Life in the small intestine: the forgotten microbiome?



Erin R Shanahan[A,B,C], *Gerald Holtmann*[A] *and Mark Morrison*[B]

[A]Department of Gastroenterology and Hepatology, Princess Alexandra Hospital and Faculty of Medicine, The University of Queensland, Brisbane, Qld, Australia

[B]The University of Queensland Diamantina Institute, Brisbane, Qld, Australia

[C]Translational Research Institute, 37 Kent Street, Woolloongabba, Qld 4102, Australia. Tel: +61 7 3443 8022, Email: e.shanahan@uq.edu.au

The gastrointestinal (GI) microbiota is now widely accepted to be an important modulator of our health and well-being. The microbes colonising the GI tract aid in promoting gut and immune homeostasis, while alterations in the composition and/or density of these microbes, often referred to as dysbiosis, have been implicated in many intestinal and extra-intestinal disorders. As a result, the GI microbiota is of increasing interest as a therapeutic target. This is particularly the case in the context of GI disorders linked to chronic inflammation of the mucosa. In this article, we focus on the small intestinal microbiota, which in many senses can be considered the 'forgotten' gut microbiome.

Much of the current knowledge of the microbiota is from studies of faecal samples, which are relatively easy to access, and contain a high microbial biomass that is amenable to widely used culture-independent approaches. However, the faecal microbiota may be subject to physiological and environmental factors such as intestinal transit time and dietary variation. In contrast, those microbes that colonise the GI mucosa may be less subject to short term environmental variations, and have greater capacity to affect mucosal barrier functions and host immune responses. Assessment of the mucosa-associated microbiota (MAM) has therefore received increasing attention, and these sub-communities in the large bowel show a defined biogeography in healthy subjects[1] as well as variations associated with particular disease states[2].

Conversely, there has been relatively scant attention paid to the small intestine, particularly the proximal regions (duodenum and jejunum). This is despite the fact that the small intestine represents the greatest surface area of the bowel, estimated to be ten times greater than that of the large intestine[3]. The small intestine is also an important site for digestion, nutrient absorption and immunological functions. Clinically, up to one-third of the population experience some form of upper GI digestive disease or disorder. Yet we understand very little about the structure-function relationships inherent to the proximal GI microbiota, constraining our diagnosis and treatment of these conditions, many of which are associated with chronic inflammation. If we are to fully appreciate the role and potential clinical management of the gut microbiota, we need to better understand the host-microbe interactions of the small intestine.

The small intestinal niche and its 'forgotten microbiota'

The small intestine represents a unique series of niches within the digestive tract (Figure 1). An important difference relative to the large intestine is the existence of an oxygen gradient, with the

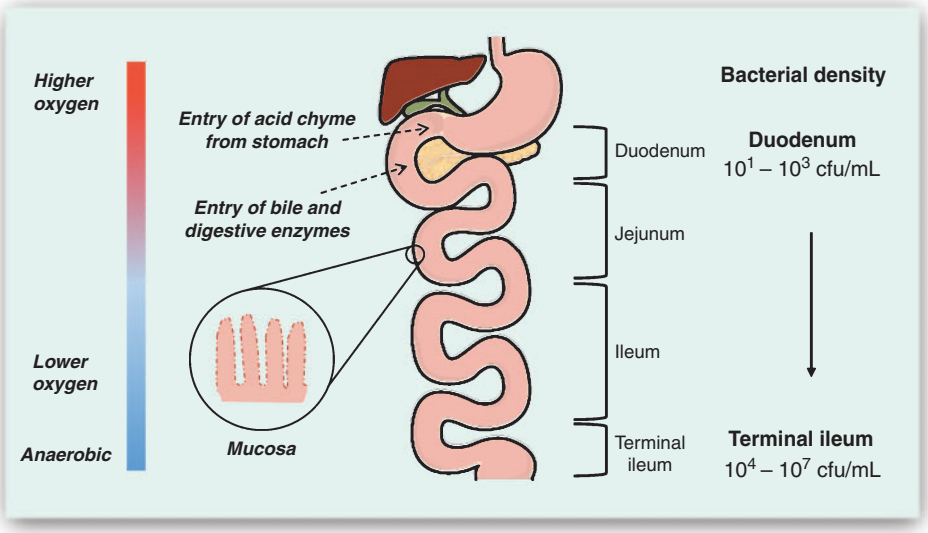

Figure 1. The small intestine. A gradient in oxygen levels and bacterial load exists along the length of the tract. The proximal small intestine (duodenum and jejunum) are particularly influenced by the entry of acid chyme, as well as bile and digestive enzymes. The small intestinal mucosa is lined with villi, which project into the lumen and create a vastly increased surface area.

proximal regions more oxygenated, and only the terminal portion being anaerobic, sharing greater similarity with the large bowel. It is a relatively harsh environment characterised by influxes of acidic chyme from the stomach, and the presence of bile and digestive enzymes. The effect of gut motility on digesta residence time is also a strong selective pressure on the microbiota. Thus microbial colonisation of the small intestine, and the proximal regions in particular, is a challenge in comparison to the more hospitable environments of the lower gut. These factors, along with the sporadic presence of host ingested nutrients, mean mucosal colonisation is of particular importance for small intestinal microbes.

For these reasons, the proximal small intestine, was long considered sterile. Early culture-based studies found that in most cases, very limited numbers of bacteria could be isolated[4], and were often speciated as either oral or colonic/faecal bacteria[5–7]. The general consensus was that bacteria were not resident but rather contamination from other sites, such as the oral cavity, and were of little relevance. These assumptions were also driven, in no small part, by the fact that the proximal small bowel is difficult to reach and therefore sample. It is only possible to sample this site during medical procedures, primarily endoscopy. Standard endoscopic procedures are only able to reach the duodenum and collection of tissues during these procedures is also inherently susceptible to contamination, via exposure to the oral cavity and lumen. Thus it can be difficult to distinguish between the MAM and non-resident organisms picked up during sampling[8].

However, as the *Helicobacter pylori* story so aptly demonstrates, the mucosa of such harsh regions of the GI tract can be stably colonised by niche adapted organisms, and these previously overlooked microbes can have important implications for health, with the role of *H. pylori* in ulcers and gastric cancer clearly established. Interestingly, emerging evidence also suggests the importance of other microbes, resident in the gastric mucosa, along with *H. pylori*, in the progression to gastric cancer[9]. Thus it is clear that the mucosal microbiota of the upper GI tract cannot be assumed to be flow through or contamination.

A developing picture of the small intestinal microbiota

The development of new molecular, sequencing and computational technologies has driven the renewed interest in the complex communities of microbes associating with the human body. In particular, these technologies are facilitating analysis of previously overlooked body sites. Thus there are emerging data describing the microbiota present in the small intestinal mucosa, in both an inflamed and non-inflamed gut. These studies have revealed the proximal bacterial community is principally comprised of Firmicutes, Bacteroidetes and Proteobacteria affiliated taxa, dominated by the genera *Streptococcus, Prevotella, Granulicatella, Veillonella, Neisseria, Haemophilus, Gemella* and *Rothia*[10–17]. From these studies there appear to be overlaps with the oral microbial community, at broader taxonomic levels[18], as well as some similarity to the microbiota of the large bowel, although few studies have directly compared the two regions in the same patients[13]. While the proximal small bowel lacks many of the highly abundant large bowel groups including *Faecalibacterium*, *Ruminococcus*, *Bacteroides* and *Clostridium*, there are some overlaps, notably *Prevotella*. However, whether the *Prevotella* spp. observed at the

two sites are the same, or represent unique/niche-adapted strains, has not been characterised.

In the context of disease, a primary focus for the small bowel has been coeliac disease. When comparing patients to non-coeliac controls, lower microbial diversity and reductions in the relative abundance of *Streptococcus* and *Prevotella* have been reported, along with higher levels of Proteobacteria[10,12,14], although not all studies have identified such differences[11,16]. In irritable bowel syndrome, a single study has compared the jejunal mucosal microbiome, which is similar in composition to that of the duodenum, in patients and healthy volunteers, however no substantive differences in microbial profiles were observed[19].

In other regions of the GI tract, increasing interest and attention is being directed towards the further components of the microbiota: fungi, archaea, viruses, bacteriophage and protozoa[20]. However, in the small intestine, our understanding of these microbes as interacting members of the mucosal microbiota is very limited. In the context of the proximal small bowel, we are aware of only one study to date that has profiled the non-bacterial mucosal community. This study focused on the mycobiome (the fungal microbiota), and identified two predominant phyla, Ascomycota and Basidiomycota, with the most represented classes being *Tremellomycetes*, *Saccharomycetes*, *Basidiomycetes*, *Agaricomycetes* and *Dothideomycetes*[21]. Given the recent identification of small intestinal fungal overgrowth, associated with otherwise unexplained gastrointestinal symptoms[22], further efforts to characterise the mycobiome, as well as other microbiota components, in the proximal gut mucosa are warranted.

Ongoing challenges

Such profiling studies are not without their challenges, and this is particularly the case when working with human tissue samples, of limited size, that have relatively low microbial loads and high levels of human DNA. Typically, these tissues are subject to profiling studies that involve amplification of a marker gene, such as the bacterial 16S rRNA gene, next-generation sequencing, and bioinformatics procedures to align sequence reads to a database and assign taxonomy. However, bias may be introduced through sample collection methods, primer choices and bioinformatics methodologies[23,24], taxonomic assignment beyond genus level can be problematic, and care must be taken to avoid amplification of spurious products, such as those present in common laboratory reagents[25] (Table 1).

Metagenomics, in which total DNA is sequenced, rather than an amplicon, can overcome some of these issues. The risk of amplification bias is greatly reduced, and obtaining partial, to near complete, microbial genomes can enable more specific taxonomic assignments, such as to species level; as well as facilitate assessment of the functional capacity of the microbiota[24]. However, in low microbial load mucosal samples, performing metagenomics is technically challenging[26] and it can be difficult to obtain sufficient microbial biomass for accurate profiles.

The limited number of studies investigating the proximal small intestinal MAM are constrained by a number of other factors, including relatively small numbers of participants. Factors such as diet and medication use have also not yet been considered in detail in these studies. In particular, the widespread use of proton pump inhibitors (PPIs), their impact on gastric acid secretion, and recent observations that the use of PPIs results in changes to the stool microbiota, namely increases in the presence of oral-like organisms[27,28]; indicates these are likely to also impact the proximal small intestinal microbiota. A further complication when investigating the healthy microbiota in these sites is the unlikelihood of a symptom-free, healthy individual undergoing a medical procedure such as an endoscopy. Understanding the relative contributions of the microbiota in the various niches of the GI tract, to various disease states, is also an important consideration, which has not been well addressed. Given the burden of GI disorders in which the small intestine is implicated, more comprehensive studies are required to address these issues.

The 'Brisbane approach' to the small intestinal microbiota

We are interested in the mucosal microbiota in the proximal small bowel in the context of functional gastrointestinal disorders, specifically functional dyspepsia (FD) and irritable bowel syndrome. Patients with these disorders experience often severe GI symptoms such as meal related pain, fullness, indigestion, bloating and altered bowel habits. These symptoms are associated with mild chronic inflammatory responses but the drivers of inflammation are unknown[29,30]. The microbiota is suggested to play a key role. However, to understand the potential of host-microbe interactions in driving these symptoms, the constraints limiting the prior studies of the proximal small intestinal MAM must be overcome.

We have recently described an approach in which a sheathed biopsy forceps, designed to remain free of luminal and oral material during sampling of the mucosa, was utilised during endoscopy procedures to sample the small intestinal mucosa[31]. This type of technique is not routinely utilised when sampling in the GI tract, and has facilitated a targeted assessment of the duodenal MAM using molecular and culture-based approaches (Figure 2). Through these

Table 1. Challenges associated with microbial profiling studies based on amplicon sequencing. Working with human tissues, with low microbial load, presents additional challenges compared to high biomass samples such as stool.

	General considerations	Additional considerations for low microbial load tissue samples
Sampling	– Consistency in sample collection and storage prior to processing – Collection of sufficient number of samples to accurately profile tissue – Inclusion of sufficient study participants to overcome inter-individual variations	– Collection method that specifically samples intended microbiota target
Nucleic acid extraction	– Cell lysis methods that can effectively lyse all members of the microbial community – Avoid introduction of batch effect (e.g. avoid processing all control samples in a single batch)	– Strategies to avoid contamination of low microbial load samples from the laboratory environment – Methods to enrich for microbial DNA
Amplification and Sequencing	– Choice of primer set targeting region of interest (e.g. 16S rRNA gene variable region) – Amplification bias	– Primer set that avoids amplification of human DNA – Include multiple negative controls to account for potential amplification of low level nucleic acids in laboratory reagents
Bioinformatics	– Pre-processing to retain only informative, high quality sequence read data – Methods for alignment of sequences and database used for taxonomic assignment – Accuracy of taxonomic assignment from short sequence reads – Assessment of required sequencing depth	– Removal of non-target reads
Statistical analyses	– Accounting for differences in sequencing depth (number of sequence reads per sample) – Methods for data normalisation – Applying statistical methods to sparse (many zeros) and relational (relative abundance) data – Integrating microbial profiles with clinical data and patient characteristics	

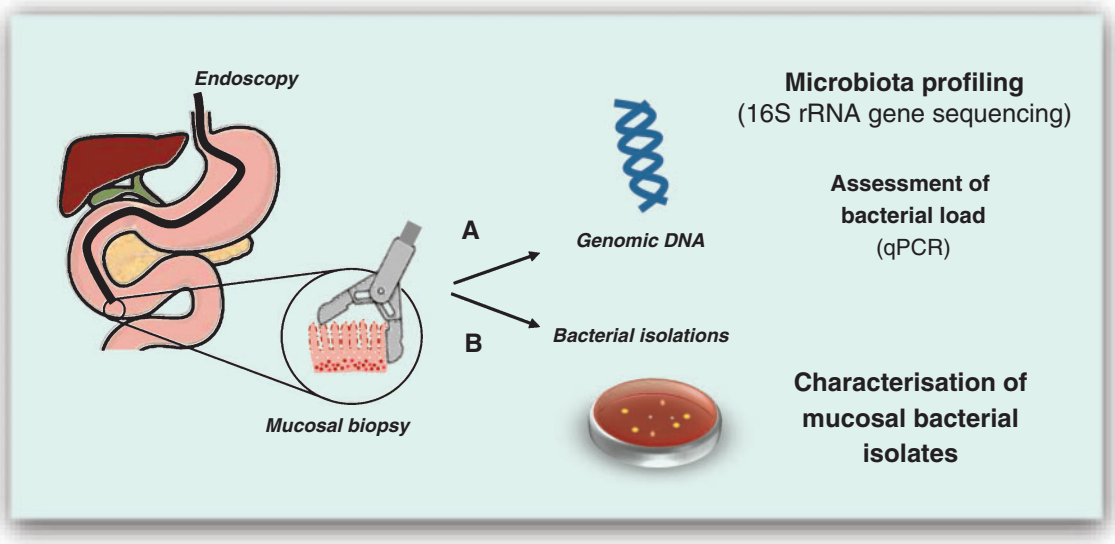

Figure 2. Analysis of duodenal mucosal biopsies. The mucosa of the small intestine is sampled using a biopsy forcep during a patient's endoscopy procedure. The mucosal biopsies are processed via two methods. (A) For nucleic acid based analyses, the biopsy is stored frozen until use. Genomic DNA is extracted utilising optimised methods and utilised for Illumina 16S rRNA gene amplicon sequencing, and quantitative PCR analysis for approximation of bacterial load. (B) For culture based analyses, the biopsy is transferred, within the biopsy forceps, immediately to an anaerobic chamber, homogenised and stored frozen in anaerobic glycerol. A variety of rich culture media are utilised, as well as aerobic and anaerobic incubation conditions, to isolate bacteria directly from the biopsy tissue.

techniques, we have identified correlations between bacterial load and the specific symptoms experienced by FD patients[32]. Specifically, the density of mucosal bacteria in the duodenum was positively correlated with meal-related symptoms and negatively correlated with patient quality of life scores. Indeed, bacterial load appears to be a stronger driver of FD symptoms than any specific alteration in the taxonomic profile of the duodenal microbiota. These findings are supported by the recent publication of a placebo-controlled trial in which treatment with the poorly absorbed antibiotic rifaximin was found to reduce symptoms in FD patients[33].

While profiling studies are useful in regards to identifying the overall structure of a microbial community, the data is limited in its ability to provide functional or mechanistic insights, or indeed reflect the presence of live, niche adapted organisms. To that end, we are currently developing a collection of contamination-free tissue for microbe isolation and culture. Using this approach, new duodenal isolates of *Streptococcus, Veillonella, Fusobacterium, Actinomyces* and *Neisseria* spp. have been obtained[34]. We are now sequencing the genomes of these isolates, and testing their pro-inflammatory activity, with a view to further defining taxonomic and functional specificities, not unlike the approaches used to date to identify colonic bacteria considered relevant to the progress of inflammatory bowel diseases[35].

Conclusion

In summary, the small intestine represents an important component of our GI tract, and yet we know relatively little regarding the microbial communities inhabiting this region. Developments in molecular microbiology have opened a window on the organisms residing in the mucosa; however, further research is required, particularly in regards to understanding the microbiota as a whole, encompassing bacteria, along with fungi, archaea, protozoa and viruses. Innovations in sampling techniques, and further well-designed studies targeting the MAM of the proximal small intestine, will allow us to better understand the niche-adapted microbes present in this region, and the host-microbe interactions that are likely to be important in disease processes. This provides potential for improved diagnoses and more targeted interventions that will enable treatment of GI disorders, rather than simply management of ongoing patient symptoms.

References

1. Aguirre de Cárcer, D. *et al*. (2011) Numerical ecology validates a biogeographical distribution and gender-based effect on mucosa-associated bacteria along the human colon. *ISME J.* **5**, 801–809. doi:10.1038/ismej.2010.177
2. Gevers, D. *et al*. (2014) The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe* **15**, 382–392. doi:10.1016/j.chom.2014.02.005
3. Helander, H.F. and Fandriks, L. (2014) Surface area of the digestive tract – revisited. *Scand. J. Gastroenterol.* **49**, 681–689. doi:10.3109/00365521.2014.898326
4. Kendall, A.I. (1915) The bacteria of the intestinal tract of man. *Science* **42**, 209–212. doi:10.1126/science.42.1076.209
5. Davison, W.C. (1925) The duodenal contents of infants in health, and during and following diarrhea. *Am. J. Dis. Child.* **29**, 743–756. doi:10.1001/archpedi.1925.04120300017002
6. Anderson, C.M. and Langford, R.F. (1958) Bacterial contents of small intestine of children in health, in coeliac disease, and in fibro-cystic disease of pancreas. *BMJ* **1**, 803–806. doi:10.1136/bmj.1.5074.803
7. Lou, M.A. *et al*. (1977) Bacteriology of the human biliary tract and the duodenum. *Arch. Surg.* **112**, 965–967. doi:10.1001/archsurg.1977.01370080063010
8. de Meij, T.G. *et al*. (2013) Composition and diversity of the duodenal mucosa-associated microbiome in children with untreated coeliac disease. *Scand. J. Gastroenterol.* **48**, 530–536. doi:10.3109/00365521.2013.775666
9. He, C. *et al*. (2016) Imbalance of gastrointestinal microbiota in the pathogenesis of *Helicobacter pylori*-associated diseases. *Helicobacter* **21**, 337–348. doi:10.1111/hel.12297
10. Nistal, E. *et al*. (2012) Differences of small intestinal bacteria populations in adults and children with/without celiac disease: effect of age, gluten diet, and disease. *Inflamm. Bowel Dis.* **18**, 649–656. doi:10.1002/ibd.21830
11. Cheng, J. *et al*. (2013) Duodenal microbiota composition and mucosal homeostasis in pediatric celiac disease. *BMC Gastroenterol.* **13**, 113. doi:10.1186/1471-230X-13-113
12. Wacklin, P. *et al*. (2013) The duodenal microbiota composition of adult celiac disease patients is associated with the clinical manifestation of the disease. *Inflamm. Bowel Dis.* **19**, 934–941. doi:10.1097/MIB.0b013e31828029a9
13. Li, G. *et al*. (2015) Diversity of duodenal and rectal microbiota in biopsy tissues and luminal contents in healthy volunteers. *J. Microbiol. Biotechnol.* **25**, 1136–1145.
14. D'Argenio, V. *et al*. (2016) Metagenomics reveals dysbiosis and a potentially pathogenic *N. flavescens* strain in duodenum of adult celiac patients. *Am. J. Gastroenterol.* **111**, 879–890. doi:10.1038/ajg.2016.95
15. Chen, Y. *et al*. (2016) Dysbiosis of small intestinal microbiota in liver cirrhosis and its association with etiology. *Sci. Rep.* **6**, 34055. doi:10.1038/srep34055
16. Nistal, E. *et al*. (2016) Study of duodenal bacterial communities by 16S rRNA gene analysis in adults with active celiac disease vs non-celiac disease controls. *J. Appl. Microbiol.* **120**, 1691–1700. doi:10.1111/jam.13111
17. Pellegrini, S. *et al*. (2017) Duodenal mucosa of patients with type 1 diabetes shows distinctive inflammatory profile and microbiota. *J. Clin. Endocrinol. Metab.* **102**, 1468–1477. doi:10.1210/jc.2016-3222
18. Dewhirst, F.E. *et al*. (2010) The human oral microbiome. *J. Bacteriol.* **192**, 5002–5017. doi:10.1128/JB.00542-10
19. Dlugosz, A. *et al*. (2015) No difference in small bowel microbiota between patients with irritable bowel syndrome and healthy controls. *Sci. Rep.* **5**, 8508. doi:10.1038/srep08508
20. Filyk, H.A. and Osborne, L.C. (2016) The Multibiome: the intestinal ecosystem's influence on immune homeostasis, health, and disease. *EBioMedicine* **13**, 46–54. doi:10.1016/j.ebiom.2016.10.007
21. D'Argenio, V. *et al*. (2016) No change in the mucosal gut microbiome is associated with celiac disease-specific microbiome alteration in adult patients. *Am. J. Gastroenterol.* **111**, 1659–1661. doi:10.1038/ajg.2016.227
22. Erdogan, A. and Rao, S.S. (2015) Small intestinal fungal overgrowth. *Curr. Gastroenterol. Rep.* **17**, 16. doi:10.1007/s11894-015-0436-2
23. Goodrich, J.K. *et al*. (2014) Conducting a microbiome study. *Cell* **158**, 250–262. doi:10.1016/j.cell.2014.06.037
24. Noecker, C. *et al*. (2017) High-resolution characterization of the human microbiome. *Transl. Res.* **179**, 7–23. doi:10.1016/j.trsl.2016.07.012
25. Salter, S.J. *et al*. (2014) Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol.* **12**, 87. doi:10.1186/s12915-014-0087-z
26. Zhang, C. *et al*. (2015) Identification of low abundance microbiome in clinical samples using whole genome sequencing. *Genome Biol.* **16**, 265. doi:10.1186/s13059-015-0821-z

27. Imhann, F. *et al*. (2016) Proton pump inhibitors affect the gut microbiome. *Gut* **65**, 740–748. doi:10.1136/gutjnl-2015-310376

28. Jackson, M.A. *et al*. (2016) Proton pump inhibitors alter the composition of the gut microbiota. *Gut* **65**, 749–756. doi:10.1136/gutjnl-2015-310861

29. Liebregts, T. *et al*. (2011) Small bowel homing T cells are associated with symptoms and delayed gastric emptying in functional dyspepsia. *Am. J. Gastroenterol.* **106**, 1089–1098. doi:10.1038/ajg.2010.512

30. Walker, M.M. *et al*. (2014) Duodenal eosinophilia and early satiety in functional dyspepsia: confirmation of a positive association in an Australian cohort. *J. Gastroenterol. Hepatol.* **29**, 474–479. doi:10.1111/jgh.12419

31. Shanahan, E.R. *et al*. (2016) Characterisation of the gastrointestinal mucosa-associated microbiota: a novel technique to prevent cross-contamination during endoscopic procedures. *Aliment. Pharmacol. Ther.* **43**, 1186–1196. doi:10.1111/apt.13622

32. Zhong, L. *et al*. (2017) Dyspepsia and the microbiome: time to focus on the small intestine. *Gut* **66**, 1168–1169. doi:10.1136/gutjnl-2016-312574

33. Tan, V.P. *et al*. (2017) Randomised clinical trial: rifaximin versus placebo for the treatment of functional dyspepsia. *Aliment. Pharmacol. Ther.* **45**, 767–776. doi:10.1111/apt.13945

34. Shanahan, E.R. *et al*. (2016) Isolation of mucosa-associated bacteria from the duodenum in functional dyspepsia patients utilising the Brisbane Aseptic Biopsy Device. *J. Gastroenterol. Hepatol.* **31**, 45[Abstract #2068]. doi:10.1111/jgh.13540

35. Sokol, H. *et al*. (2008) *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc. Natl. Acad. Sci. USA* **105**, 16731–16736. doi:10.1073/pnas.0804812105

Biographies

Dr Erin Shanahan is a post-doctoral researcher in the Gastroenterology and Hepatology Department at Princess Alexandra Hospital, and with the Faculty of Medicine, University of Queensland. She is based at the Translational Research Institute, Brisbane, Queensland. Erin is a molecular microbiologist whose focus is characterising and understanding the role of the small intestinal microbiota.

Professor Mark Morrison is Chair in Microbial Biology and Metagenomics at the University of Queensland Diamantina Institute and an Affiliate Professor with the University of Queensland School of Chemistry and Molecular Biosciences. He is based at the Translational Research Institute, Brisbane, Queensland. He and his team are using DNA sequencing technologies, as well as other microbiological and genetic methods, to produce new insights into the gut microbiota.

Professor Gerald Holtmann is the Director of the Department of Gastroenterology and Hepatology at Princess Alexandra Hospital, and Associate Dean (Clinical) in the Faculty of Medicine and Faculty of Health and Behavioural Sciences at The University of Queensland. His research focus is on the gut microbiome, mucosal and systemic inflammation and brain-gut interactions, as drivers for gastrointestinal disorders.