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Faecal microRNAs: indicators of imbalance at the host-microbe interface?

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RESEARCH ARTICLE

Abstract

The enteric microbiota is characterised by a balance and composition that is unique to the host. It is important to understand the mechanisms through which the host can maintain the composition of the gut microbiota. MicroRNAs (miRNA) are implicated in intercellular communication and have been isolated from bodily fluids including stool. Recent findings suggest that miRNA produced by the host's intestinal epithelial cells (IECs) participate in shaping the microbiota. To investigate whether miRNA expression was influenced by the gut microbiota we measured the expression of miRNAs expressed by intestinal epithelial cells in faeces. Specifically, we measured miRNA expression in faeces from germ-free (GF) and conventional mice and similarly in a rat model of antibiotic-mediated depletion of the gut microbiota control rats. In adult male GF and conventional mice and adult Sprague Dawley (SD) rats were treated with a combination of antibiotics for 8 weeks; total RNA was extracted from faecal pellets taken at week 0, 2, 4, 6 week 8 and the expression of let-7b-3p, miR-141-3p, miR-200a-3p and miR-1224-5p (miRNAs known to be expressed in IECs) were measured relative to U6 at each time point using qRT-PCR. In GF animals the expression of let-7b, miR-141 and miR-200a in faeces was lower compared to conventional mice. Following antibiotic-mediated depletion of gut microbiota, rats showed two divergent profiles of miRNA expression. Following two weeks of antibiotic treatment, the expression of let-7b and miR-1224 dropped significantly and remained low for the remainder of the study. The expression of miR-200a and miR-141 was significantly higher at week 2 than before antibiotic treatment commenced. Subsequently, the expression of miR-200a and miR-141 decreased at week 4 and continued to decrease at week 6. This data demonstrates that miRNAs can be used as an independent, non-invasive marker of microbial fluctuations along with gut pathology in the intestine.

Keywords: microbiota, microRNA, enteric, stool, host

1. Introduction

The gut hosts a complex and diverse population of commensal bacteria collectively known as the gut microbiota, which has increasingly been implicated in host metabolism, immunity, nervous system function and disease (Backhed *et al.*, 2005; Cryan and Dinan, 2012; Diaz Heijtz *et al.*, 2011; Grenham *et al.*, 2011). Colonisation of the human gut is post-natal and commences upon exposure to bacteria during birth. During the first year of life the microbiota has a maternal signature, however, it evolves fairly quickly and reaches adult-like complexity after 1-3 years (Grenham *et al.*, 2011; Mackie *et al.*, 1999; Palmer *et al.*, 2007). Numerous factors have been shown to influence gut microbiota composition, including diet, disease, and host age and genetics (Claesson *et al.*, 2011; Gulati *et al.*, 2012; Wu and Hui, 2011). The composition of the microbiota seems to maintain a personal 'signature' which may be transiently altered by antibiotics, infection and diseases (Forsythe *et al.*, 2010; Manichanh *et al.*, 2010). Thus, despite a significant interpersonal and inter-special variation, the enteric microbiota seems to be characterised by a balance and composition that is unique and beneficial to the individual host, and disruption of this balance causes disease susceptibility (Diaz Heijtz *et al.*, 2011). To this extent, it is important to characterise the biological mechanisms through which the host can maintain the composition of the gut microbiota and how this relationship is affected during pathological states.

MicroRNAs (miRNAs) are small, non-coding RNAs synthesised in the nucleus and functional in the cytoplasm, where they are involved in regulating transcription (Bartel, 2004). Bacteria express non-coding RNAs, but they differ from miRNA in size (50-500 nucleotides in length) and structure, including the number of stem-loops (Viegas and Arraiano, 2008). The oral pathogen *Streptococcus sanguinis* was recently shown to release a number of small RNAs in micro vesicles suggested to be involved in bacteria-to bacteria communication and in addition bacteria-to-host communication (Choi *et al.*, in press). Recently, host miRNAs have been implicated in intercellular communication and have been isolated from body fluids including stool (Ahmed *et al.,* 2012, Mittelbrunn and Sánchez-Madrid, 2012, Weber *et al.,* 2010). The functional role of miRNAs in host-microbe communication is only beginning to be understood. It has recently been shown that the microbiota regulates gene expression in the colon of mice (Dalmasso *et al.*, 2011) and recent findings seem to suggest that miRNAs produced by the host's intestinal epithelial cells IECs participate in shaping the gut microbiota and affect bacterial growth (Liu *et al.,* 2016). Detection of miRNA isolated from the faeces of rodents may offer the potential to monitor this relationship in live animals in a temporal fashion. To further investigate this possibility, we aimed to determine the effect of bacterial status on the expression of key microRNAs expressed by IEC's in GF mice and in a rat model of antibiotic-mediated depletion of the gut microbiota.

2. Materials and methods

Germ-free mice

All experiments were conducted in accordance with European Directive 86/609/EEC. Approval by the Animal Experimentation Ethics Committee of University College Cork was obtained before commencement of all animal related experiments. Adult male germ-free (GF) and conventional (CON) C57/Bl6 breeding pairs were obtained from Taconic (Germantown, NY, USA) and first generation mice were used. GF mice were housed in gnotobiotic isolators under a strict 12-h light/dark cycle in cages of 4-5 animals. CON mice are similarly housed under regulated conditions (temperature 20-21 °C, 55-60% humidity), under the same 12 h light/dark cycle and housed 4-5 per cage. Autoclaved, pelleted diets were the same for both groups (Special Diet Service, Essex, UK, product code 801010). Faecal pellets were obtained at 8 weeks old. Fresh faecal pellets were collected and stored at -80 °C.

Animals, antibiotic treatment

Adult male Sprague Dawley rats (n=10/group) (Envigo, Alconbury, UK) were housed 5 per cage in standard rat cages. All animals were housed in a standard animal facility under a strict 12-h light/dark cycle. All experimental groups received the same autoclaved diet (Teklad Global 18% Protein Rodent diet, Product code 2018S). Approval by the Animal Experimentation Ethics Committee of University College Cork was obtained before commencement of all animal related experiments. In order to sufficiently deplete the gut microbiota rats were treated with a combination of antibiotics for 8 weeks at adulthood (10 weeks old at start of treatment). The antibiotic cocktail was administered in drinking water to avoid possible adverse effects from chronic stress induced by alternative administration methods such as oral gavage. The antibiotic cocktail consisted of ampicillin (1 g/l), vancomycin (500 mg/l), and ciprofloxacin HCL (20 mg/l), imipenem (250 mg/l) and metronidazole (1 g/l). The antibiotic cocktail was chosen based on published protocols and was made up in autoclaved water and changed every 3 days (Hoban *et al.*, 2016). Animal weights were taken every 3 days to ensure animals were not losing excessive body weight. Along with regular weight monitoring, weekly fresh faecal pellets were collected to ensure microbiota depletion. Control animals received autoclaved water without any antibiotics which was also changed every 3 days. Fresh faecal pellets were collected once a week for 8 weeks and stored at -80 °C.

Total RNA extraction and quantitative real-time PCR

100 mg of faecal pellets from each animal were homogenised in miRVana™ lysis buffer using a bead beater. Total RNA (including miRNAs) was extracted using the miRVana™ Total RNA Isolation kit (Ambion; Thermo Fisher, Waltham, MA, USA) as per the manufacturer's instructions. Purity and quantity were assessed using the Agilent bioanalyzer and a Nano-drop spectrophotometer (Agilent, Santa Clara, CA, USA). mRNA was reverse transcribed to cDNA using the miRCURY LNA™ cDNA synthesis kit II and cDNA was amplified using probe specific primers (Table 1) and Exilent SYBR green (all Exiqon, Vedbaek, Denmark). Assay and spike-in controls were used for normalisation and all assays were run in triplicate and expressed relative to U6 at each time point. Melt-curve analysis was performed to ensure that only one source of amplification was present. Using the Grubbs test any outliers were removed and all data were analysed using the $2^{-\Delta\Delta Ct}$ relative quantitation method (Livak and Schmittgen, 2001) and expressed as fold change compared to control values.

In-silico analysis of faecal miRNA target gene function

Pathway analysis was carried out with DIANA-miRPath v3.021 (Vlachos et al., 2015), using predicted microRNA targets from the DIANA-microT-CDS v5.0 algorithm49 and Gene Ontology genesets derived from KEGG. The *P*-value threshold was set to 0.05 and MicroT threshold to 0.8, statistical correction for multiple comparisons was used.

Table 1. miRNA probe details and sequence information.

Probe	Product code	Sequence	Sequence reference
U6 snRNA	203907	CACGAATTTGCGTGTCATCCTT	NR_004394.1 (primer detects mouse and rat transcripts)
let-7b-3p	205653	CUAUACAACCUACUGCCUUCCC	MIMAT0004482
miR-141-3p	204504	UAACACUGUCUGGUAAAGAUGG	MIMAT0000432
miR-200a-3p	204707	UAACACUGUCUGGUAACGAUGU	MIMAT0000682
miR-1224-5p	2115039	GUGAGGACUGGGGAGGUGGAG	MIMAT0005460

Statistics

Data is presented as mean ± standard error of the mean (SEM). Unless otherwise stated, data were analysed using one-way ANOVA and subsequently the unpaired Student's t-test. The level of statistical significance was set at $P \leq 0.05$. Statistical significance was indicated as follows: * indicates $P<0.05$, ** indicates $P<0.01$ and *** indicates $P<0.001$. Diana-miRPath *in-silico* analysis statistics used Fisher's exact test and false discovery rate estimation.

3. Results

Epithelial miRNAs in the faeces of germ-free mice are significantly reduced

MicroRNAs are stably expressed and secreted in the faeces of mice (Liu *et al.*, 2016b). In an effort to identify if miRNA present in the faecal pellets of mice are affected by absence or depletion of the microbiota we performed a literature search and chose to measure the expression of four miRNAs previously shown to be expressed in the epithelial cells of mice (Lee *et al.*, 2015). We initially chose 4 miRNAs that were previously shown to be expressed in the intestinal epithelium (Lee *et al.*, 2015). Expression of let-7b-3p, miR-141-3p, miR-200a-3p and miR-1224-5p were measured in faeces at 8 weeks of age and were chosen based on the literature and were measured relative to U6 snRNA in conventional and germ-free mice. Mice born in a germ-free environment showed a significant reduction in expression of let-7b-3p, miR-141-3p and miR-200a-3p (Figure 1, $P<0.05$) compared with conventionally raised mice. The expression of miR-1224-5p was also lower in germ-free animals, however, this failed to reach statistical significance.

Antibiotic mediated depletion of the gut microbiota influences faecal miRNA expression in diverse patterns

Recognising that miRNA expression in the faeces of mice was influenced by the microbiota, we investigated if absence of bacteria from birth was required to see such reductions in miRNA expression. To this end we depleted the microbiota in rats using a cocktail of antibiotics previously described (Desbonnet *et al.*, 2015). No weight loss was observed in control or antibiotic treated rats. We were able to detect miRNAs in the faecal pellets of rats, as others had previously shown was possible (John-Baptiste *et al.*, 2012). We subsequently measured the expression of the same miRNAs (let-7b-3p, miR-141-3p, miR-200a-3p and miR-1224-5p) in the faeces of rats at 8 weeks while undergoing antibiotic-mediated depletion of the gut microbiota over a 13 week period. Interestingly, miRNA expression displayed a divergent pattern in response to the antibiotic treatment. Following two weeks of antibiotic treatment, the expression of let-7b-3p and miR-1224-5p was significantly lower (Figure 2A-B, $P<0.001$) and remained at the same level during antibiotic treatment for four, six and eight weeks of antibiotic treatment. In contrast to this expression pattern; following two weeks of antibiotic treatment, the expression of both miR-200a-3p and miR-141-3p was significantly higher than before the treatment (Figure 2C-D, $P<0.05$). At week 4 though, the expression of both miRNAs had returned to baseline and maintained this level until the end of the experiment.

Functionality of faecal miRNA target genes

MiRNAs negatively regulate target mRNA expression via repression or degradation (Bartel, 2004) and to verify the functionality of the miRNAs measured in this study we performed *in silico* analysis on the target genes of these miRNAs. Using DIANA-miRPath (Vlachos *et al.*, 2015) we functionally annotated the target genes relevant to let-7b-3p, miR-141-3p, miR-200a-3p and miR-1224-5p. DIANA-miRPath assigns experimentally supported target genes of miRNAs (from DIANA-microT-CDS (Paraskevopoulou *et al.*, 2013)) to KEGG pathways which allows a functionality of the miRNA to be inferred. This bioinformatic approach revealed a number pathways of functional relevance in our study. Specifically, a number of pathways relevant to the intestinal environment were significantly over-represented (Table 2). These included bacterial invasion of epithelial cells (let-7b-3p), pathways involved in mucin production (mmu-1224-5p) and both inflammable bowel disease (IBD) and immunoglobulin A (IgA) synthesis (miR-200a-3p). Furthermore, they provide a rational contact for future work in this area.

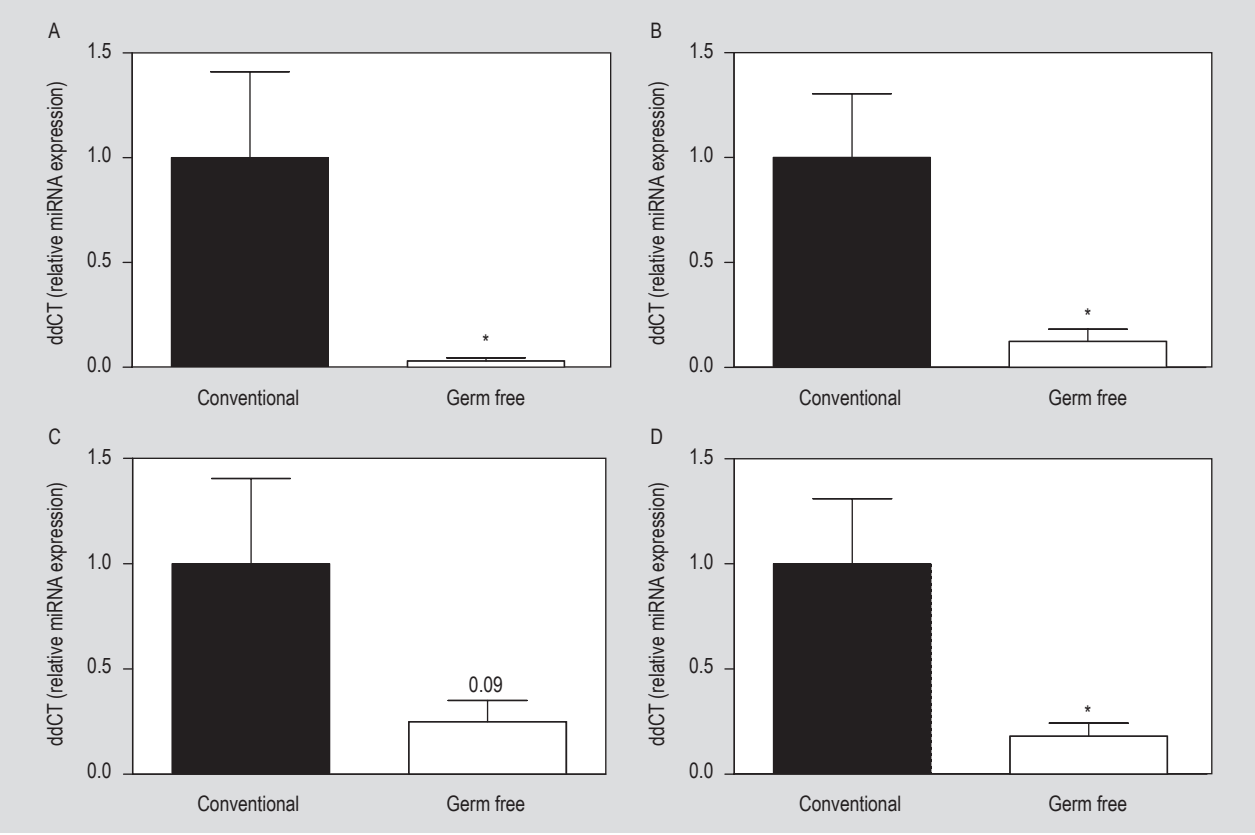

Figure 1. Host miRNA expression is altered in the faeces of germ-free mice. Total RNA is extracted from faecal pellets from germ-free and conventional mice and gene expression for (A) let-7b-3p, (B) miR-200a-3p, (C) miR-1224-5p and (D) miR-141-3p were measured by qRT-PCR relative to U6 gene expression (n=5-6 per group). The expression of miR-200a, miR-141 and let-7b was significantly lower in germ-free animals (t9=2.571, *P*=0.0301; t9=2.351, *P*=0.0432; t7=2.685, *P*=0.0313). The expression of miR-1224 in germ-free animals was also lower, without reaching statistical significance (t9=2.003, *P*=0.0853). Data is presented as mean ± standard error of the mean (SEM). * indicates *P*<0.05.

Using metagenomic data recently published from our group (Hoban *et al.*, 2016) we were able to correlate faecal miRNA expression in antibiotic-treated rats with relative abundance data from the faeces of the same rats at week 4 of the experiment (4 weeks after the start of antibiotic treatment) (Table 3). No correlation between the relative abundance of bacterial families was observed (data not shown), interestingly though, there was significant correlation between relative abundance of the phyla *Bacteroidetes*, and *Firmicutes* with miR-141-3p. Similarly, the phyla *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes* and *Proteobacteria* were significantly correlated with miR-200a-3p (Table 3). Interestingly, correlations were only observed in those miRNAs that increased significantly following the commencement of antibiotic administration.

4. Discussion

Recent studies have shown that host miRNAs exist extracellularly and may be indicative of specific disease states (Weber *et al.*, 2010). Additionally, recent work has identified host faecal miRNAs as a normal component of both human and mouse faeces (Liu *et al.*, 2016). It has only recently become clear that miRNAs may be altered in the presence of a disturbed gut microbiota (Dalmasso *et al.*, 2011). To this end we sought to identify if the expression of host miRNAs in the faeces were altered in germ-free mice and in rats whose microbiota has been depleted by antibiotics using qRT-PCR. We chose to analyse the expression of 4 key miRNAs; miR-1224-5p, miR-141-3p, miR-200a-3p and let-7b-3p. We selected these miRNAs on the basis that they are highly expressed and abundant in mouse faecal samples (Lee *et al.,* 2015). In addition, the miRNA selected were also among the most abundant miRNAs isolated from human faecal samples and thus of further translational interest (Liu *et al.*, 2016). Furthermore, these miRNAs are constitutively expressed in murine IECs, the most likely source of faecal miRNAs (Lee *et al.*, 2015).

The interaction between the host and its resident microbiota is an intriguing one, and miRNAs are known to operate at this interface (Runtsch *et al.*, 2014). Defining the molecular pathways involved in host-microbe crosstalk are essential for translational research and the search for

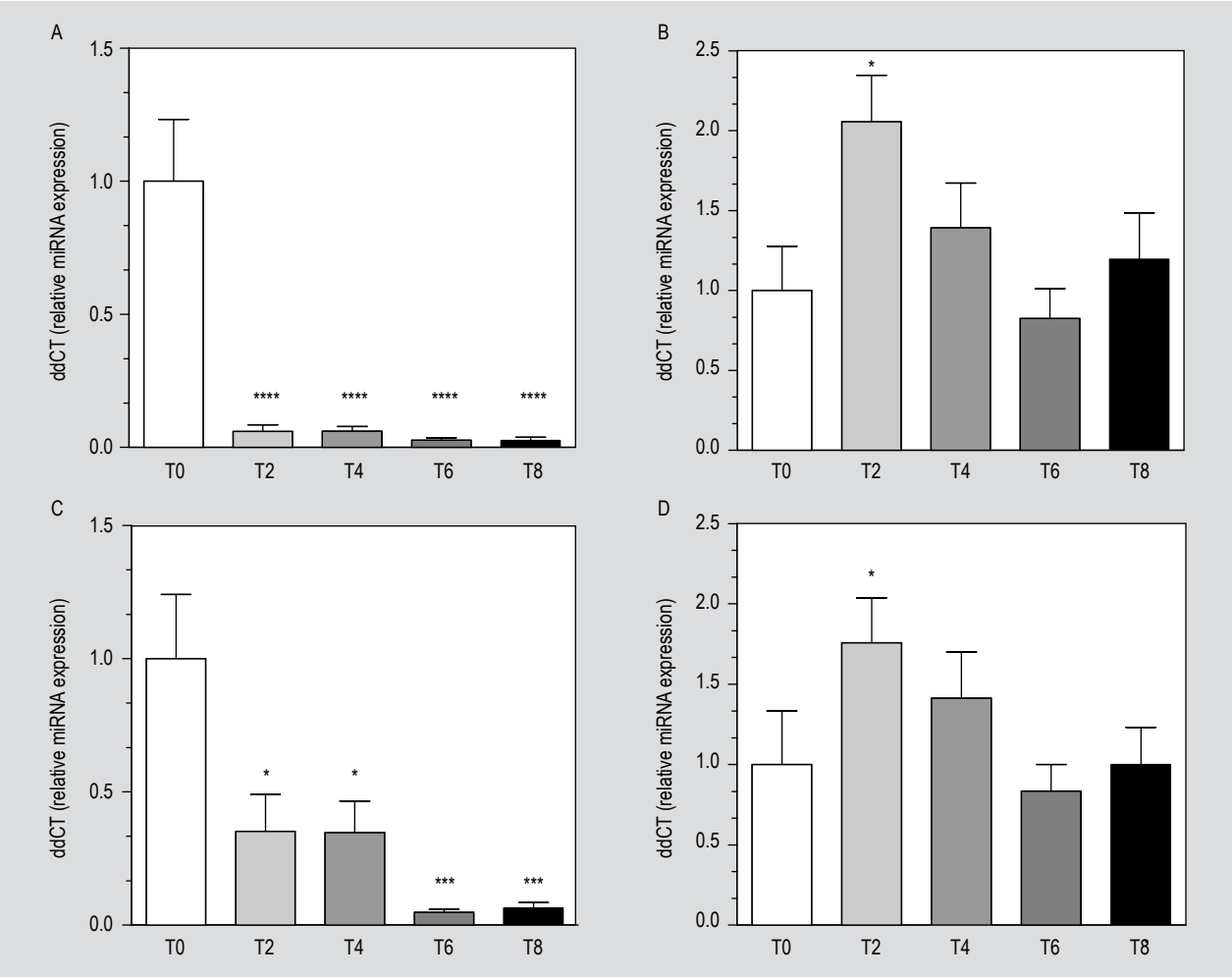

Figure 2. Depletion of the gut microbiota with antibiotics has divergent effects on host-miRNA secretion in faeces. Total RNA is extracted from faecal pellets from mice treated with an antibiotic cocktail and untreated mice and gene expression for (A) let-7b-3p, (B) miR-200a-3p, (C) miR-1224-5p and (D) miR-141-3p were measured by qRT-PCR relative to U6 gene expression over an 8 week treatment period at 2 week intervals (n=7-10 per group). Data is presented as mean ± standard error of the mean (SEM). * indicates *P*<0.05, ** indicates *P*<0.001.**

alternative therapies for conditions in which changes in the microbiota are implicated. Dalmasso et al. (2011), recently demonstrated that in the colon of germ-free mice, a number of miRNAs are differentially expressed and that these miRNAs interact with epithelial cell ion transporters in a mechanism which may regulate host gene expression (Zhang *et al.*, 2016). It is intriguing to the think that miRNAs in the faeces may represent a method of analysing host-microbe interactions in a live animal. In germ-free animals we observed significant reductions in miR-141-3p, miR-200a-3p and let-7b-3p, respectively. This data is in partial agreement with data reported by Dalmasso *et al.* (2011) in which the expression of a number of miRNAs different to those measured here were reduced in the germ-free colon. However, this data is at odds with recent sequencing data which found increased abundance of miRNA transcripts in GF mice. Using RNA sequencing Liu *et al.* (2016) found increases in global miRNA expression, including the miRNAs measured here albeit using a different technique. Reasons for this may be due to technical differences, this paper measured all detectable miRNAs in one sample whereby we have compared singular miRNA expression using a sensitive and quantitative method (Liu *et al.*, 2016).

Germ-free rodents are different from antibiotic treated rodents in many physiological aspects as we and others have shown in regards behaviour (Hoban *et al.*, 2016), the immune system and metabolism (Sudo *et al.*, 2004). In addition, antibiotic treatment will eliminate different bacteria at different times. This suggests that miRNAs and bacteria may interact indirectly, such that genes targeted by specific miRNAs may be translationally repressed which subsequently influences epithelial cell structure and function which effects the microbiota (Goodrich *et al.*, 2014). Moreover, eliminating the microbiota with

Table 2. Functional characterisation of faecal microRNAs.

	KEGG pathway	*P*-value
let-7b-3p		
1	Pantothenate and CoA biosynthesis (mmu00770)	0.00389
2	Cell adhesion molecules (CAMs) (mmu04514)	0.00389
3	Bacterial invasion of epithelial cells (mmu05100)	0.00689
4	Glycerolipid metabolism (mmu00561)	0.02292
5	Biosynthesis of unsaturated fatty acids (mmu01040)	0.02380
miR-141-3p		
1	Nicotine addiction (mmu05033)	0.00005
2	Gap junction (mmu04540)	0.00035
3	Thyroid hormone signalling pathway (mmu04919)	0.02383
4	Axon guidance (mmu04360)	0.04064
miR-200a-3p		
1	Glycosaminoglycan biosynthesis-heparan sulphate/heparin (mmu00534)	0.0000001
2	Intestinal immune network for IgA production (mmu04672)	0.0140674
3	Inflammatory bowel disease (mmu05321)	0.0184161
mmu-1224-5p		
1	Mucin type O-glycan biosynthesis (mmu00512)	0.003
2	Vitamin B6 metabolism (mmu00750)	0.041
3	Oestrogen signalling pathway (mmu04915)	0.049

Table 3. Correlation of faecal miRNA expression with phyla relative abundance.[1]

Phyla	*Acidobacteria*	*Actinobacteria*	*Bacteroidetes*	*Cyanobacteria*	*Firmicutes*	*Proteobacteria*
miR-141-3p						
Pearson r	0.50	0.70	0.80	0.73	0.86	0.62
P-value	0.26	0.08	***0.03***	0.06	***0.01***	0.13
R^2	0.25	0.49	0.64	0.53	0.74	0.39
let-7b-3p						
Pearson r	-0.58	-0.19	-0.22	0.40	-0.01	0.20
P-value	0.17	0.69	0.63	0.37	0.99	0.67
R^2	0.34	0.03	0.05	0.16	0.00	0.04
miR-200a-3p						
Pearson r	0.35	0.79	0.78	0.77	0.76	0.88
P-value	0.44	***0.04***	***0.04***	***0.04***	***0.05***	***0.01***
R^2	0.12	0.62	0.60	0.60	0.58	0.78
miR-1224-5p						
Pearson r	-0.27	0.15	0.07	0.62	0.21	0.55
P-value	0.52	0.72	0.87	0.10	0.62	0.16
R^2	0.07	0.02	0.00	0.39	0.04	0.30

[1] Significant correlations are marked in bold Italic.

antibiotics at different developmental stages will have very distinct effects in the molecular architecture of the intestinal epithelium.

The detection of miRNAs in the faeces of rodents may offer a new method for measuring signalling events between the microbiota and the host epithelium on a temporal basis in live animals. To examine this, we measured the expression of miR-1224-5p, miR-141-3p, miR-200a-3p and let-7b-3p

in the faeces of rats before antibiotic treatment and every 2 weeks thereafter up to 8 weeks. Previous work by Liu et al. (2016) has shown in mice using RNA sequencing that almost all luminal miRNAs are increased significantly following 1 week of antibiotic treatment compared with specific pathogen free mice, albeit with a different antibiotic cocktail (ampicillin 1 mg/ml, vancomycin 500 mg/ml, neomycin 1 mg/ml, metronidazole 1 mg/ml, and streptomycin 1 mg/ml). Similarly, in rats, we found that the expression of 2 of the miRNAs examined, miR-141-3p and miR-200a-3p were significantly increased in faeces after 2 weeks. Subsequently though the expression of these miRNAs decreased to baseline levels at 6 weeks and remained at this level for the rest of the study. MiR-1224-5p and let-7b-3p on the other hand displayed a rapid reduction in expression following 2 weeks of antibiotic treatment, a reduction that persisted up to 8 weeks. Our analysis of miRNA expression was conducted over an 8 week period, whereas Liu et al. (2016) measured miRNA expression at just 1 week. MiRNAs are expressed on numerous epithelial cell lineages in the intestine and these cells may be shed at different rates following antibiotic treatment and this may be reflected in the divergent pattern of miRNA secretion in faeces. MiRNA may exist in the lumen in 2 ways, firstly, exosomes released from almost all cell types and secondly 'naked'. All exosomes bare the molecular hallmarks of the cell from which they are derived. For example, miRNA derived from exosomes of epithelial origin will contain proteins, genes and miRNAs specifically associated with the epithelial cell (Schorey *et al.*, 2015).

Let-7b-3p belongs to the let7 family of miRNAs which all show a generic response during bacterial infection of macrophage and epithelial cells to reduce the expression (post-transcriptionally) of IL-10 production (Schulte *et al.*, 2011). Mmu-let-7b-3p has also been shown to mediate the epithelial innate immune response via TLR signalling (Hu *et al.*, 2009). Furthermore, let-7b is also known to target Tlr4 and regulates the activation of NF-κB and the expression of downstream genes related to immune responses in *Helicobacter pylori* infection (Teng *et al.*, 2013) while also binding to the Muc1 gene in epithelial cells (Inyawilert *et al.*, 2015). miR-141-3p is expressed in immune cells (natural killer cells, leukocytes) and both inflamed and non-inflamed epithelial cells in the small and large intestine (Lee *et al.*, 2015). Similar to let-7b-3p, miR-141-3p is thought to target chemokines, increasing the production of Cxcl16 and Cxcl9 which drive inflammatory progression in IBD (Uza *et al.*, 2011). miR-200a-3p belongs to the miR-18 family (of which miR-141-3p is a member also) and is expressed in epithelial cells throughout the body. Much of the biological function of this miRNA has been linked with cancer and in metastasis (Zang *et al.*, 2016) and along with miR-141-3p are enriched in the epithelial tissue. miR-1224-5p is expressed in the epithelial cells of both the small and large intestine where it is predicted to target the genes Aqp8 and Abcg2 in colitis (Lee *et al.*, 2015). miR-1224-5p has been shown to be induced by lipopolysaccharides *in vitro* in a macrophage cell line (Niu *et al.*, 2011) where it subsequently suppresses tumour necrosis factor alpha expression.

While faeces is considered a material waste it is also an extremely useful matrix for biological discovery. Firstly, it is consistently used as a source of biomarkers of diseases, such as IBD (calprotectin) and colon cancer (faecal occult blood). Secondly, it is non-invasive and is currently the source material for all faecal microbiota transplants in humans (and rodents) and finally, detection of mRNA in stool from infants has been used to monitor gastrointestinal development and maturation (Chapkin *et al.*, 2010). We and other groups have shown that detection of miRNA in the faeces of rodents and humans is possible and quantitative (John-Baptiste *et al.*, 2012; Liu *et al.*, 2016; Weber *et al.*, 2010). Furthermore, specific cells in the intestinal crypt express a distinct repertoire of miRNAs that allow each cell type to be distinguished (Peck *et al.*, 2017). The composition and expression levels of these miRNAs and how they relate to specific intestinal crypt cell types may indicate barrier function defects in pathological conditions. In fact, McKenna et al demonstrated using Dicer 1 (an enzyme essential for miRNA biogenesis) knock-out mice that miRNAs were essential for epithelial cell proliferation, differentiation, nutrient absorption, and that defective miRNA biogenesis was also responsible for impaired intestinal barrier function (McKenna *et al.*, 2010). Further work is required to confirm this but we feel that given the central role miRNAs play in intestinal barrier function that this hypothesis is relevant. More evidence is required before measuring miRNAs in faeces provides adequate information in regards intestinal barrier function and integrity.

It has been shown that faecal miRNAs are present in extracellular vesicles, and that these vesicles are shed primarily from IECs and +4 niche-derived Hopx-expressing cells, such as Goblet and Paneth cells (Liu *et al.,* 2016). In addition, recent work has demonstrated the use of faecal miRNAs as biomarkers of colorectal cancer (John-Baptiste *et al.*, 2012), further investigation is warranted in this area but with the caveat that faecal miRNAs may be effected by antibiotic treatment as we have shown here. Further work is needed to determine the precise source of the miRNAs within the faeces, they may be exosomal or epithelial cell in origin and we have only identified alterations in four miRNAs. Both models used in this study are extreme models in regards microbial depletion, further work should look at less severe models of microbial perturbation, specifically, it would be interesting to profile miRNAs in a monoassociated germ-free model and also, is the pattern of miRNA expression changed based on whether the bacteria is commensal or pathogenic. Furthermore, further work should characterise the temporal pattern of all miRNAs, both abundant and rare transcripts. The work presented

here offers a potential method for screening the viability of the intestinal barrier in live animals and how it is effected in numerous pathological conditions. How the host interacts and tolerates the gut microbiota is a key tenet of research at the moment, we believe this method will contribute critical insights into this mutualism and demonstrate previously unknown cooperation between host and microbe.

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