended in clarified rumen fluid. Using a Helber counting chamber (Weber Scientific Instruments, West Sussex, UK), the numbers of bacterial cells were determined under a microscope.

The real-time PCR cycling was as follows: one cycle of 50 °C for 2 min followed by an initial denaturation at 95 °C for

2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min to allow primer annealing and product elongation. Detection of the fluorescent product was set at the end of each denaturation and extension cycle. The specificity of the amplicon was determined by continuously monitoring the dissociation melting curve of the PCR analysis of the end products by increasing the temperature at a rate of 1 °C every 30 s from 60 to 96 °C. Threshold cycles (Ct) were automatically calculated by the Icyler software (version 3.5) (Bio-Rad, Hercules, CA, USA).

### Statistical analysis

Statistical significant differences were analysed by ANOVA/general linear model using Tukey's test. Minitab 16 statistical software (Minitab Incorporation, State College, Pennsylvania, USA) was used for all analyses. Data were analysed using the model

$$q_s = (YA - YB) / SE$$

where

- $q$  studentized range
- $s$  standard deviation of the second sample
- $YA$  the larger of the two means being compared
- $YB$  the smaller of the two means being compared
- $SE$  the standard error of the estimates

### Results

Quantification of the total bacterial community and the two of the fibre-degrading bacterial species known to be predominant in the rumen of cattle was successfully performed using an absolute quantification by real-time PCR of the *rrs* gene. The gene originated from each bacterial species through using the previously published primers that were shown to be species-specific (Table 1). Estimates of the total bacteria, *F. succinogenes* and *R. flavefaciens* cell numbers are presented in Table 2. The numbers for total bacteria in all samples

**Table 1** Primers for real-time PCR analysis (Denman and McSweeney 2006)

Target species	Forward primer	Reverse primer	Size (bp)
General bacteria	5'-CGGCAACGAGCGCAACCC-3' (1,114) <sup>a</sup>	5'-CCATTGTAGCACGTGTGTAGCC-3' (1,275)	130
<i>F. succinogenes</i>	5'-GTTCGGAATTACTGGGCGTAAA-3' (586)	5'-CGCCTGCCCTGAACTATC-3' (706)	121
<i>R. flavefaciens</i>	5'-CGAACGGAGATAATTTGAGTTTACTTAGG-3' (96)	5'-CGGTCTCTGTATGTTATGAGGTATTACC-3' (220)	132

Numbers in bracket represent *Escherichia coli* numbering positions

<sup>a</sup> Modified from Lane (1991)

**Table 2** Quantification of general bacteria and *F. succinogenes* and *R. flavefaciens* (cells ml<sup>-1</sup>±SEM)

Sample	General bacteria	<i>F. succinogenes</i>	<i>R. flavefaciens</i>
Rumant content	1.13×107±3.18×106	2.03×103±2.85×102	9.83×103±1.69×103
Cotton thread media	2.73×109±4.84×108a	1.80×103±2.65×102	5.78×101±5.61×101
Filter paper media	4.07×106±2.60×105	2.17×105±2.03×104a	1.00×102±8.02×101
NDF media	1.43×109±1.20×108a	1.34×103±1.23×103	3.53×104±5.36×103a

Values are means of three determinations±SE (cell numbers) of triplicate and within the same column not bearing a common lowercase letter differ ( $p<0.001$ )

ranged from  $4.07\times 10^6$  to  $2.73\times 10^9$  cells ml<sup>-1</sup>; as for *F. succinogenes* and *R. flavefaciens*, the range was from  $1.34\times 10^3$  to  $2.17\times 10^5$  and  $5.78\times 10^1$  to  $3.53\times 10^4$  cells ml<sup>-1</sup>, respectively. The mean cell number for total bacteria was higher in the CT enrichment media at 242-fold than the foregut content. The FP enrichment media, at approximately 107-fold, had a higher mean cell number of *F. succinogenes* than the rumen content, whereas for the *R. flavefaciens* targeted primer, the NDF enrichment media had a higher mean cell number, at approximately 4-fold, than that of the rumen content.

The cell numbers of *F. succinogenes* and *R. flavefaciens* in the foregut content of the dromedary camels, estimated by real-time PCR, were  $2.03\times 10^3$  and  $9.83\times 10^3$  cells ml<sup>-1</sup>, respectively (Table 2). The percentage of *F. succinogenes* and *R. flavefaciens*, based on the total number of bacteria, has demonstrated that both fibre-degrading bacteria were found to be present in less than 0.01 % in all samples, except in the FP enrichment media, where *F. succinogenes* was present at 5.3 % of the total bacterial community. Moreover, *F. succinogenes* cell numbers were found to be higher than those of *R. flavefaciens* in the CT and FP enrichment media, whereas the rumen content and the NDF enrichment media were dominated by *R. flavefaciens*.

## Discussion

The density of total and fibre-degrading bacteria in the fibre enrichment cultures was quantified using a species-specific primer to target certain bacterial species in the extracted DNA. In this study, the use of different fibre types, as a carbon source in the enrichment media, was to stimulate the growth of fibre-degrading bacterial communities that otherwise would be found to be low in the foregut contents of the dromedary camel. The primer sets used to quantify the fibrolytic bacteria in this study were developed by Denman and McSweeney (2006), while the primer set used for total bacteria was modified from Lane (1991). The primers have demonstrated high specificity for *F. succinogenes* and *R. flavefaciens* as shown by the dissociation curve profiles

that were firm. Furthermore, they also did not detect any cross-reactivity with other species tested. Any nonspecific amplification from other rumen microbial populations would have resulted in invalid dissociation curves. To overcome amplification problems associated with larger fragments, the size of primer sets should be concise. Denman and McSweeney (2006) also demonstrated that, by reducing the amplicon sizes of the *F. succinogenes*, the amplification of the selected bacteria from the rumen samples was greater when the entire 16S ribosomal RNA (rRNA) gene was amplified, compared to the amplification results gained by using a primer set used by Tajima et al. (2001).

The fibre-degrading species from the camels were studied after 21 days of in vitro incubation except for the original foregut content. This long exposure to incubation conditions, largely different from that established in the camel foregut, may affect microbial communities and some species (unculturable or culture sensitive) may disappear from the media, while other opportunistic bacteria may proliferate and reach detectable numbers. Consequently, experimental differences observed can be at least partly affected by this fact. This is an unavoidable problem considering the origin of the inocula (feral animals slaughtered).

The mean density of both fibre-degrading bacteria described in the present study was relatively lower than in cattle, sheep and buffalo (Tajima et al. 2001; Denman and McSweeney 2006; Mosoni et al. 2007; Cherdthong et al. 2010). The density of *F. succinogenes* detected in the foregut of the Arabian camel was 0.02 % of the total bacterial population. Similar studies on cattle revealed that *F. succinogenes* exist as the predominant fibrolytic species in the rumen of cattle, ranging between 0.1 and 6 % with respect to the total microbial rRNA gene signal (Tajima et al. 2001; Koike et al. 2003; Denman and McSweeney 2006). The result in the present study demonstrated that the bacterial cell density of *F. succinogenes* in the foregut content of the Arabian camel estimated using real-time PCR was lower than that of other domesticated ruminants. Only FP enrichment media yielded a relatively high percentage (5.3 %) for *F. succinogenes* of the total bacterial community. Similarly, a study done by Varel et al. (1991) has also demonstrated that *F. succinogenes* was



the predominant fibre-degrading bacteria in the FP enrichment media when it was cultured together with two phenolic compound-degrading bacteria (*Eubacterium oxidoreducens* and *Syntrophococcus sucromutans*) to measure the extent of fibre degradation by ruminal cellulolytic bacteria.

The degradation rate of fibre by fibre-degrading bacteria in the rumen is known to be affected by the nature of the substrate (Dehority and Scott 1967; Miron et al. 1994). The number of fibre-degrading bacteria could increase if the surface area of the cellulosic substrate increases (Miron et al. 2001). The filter paper strip used in the enrichment media provided a large surface area for the adhesion of *F. succinogenes*. This explains why *F. succinogenes* was found in high numbers only in the FP enrichment media and not in other enrichment cultures. On the other hand, the *R. flavefaciens* population was dominant when NDF was used as a substrate in the enrichment culture. This is in agreement with several studies that reported that *R. flavefaciens* has the ability to degrade grass and straw cell-wall polysaccharides more efficiently than *F. succinogenes* and *R. albus* (Latham et al. 1978; Miron et al. 1994). Moreover, when the plant cell wall is available in the culture, *R. flavefaciens* will adhere instantly and more tightly bind to the fibrous plant particles than the other fibrolytic bacteria species.

The main factors affecting the population and the abundance of the bacterial community, particularly fibre-degrading bacteria, in the rumen were heavily influenced by the dietary conditions. Miron et al. (2001) suggested that factors like the pH of the rumen, substrates, temperature, presence of cations and soluble carbohydrates were vital in governing bacterial attachment to the fibre particles. Among the factors described above, ruminal pH is the most important environmental parameter because the numbers of fibre-degrading bacteria could be significantly reduced due to the sensitivity of these bacteria towards pH change (Russell and Wilson 1996).

Moreover, the different liquid turnover or, perhaps, the prolonged retention time of fibre in the foregut of the dromedary camel when compared to ruminants could contribute to a high density of the bacterial community (Lechner-Doll et al. 1991; Kayouli et al. 1993). This can partly explain why dromedary camels, when compared with cattle and sheep, are highly selective towards low degradable forages that contain a high level of lignocellulosic compounds.

Theoretically, the predominant fibre-degrading bacteria should be revealed as high in number by real-time PCR if it were supported by the abovementioned conditions. However, the data gathered in this study did not support that theory. The populations of fibre-degrading bacteria in this study were relatively low. Samsudin et al. (2012) found that a very low number of clones of *F. succinogenes* were detected in the 16S rRNA gene library derived from the FP enrichment medium, and no cellulolytic cocci were detected at all from the other clone libraries (NDF and CT enrichment medium). This might

be due to the presence of anti-nutritional compounds in the native vegetation that was the main feed consumed by the feral camels in Australia. The anti-nutritional compounds (e.g. tannins) present in the plant cell wall is believed to have an inhibitory effect on the growth of fibre-degrading bacteria and forage digestion and also inhibited the attachment of the fibrolytic bacteria to fibre particles (Akin et al. 1988; Varel et al. 1991). Furthermore, it has the ability to incorporate with dietary proteins; polymers of carbohydrates such as cellulose, hemicelluloses and pectin; and with minerals, thus slowing down their digestion by the rumen bacteria (McSweeney et al. 2001). It can be detected in nearly all of *Acacia* sp. that consists of the most favourable diet consumed by feral camels in Australia (Philips et al. 2001).

## Conclusion

The application of real-time PCR techniques to quantify the population of the predominant fibre-degrading bacteria present in the foregut of the camel and three fibre enrichment media proved to be very effective and provided useful data. The average density of the fibre-degrading bacteria in all samples revealed that *F. succinogenes* was found to be more dominant than *R. flavefaciens*. The dynamics of the fibre-degrading bacteria in the foregut of the camel is highly influenced by the type of fibre supplied in the enrichment media when used as a carbon substrate. The presence of plant secondary metabolites in the foregut content of Arabian camels has possibly reduced the population of the two fibre-degrading bacteria. Despite having a low population density in the foregut samples compared to cattle and sheep, the data presented here would help us understand the interaction between the diet consumed and foregut microbes, and the impact of such interaction on feeding behaviour, survival and adaptation to seasonal changes especially during drought seasons.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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