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Differences down-under: functional and comparative studies of the adaptations of heterotrophic methanogens to ruminant and other gut environments

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Executive summary

The methanogenic archaea are responsible for maintaining an efficient scheme of fermentation in many environments and habitats, including the gastrointestinal tracts of animals and humans. The principal members of gut methanogenic communities are members of the *Methanobrevibacter* genus, with lesser numbers of *Methanosphaera* and *Methanomassiliicoccus* spp. Much of our understanding of gut methanogens has been produced using axenic cultures and genomic data for ~30 *Methanobrevibacter* spp. but the functional relevance of these other archaeal lineages to digestive function remains poorly understood. With this background, the goals of my PhD research are: i) to increase the biotic representation of the *Methanosphaera* genus through the recovery of axenic isolates from different environments; ii) characterise the metabolic properties of these isolates in terms of their methanogenic pathways and; iii) expand our functional understanding of this genus via reconstruction of “population genomes” from existing metagenomic datasets and comparative genome analyses. During the latter stages of my PhD, I chose to make a transition in my research activities to include some biomedical focus and take advantage of my relocation to the University of Queensland Diamantina Institute. Here, I have examined variations in methanogenic archaeal populations in some clinical studies, as well as evaluated the immunostimulatory properties of some gut archaea. This aspect of my work aims to not only benefit people suffering from gut dysbiosis, but also through the management of methane emissions from livestock by improving the effectiveness of anti-methanogen vaccine-based approaches.

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1 Background

1.1 Introduction and literature review

1.1.1 Methanogens, their niche and biochemistry

The world's societies are being challenged to reduce their greenhouse gas emissions, in response to global concerns about our impacts on the environment and climate change. Methane is recognized to be a potent greenhouse gas, and much of it arises during anaerobic decomposition of organic matter by the microbial world. The processes governing methanogenesis reside within members of the Archaea and more specifically, the phylum Euryarchaeota. Five orders of methanogens have long been recognized: Methanopyrales, Methanococcales, and Methanobacteriales (class I); the Methanomicrobiales (class II) (Bapteste et al., 2005); and the Methanosarcinales (class III) (Anderson et al., 2009). However, this has since expanded to recognise Methanocellales (Sakai et al., 2008) as well as the recently proposed seventh order of methanogens provisionally named "*Methanoplasmatales*" (Paul et al., 2012). The role methanogens play within microbial communities supports the recycling of complex organic materials through a 'three-step process' of bioconversion in which methanogenesis is the final step (Liu and Whitman, 2008).

While the metabolic versatility of the members of each order of methanogens may differ, methanogenesis is only known to occur through three different processes: the carbon dioxide-reduction, methyl-group reduction and acetic acid cleavage (acetoclastic) reactions (Liu and Whitman, 2008, Thauer et al., 2008). Not surprisingly then, methanogens are found in many environments, especially those where sulphate is limiting; including fresh water sediments and rice paddies, landfills, moist soil biomes, the human, animal and some insect gastrointestinal tracts and their waste streams (Edwards and McBride, 1975, Liu and Whitman, 2008, Evans et al., 2009). Although the presence and role of methanogenic archaea in gut environments has long been recognized, greater attention has been directed to studying methanogens from other environments. It is only in recent years that there has been a renewed interest in gut methanogens, driven in part by the desire to reduce livestock methane emissions, as well as the possibility that methanogens might influence human gut function and health.

1.1.2 Methane and ruminants

Various studies have reported that the global annual methane production ranges from 500-600 Tg (trillion grams); as much as 20% of this amount is currently attributed to ruminant livestock such as cattle, sheep, deer and goats (Lowe, 2006). The global demand for meat and milk is predicted to increase by 60% before 2050, in response to the world's growing human population (FAO, 2012). Australia in particular has been identified as one of the world's largest producers of greenhouse gases on a per-capita basis (Garnaut, 2008) and methane emissions from ruminant livestock are now being targeted as a critical control point. There is an increased focus on the development of novel approaches and technologies to manage livestock methane emissions, so it seems logical that without a thorough characterisation of the gut methanogenic archaea responsible, it will be difficult to achieve success. Ruminants rely upon their microbial community to breakdown plant biomass in a manner that is consistent with that illustrated in Figure 1 (13, 14). Upon ingestion of plant

biomass by the host, it is initially broken down by hydrolytic enzymes, then fermented by a diverse community of prokaryote and eukaryote microbes (Bryant, 1979); subsequently synthesizing short chain fatty acids, hydrogen and carbon dioxide (Karasov and Carey, 2009). Within this community hydrogen accumulation can result in the inhibition of further metabolism of certain microorganisms (Hobson, 1988); so hydrogen utilising microbes are very important, as they allow the rest of the rumen microbial community to function most efficiently (Hobson and Stewart, 1997). Within the rumen, this niche is most often filled by methanogens; demonstrating this symbiotic relationship, of "interspecies hydrogen transfer" (IHT) as first described by Bryant and Wolin (1975). Since that time, much of the research has focused on confirming the class I methanogens, and especially the autotrophic *Methanobrevibacter* spp. are numerically most predominant (~6.0 x 10^8 cells per gram of sample) (Janssen and Kirs, 2008, Evans et al., 2009). Despite the current research interests seeking abatement of livestock methane emissions, only seven types of rumen methanogens (*Methanobrevibacter* spp., *Methanobacterium* spp., *Methanomicrobium* sp., and *Methanoculleus* sp.) have been described in any detail (Attwood et al., 2011, Janssen and Kirs, 2008) and relatively little attention has been paid to the heterotrophic members of the community.

1.1.3 Methanogens and macropodids

Australia's native macropodids (kangaroos and wallabies) are similar to ruminants in that they rely on a fore stomach colonized by microbes for the breakdown of plant material, and the production of protein- and energy-yielding nutrients. Interestingly, two studies in the late 70's suggested the scheme of anaerobic digestion in macropodids results in relatively low methane emissions per unit of digestible energy intake compared to sheep (Madsen and Bertelsen, 2012, Engelhardt et al., 1978). These earlier observations have recently been validated using a colony of kangaroos and wallabies from a zoo in Denmark (Madsen and Bertelsen, 2012). Macropodids have been shown to harbour methanogenic archaea in their foregut; with Evans et al. (2009) demonstrating the methanogen-specific 16S rRNA gene clone libraries produced from macropodids are taxonomically similar to those produced from numerous rumen and human microbiota, namely *Methanobrevibacter, Methanosphaera*, and uncultured *Methanoplasmatales* archaea. However, their qPCR analyses established that the abundance of methanogens were substantially less in macropodids (7.0 x 10^5 to 3.9 x 10^6 cells per gram of sample) than that of ruminant livestock (~9.8 x 10^8 cells per gram of sample) (Evans et al., 2009). Additionally, the recent identification of both acetogenic bacteria (Ouwerkerk et al., 2009) and Succinivibrionaceae WG-1 (Pope et al., 2011) suggests these bacteria could act as hydrogen sinks and effectively compete with methanogens for hydrogen, in a manner similar to that observed with some species of termites, and ostriches (Breznak and Switzer, 1986, Fievez et al., 2001).

The goal of my Honours project was to initiate a better characterization of the metabolic capabilities of macropodid methanogens, in which I successfully produced an axenic culture of a strain assigned to the genus *Methanosphaera* (hereafter referred to as strain WGK6) from a digesta sample collected from a Western grey kangaroo (*Macropus fuliginosus,* see Hoedt, 2011). *Methanosphaera* spp. is defined by the literature as methyl-group reducing methanogens limited to the utilisation of methanol, through a hydrogen dependant process. The pathway is initiated by coenzyme M methyltransferase (MtaABC). The reduction of methanol (CH_3OH) by coenzyme M (HS-CoM) produces 2-(methylthio)ethanesulfonic acid

(CH_3-S-CoM) and water. Methyl-coenzyme M reductase (MrtABG) reduces the product CH_3-S-CoM with coenzyme B (HS-CoB) to methane and coenzyme M-HTP heterodisulfide (CoM-S-S-CoB). Heterodisulfide reductase (HdrABC) then acts on CoM-S-S-CoB using $2e^-$ and $2H^+$ to reduce the disulfide bond thus regenerating HS-CoM and HS-CoB. The necessary electrons and hydrogen protons are generated by non-F420-reducing hydrogenase (MvhADG) from hydrogen. Methane production drives proton motive force through proton-translocating ATPase, resulting in the energy metabolism by the conversion of ADP and phosphate to ATP (Fricke et al., 2006).

Preliminary studies from my Honours project also suggested that strain WGK6 is capable of using either hydrogen or ethanol as a source of reducing power during growth and methanogenesis with methanol. Such findings raise important questions relevant to the colonization and persistence of methanogens in the macropodid foregut. Have at least some of these methanogens evolved to survive by using H_2-independent pathways for methane production and growth? Furthermore, is it possible that other gut *Methanosphaera* spp. also possess the same metabolic attributes? These questions can be addressed if more methanogen isolates are recovered from native Australian herbivores, in addition to comparing strains from multiple gut environments.

1.1.4 Methanogens and humans

Methanogenic archaea have also been identified within human hosts through use of culture, PCR, metagenomic and methane formation confirmed clinically by breath test techniques (Scanlan et al., 2008). Much of the early studies used stool samples and like the rumen and macropodid foregut, the two major groups identified were the autotrophic *Methanobrevibacter spp.* (principally *Mbb. smithii)* and the methylotrophic *Methanosphaera* spp. (principally *Msp. stadtmanae*). More recently, the analysis of additional human microbiota samples from subgingival, intestinal or vaginal mucosae have expanded the diversity of methanogenic archaea found (Dridi et al., 2012, Borrel et al., 2012). In addition to the identification of a new species of *Methanobrevibacter (Mbb. oralis)*, two isolates of methylotrophic archaea *Candidatus '*Methanomethylophilus alvus', and *Methanomassiliicoccus luminyensis,* are affiliated with the newly defined order *Methanoplasmatales.* Many studies have now been conducted to determine whether an association between the presence/absence of specific microbes (including archaea) and healthy/disease states exists (e.g. Furnari et al., 2012, Pimentel et al., 2003, Lepp et al., 2004). For instance, the subgingival plaque collected from patients with periodontitis have been found to harbour large numbers of total bacteria including methanogenic archaea, acetogenic bacteria and sulphate- reducing bacteria (SRB) (Vianna et al., 2008). A study of irritable bowel syndrome patients showed there was a positive correlation between a positive breath methane test and constipation frequency; while another study of patients suffering from Crohn's disease or ulcerative colitis appear to have suppressed methanogen populations in their gut (Pimentel et al., 2003, Pimentel et al., 2012). Such findings suggest that fluctuations in the abundance of methanogenic archaea within human microbiomes might be associated with alterations in fermentation schemes that impact on gut function and health (Hajishengallis et al., 2012). Additionally, research completed by Blais Lecours, et al. (2011) has confirmed that both *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* can be immunostimulatory in animal models of respiratory disease, with the latter provoking a stronger immune response (Blais Lecours et al., 2011). Furthermore, Blais

Lecours et al. (2014) have reported that while the total numbers of methanogenic archaea are less in patients suffering from inflammatory bowel disease (IBD), the prevalence of *Msp. stadtmanae* was greater in these patients, and healthy and diseased human subjects produced an increased antigen-specific IgG response to this archaeon when present in stool (Blais Lecours et al., 2014). These results suggest that *Msp. stadtmanae* prevalence and/or abundance may be a biomarker of gut dysbiosis, being more prevalent in persons with an altered “low hydrogen” fermentation scheme. This hypothesis warrants more detailed examination and as part of my clinical investigations of methylotrophic archaea the opportunity exists to further investigate the abundance and differences in host immune response elicited by these methanogenic archaea. The knowledge gathered for the immune response to methanogenic archaea might allow us to make progress with the development of anti-methanogen vaccines for livestock to reduce methane emissions.

1.1.5 Summary

Despite the widespread recognition of the roles methanogenic archaea may play in gut environments, very little is known about their metabolic versatility or host adaptation. With the exception of some detailed examination of the physiological and genetic potential of the autotrophic *Methanobrevibacter* spp. (Leahy et al., 2010), much of the past efforts have been directed towards examining the ecological and taxonomic variations among these microbes in gut environments. To date, little is understood about the heterotrophic methanogens present in these environments; although my Honours research suggested that these archaea may possess novel metabolic features associated with host adaptation. Furthermore, the recent identification and draft genome sequences for several members of the *Methanoplasmatales* recovered from gut environments further highlights that much remains to be learned about these archaea.

The fields of microbial genomics and metagenomics offer the potential to examine, and obtain a better understanding of the many microbes present in complex microbial communities, like those present in the bovine rumen, macropodid foregut and large bowel of humans.

2 Project bjectives

2.1 Characterization of the new methylotrophic methanogenic archaeon from Western grey kangaroo (*Macropus fuliginosus*).

Methanosphaera sp. WGK6 was isolated and characterised from the foregut digesta of Western Grey kangaroo. The work has been accepted and published by *ISME Journal* (Hoedt et al., 2016; Nature Publishing Group, 2014 Impact Factor 9.302).

2.2 Comparative genome analysis of *Methanosphaera* spp.

Isolation of bovine *Methanosphaera* spp. from Australian bovine combined with whole genome sequencing and metagenomic population genome recover of other *Methanosphaera* spp. Work in preparation for submission to *Genome Research* (Cold Spring Harbour Laboratory Press, 2014 Impact Factor 14.63).

2.3 Investigation of the immunomodulatory potential of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*

Inflammatory profiling for methanogens *Methanobrevibacter* spp. and *Methanosphaera* spp. in respect to the human host.

3 Methodology

3.1 Isolation and characterization of a new methylotrophic methanogenic archaeon from Western grey kangaroo (*Macropus fuliginosus*).

3.1.1 Methanogen enrichment and isolation

Digesta samples taken from killed Western Grey kangaroos, and a rumen-fistulated Brahman steer grazing native forage, and were stored anaerobically as 30% (v/v) glycerol stocks at -80°C. A basal anaerobic medium containing 30% (v/v) clarified rumen fluid (RF30) (Joblin et al., 1990, Skillman et al., 2004) was dispensed in 10 ml volumes into Balch butyl rubber sealed tubes (Hungate et al., 1966, Balch and Wolfe, 1976) within an anaerobic hood was used for the first stage of enrichment. The medium was prepared to encourage methanogen growth by providing a variation of substrates suitable for methanogenesis, including a mixture of hydrogen and carbon dioxide (80:20 v/v, pressurized to 100 kPa); methanol (1% v/v, 0.25M); methanol and ethanol (1% v/v each, 0.2M and 0.17M, respectively); formate (50mM); and acetate (20mM). Additionally, a mixture of penicillin and streptomycin sulfate (to provide final concentrations of 5 mg/mL and 80 µg/mL, respectively) was added to replicate tubes of the different substrate combinations to further suppress bacteria. Microbial growth was monitored by twice-daily spectrophotometric measurements of optical density at 600 nm (hereafter referred to as OD600).

The presence of methanogenic archaea in these enrichment cultures was determined by measuring methane concentrations in headspace gases using a gas chromatograph (GC-2014 Shimadzu) fitted with a flame ionization detector (Gagen et al., 2014). Additionally, methane positive cultures were sampled and microscopically examined for fluorescent cells upon UV illumination. In many methanogenic archaea, the reduced form of coenzyme F420 acts as the direct electron donor for two reducing steps of methanogenesis, with the oxidised form displaying fluorescence (Edwards and McBride, 1975). The negative controls used throughout these steps were uninoculated medium; and the positive controls were *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium*.

Methanogen-positive cultures were then serially diluted, and aliquots of these dilutions were used to inoculate Hungate tubes containing the basal medium with added agar, which had set around the inner surface of the tube (Hungate roll tube) (Hungate et al., 1966, Ouwerkerk et al., 2005). After incubation for 7 days at 39^{o}C, the tubes were placed inside the anaerobic chamber and single colonies were picked and removed from the tubes with a sterile glass pipette, modified by Bunsen burner to possess a 90^{o} end. These colonies were then used to inoculate fresh tubes containing the same medium. The culture was deemed to be pure by microscopic examination and PCR with archaea specific primers 86F/1340R (Wright and Pimm, 2003). Sequencing of this PCR product suggested a single archaea species was

present. In parallel, PCR bacterial specific primers 27F/1492R (DeLong, 1992) yielded a negative result supporting the attainment of an axenic culture.

3.1.2 Substrate utilization profile testing of isolates and growth studies.

The growth and substrate-utilization profiles of the macropodid-, ruminant- and human-derived isolates of Methanosphaera spp. were examined using the BRN-RF10 basal medium described by Balch et al. (Balch et al., 1979) with the modifications described by Wright and Pimm (Wright and Pimm, 2003). The medium was prepared similarly to that of RF30, as described above, with the exception that before autoclaving the headspace gases were purged with CO_2 to remove any residual H_2. After autoclaving, the following substrate mixtures were added to triplicate individual tubes using aseptic techniques: ethanol alone, ethanol plus H_2:CO_2 (80:20 vol/vol) pressurised to 202 kPa, methanol alone, methanol plus H_2:CO_2, methanol (0.25M) and ethanol (0.17M), 20 mM each of mono-, di- and trimethylamine, propanol (0.13M), and H_2:CO_2. Optical density was measured three times daily (0800, 1300 and 1800) until the stationary phase of all cultures was reached, at which time samples for headspace gas analysis were also collected.

More detailed growth studies were then conducted using larger cultures from which multiple samples could be collected over time. For this purpose, 1.2 L bottles modified with a serum bottle type closure were used. Each bottle contained 500 mL of the medium and was inoculated with 10 ml of mid-log phase cultures, which produced a starting OD600 ~0.02. The bottles were placed within a shaking incubator cabinet at 39^{o}C and rotated at 100 rpm for 7 days. At 0800 and 1300 each day, 1 mL was aseptically removed from each culture, and used for an OD600 measurement. At 1800 each day, 2 ml of each culture was collected. A 1 ml aliquot was used for OD600 measurement and the remainder was subjected to centrifugation at 13,000 rpm for 15 minutes at room temperature (Eppendorf). The supernatant was then carefully removed and stored at -40^{o}C prior to analyses for residual alcohol substrates (methanol and (or) ethanol) by the GC methods described below. Sampling of the headspace gases was performed by aseptic removal of 2 mL samples at 1800 each day, and their composition also analysed by GC.

3.1.3 Gas chromatography analyses of substrates, metabolites and gases

To allow for the measurement of short chain alcohols (methanol and ethanol) and volatile fatty acids within culture medium the analyses was performed using a CSIRO unpublished gas chromatography (GC) modified running conditions (Pontes et al., 2009). The column was ZEBRON-ZB-FFAP capillary column (Nitroterephthalic acid Modified Polyethylene Glycol), 30 m x 0.25 mm x 0.25 µm. The temperature of the flame ionization detector was 230^{o}C, and the injector temperature was 220^{o}C. The oven temperature was programmed to 35^{o}C, 12 min; 35-60 (3^{o}C /min) ; 60-220 (10^{o}C /min); 220^{o}C , 5 min gradient column temperature. The carrier gas was N2 with a flow of 5 ml/ min and volume of injection was 1.0 µL; sample analysis was approximately 45 min. The concentrations of methanol and ethanol were varied (2mM, 4mM, 8mM, 16mM, 32mM, and 64mM) and used to generate a standard curve. Methanol and ethanol of concentrations to be used during culture work (see above) were used to spike clarified rumen fluid, RF30 and BRN-RF10 medium to run as initial test samples. For all samples 500µL volumes were prepared for each standard, control and culture supernatant; placed in 2mL screw cap vials (Agilent Technologies) with an additional 50µL of analytical grade isopropanol (13.1 M) added, to act as an internal standard.

3.1.4 Quantitative analysis of dehydrogenase gene expression in strain WGK6

The isolate *Methanosphaera* sp. WGK6 from the Western grey kangaroo was found to be capable of using ethanol as a source of reducing power to convert methanol to methane. Primers were then designed to target the candidate alcohol and aldehyde dehydrogenase genes identified in the WGK6 genome, believed responsible for the oxidation of ethanol. Total RNA was extracted from WGK6 cells cultured with either ethanol: methanol, or H_2: methanol and RT-PCR performed with specific primers.

3.2 Comparative genome analysis of *Methanosphaera* spp.

3.2.1 Bovine *Methanosphaera* spp. enrichment and isolation

Enrichment and isolation techniques described above were employed for the isolation of a bovine originating *Methanosphaera* spp.

3.2.2 Genome analysis and characterisation of *Methanosphaera* sp.

The genome of *Methanosphaera* sp. strain BMS was sequenced using the PacBio RS2 system at The University of Queensland Diamantina Institute.

3.2.3 Metagenomic *Methanosphaera* spp. population genome recovery

Methanosphaera spp. population genomes were recovered through bioinformatics pipelines from publically available metagenomic datasets.

3.2.4 Pan-genomic analysis

Various software platforms were employed to determine the pan and core genome selection for all *Methanosphaera* genomes, as well as the whole genome phylogeny.

3.3 Investigation of the immunomodulatory potential of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*

3.3.1 Monitoring immunomodulatory potential

A combination of immune cell reporter systems and peripheral blood mononuclear cells (PBMC) have been challenged with cell preparations from *Methanobrevibacter* and *Methanosphaera* spp., to evaluate their responses in terms of cytokine profiles (types and amounts) as well as the regulation of the universal stress-related pathway coordinated by the NF-KB transcription factor. These results will be considered with respect to the isolates' immunomodulatory potential of these archaea, in terms of their capacity to stimulate an immune response.

4 Results

4.1 Isolation and characterization of a new methylotrophic methanogenic archaeon from Western grey kangaroo (*Macropus fuliginosus*).

- *Methanosphaera* sp. WGK6 utilises ethanol (or hydrogen) and methanol to grow and produce methane, making this species metabolically more versatile than other cultured *Methanosphaera* spp.
- WGK6 alcohol-fuelled methanogenesis is coupled with acetate formation from the oxidation of ethanol.
- The WGK6 genome encodes dehydrogenase genes that are absent from the cultured human type strain *Methanosphaera stadtmanae*.
- The dehydrogenase genes are constitutively expressed and co-transcribed.
- Orthologs of the dehydrogenase genes from strain WGK6 are also present in other members of the Methanobacteriales (*Methanobrevibacter* spp.) suggesting that the metabolic versatility of *Methanosphaera* is broader than previously documented.

4.2 Comparative genome analysis of *Methanosphaera* spp.

- Isolate of a bovine originating *Methanosphaera* sp. recovered.
- *Methanosphaera* sp. BMS genome sequenced.
- Larger genome for *Methanosphaera* sp. BMS compared to other *Methanosphaera* sp. isolates and is not a result of recent gene acquisition or chimeric assembly.
- Another late stage enrichment of a hydrogen-dependent *Methanosphaera* sp. was produced during my 1-month visit to the University of Illinois.
- *Methanosphaera* sp. population genomes have been recovered from metagenomic datasets from human (Europe), sheep (NZ) and bovine (Aus.).
- The *Methanosphaera* pan-genome calculated and core-genome consists of genes supporting methanogenesis, anaerobic metabolism, related functions and hypothetical genes.
- Whole genome phylogeny shows a monophyletic origin for the genus *Methanosphaera*.

4.3 Investigation of the immunomodulatory potential of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*

- Preliminary assay of immunomodulatory capability of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* using a mouse macrophage cell line.
- Pilot study of PBMC stimulation protocol and TNF-α ELISA assay was completed using different cell preparations of the archaea outlined above.
- Preparations of PBMCs from healthy subjects were stimulated with cell biomass of either *Methanobrevibacter smithii* or *Methanosphaera stadtmanae* and the released cytokines have been profiled to indicate the nature and degree of immune response.

5 Conclusion

The findings arising from my research provide a deeper insight into the genus *Methanosphaera*, in terms of its evolutionary development, nutritional ecology, host adaptation, and role in ruminant gut function. These findings are both novel and provide new opportunities to more effectively target methanogenic archaea in livestock, and broaden the scope of the interventions needed and used to successfully redirect fermentation away from a methane-producing endpoint. My research has also provided some new insights into the nature of the immune response that gut methanogens can stimulate in their host. These findings could provide researchers with the opportunity to use methanogenic archaea as a biomarker for gut dysbiosis, and perhaps, improve the selection of cellular components that are most immunostimulatory, which can then be targeted for the development of anti-methanogen vaccines. As part of my PhD I have also published and contributed to literature through review articles discussing the impact of methanogenic archaea in respect to the host and their contribution to greenhouse gas emissions within livestock (Hoedt et al., 2015, Burman et al., 2016). A third perspective piece is due for release in July (2016) by the American Society of Animal Science, Animal Frontiers Journal.

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