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Loss of microRNA-21 influences the gut microbiota causing reduced susceptibility in a murine model of colitis.

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Short title: microRNA-21 influences the gut microbiota and colitis.

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Abstract:

Background and aims: microRNAs regulate gene expression and influence the pathogenesis of human diseases. The present study investigated miR-21 in the pathogenesis of intestinal inflammation, as miR-21 is highly expressed in Inflammatory Bowel Disease (IBD). IBD is associated with intestinal barrier dysfunction and an altered gut microbiota. Recent studies have demonstrated that host microRNAs can shape the microbiota. Thus, we determined the influence of miR-21 on the gut microbiota and subsequent impact in a dextran sodium sulphate (DSS)-induced colitis model.

Methods: The influence of miR-21 on the gut microbiota and inflammation was assessed in wild-type (WT) and miR-21$^{-/-}$ mice, in co-housed mice, following antibiotic depletion of the microbiota, or by colonization of germ-free (GF) mice with fecal homogenate, prior to DSS administration. 16S rRNA sequencing was performed on WT and miR-21$^{-/-}$ mice to dissect potential differences in the gut microbiota.

Results: miR-21$^{-/-}$ mice have reduced susceptibility to DSS-induced colitis compared to WT mice. Co-housing conferred some protection to WT mice, while GF mice colonized with fecal homogenate from miR-21$^{-/-}$ were protected from DSS colitis compared to those colonized with WT homogenate. Further supporting a role for the microbiota in the observed phenotype, the protection afforded by miR-21 depletion is lost when mice are pre-treated with antibiotics. 16S rRNA sequencing revealed significant differences in the composition of WT and miR-21$^{-/-}$ intestinal microbiota.

Conclusions: These findings suggest that miR-21 influences pathogenesis of intestinal inflammation by causing propagation of a disrupted gut microbiota.

Keywords:

MicroRNA

Microbiota

Inflammation

1. Introduction

IBD, which includes Crohn's disease (CD) and Ulcerative colitis (UC), is a lifelong chronic inflammation of the gastrointestinal tract. IBD is a complex and multifactorial disease of unknown etiology, however it is generally accepted to arise from a complex interaction between genetic factors, environmental factors and the host immune system. It is thought that abnormal interaction of the gut microbiota with the underlying mucosal immune system leads to an aberrant immune activation and chronic inflammation[1]. Indeed IBD patients are associated with an altered gut microbiota (dysbiosis) and have an impaired intestinal barrier which either

contributes to the disease or arises as a result of inflammation, subsequently exacerbating the disease. Indeed, whether microbial dysbiosis causes impaired barrier function and development of inflammation, or if dysbiosis is a consequence of an altered barrier, remains to be determined. Recently, there has been increasing evidence that this inflammation is associated with the altered expression of miRNAs. MiRNAs are short ~22 nucleotide non-coding RNAs that act as post-transcriptional regulators of mRNA function by targeting specific RNAs for destruction or by repressing their translation[2-4]. miRNAs regulate many cellular processes in response to a wide variety of stimuli, including processes associated with the progression of IBD such as barrier function, mucin secretion, apoptosis and immune cell activation and function[5]. However, although altered miRNA expression profiles have been identified in serum and tissue from IBD patients the mechanistic basis underlying some of these associations is lacking[4].

MicroRNA-21 (miR-21) is overexpressed in both IBD patients and experimental models of IBD[6-9]. It has several reported functions that may impact on the disease, including roles in apoptosis and cytoskeletal rearrangement in the context of the barrier[8,9]. It also has functions in both innate and adaptive immunity. In particular, miR-21 has been shown to modulate the responses of macrophages to bacterially derived Toll-like receptor (TLR) agonists including LPS[10]. Recently the role of miRNAs in interactions between the host and its microbiota has begun to emerge, with the miRNA profile being dependent on the presence of an endogenous microbiota, while furthermore host miRNAs can influence the activity of the gut microbiota[11-13]. Given these observations and the ever-increasing evidence implicating the intestinal microbiota in the pathogenesis of IBD, we questioned whether miR-21 expression may influence the composition of the microbiota and if this was contributing to the pathogenesis of IBD.

Here we demonstrate that miR-21 plays a pathological role in the development of intestinal inflammation. We show that mice deficient in miR-21 (miR-21$^{-/-}$) are less susceptible to DSS-induced colitis compared to WT mice. We demonstrate a protective microbiota in the miR-21$^{-/-}$ mice that contributes to the observed reduced susceptibility in the DSS-colitis model. Furthermore, using 16s rRNA sequencing analysis we confirm alterations in the miR-21$^{-/-}$ microbial composition which are indicative of protection including a reduced abundance of the phylum *Bacteroidetes* and increased abundance of *Firmicutes*. Interestingly, analysis also revealed an increased abundance of the protective *Clostridia* classes upon loss of miR-21. Taken together, this work identifies a novel role for miR-21 in shaping the gut microbiota and the subsequent development of intestinal inflammation such as IBD.

2. Materials and Methods

2.1 Animals

MiR-21-deficient (miR-21$^{-/-}$) mice were developed by Taconic Artemis using a Cre/*lox* approach. Briefly, miR-21 was modified by the insertion of two loxP sites that enable excision of the floxed miR-21 segment through Cre-mediated recombination. Chimeric offspring were backcrossed onto the C57BL/6J background for a total of 8 generations. Homozygous deletion of miR-21 was confirmed by PCR genotyping. Homozygous miR-21$^{-/-}$ mice and WT littermates were used for animal studies. Animals were maintained in ventilated cages at 21 ± 1 °C, humidity 50 ± 10%

and with a 12h-light/12h-dark light cycle under specific pathogen-free conditions, in line with Irish and European Union regulations. Food and water were available *ad libitum* throughout all of the experiments. All experiments were subject to ethical approval by the Trinity College Dublin's Animal Research Ethics Committee (AREC) and were carried out in accordance with the Irish Health Products Regulatory Authority. Germfree animal experiments were carried out at the Weizmann Institute, Revolt, Israel, according to The Institutional Animal Care and Use Committee (IACUC).

2.2 DSS-induced experimental colitis

Colitis was induced in age- and sex-matched WT and miR-21$^{-/-}$ mice. Mice were weighed at the start of the trial. To induce acute experimental colitis, mice were administered 2.5% (w//v) dextran sodium sulphate (DSS, 36-50 kDa; MP Biomedicals, UK) in their drinking water *ad libitum* for 5 days (unless otherwise stated in the figure legend). To assess experimental colitis and repair, a recovery model was implemented, specifically, mice were administered 2.5% (w/v) DSS in their drinking water *ad libitum* for 5 days followed by 5 days normal water. Control mice were given normal drinking water throughout. In all colitis models, mice were checked daily for morbidity and body weight was recorded. Each mouse was scored daily for pathological features, including stool consistency (diarrhoea score), presence of fecal occult (blood score), and weight loss). Individual scores were combined to generate the Disease Activity Index (DAI) which was calculated daily for each mouse. The maximum DAI score was 12 based on assigning a 1–4 scoring system for each parameter: score 0, no weight loss, normal stool and no blood; score 1, 1–3% weight loss; score 2, 3–6% weight loss, loose stool (a loose stool was defined as the formation of a stool that readily becomes paste upon handling) and blood visible in stool; score 3, 6–9% weight loss; and score 4, >9% weight loss, diarrhoea and gross bleeding. At the end of the trial, mice were euthanized by CO_2 asphyxiation. The colons were removed and measured as an indication of colonic inflammation. Subsequently, colonic sections were taken and stored in 10% formalin for histological analysis or snap frozen in liquid nitrogen and stored at -80°C.

2.3 Co-housing experiments

Prior to induction of DSS experimental colitis, sex-matched WT and miR-21$^{-/-}$ mice aged 4-weeks were co-housed for a minimum of 4 weeks, to allow natural transfer of the fecal microbiota.

2.4 Colonization of germ-free mice with fecal microbiota

A homogenate of frozen feces from WT or miR-21$^{-/-}$ mice was prepared in sterile PBS under anaerobic conditions and subsequently filtered through a sterile 70μm strainer to remove soil particles. For colonization of germfree (GF) mice, each mouse was administered 100μl fecal suspension containing 20mg feces by oral gavage. Mice were then allowed to rest for 9 days to

ensure colonization. The same dose was used to mimic fecal microbial transplant in conventional mice.

2.5 Colonoscopy

Colonoscopy was performed on experimental mice using a high-resolution mouse video endoscopic system (Carl Storz, Tuttlingen, Germany). The severity of colitis was scored in a blinded manner using MEICS (Murine Endoscopic Index of Colitis Severity), which is based on five parameters: granularity of mucosal surface; vascular pattern; translucency of the colon mucosa; visible fibrin; and stool consistency.

2.6 Antibiotic treatment of mice

Mice were put on a 4-way antibiotic treatment course *ad libitum* for two weeks prior to DSS treatment. The course of treatment consisted of vancomycin (0.5 g/l), ampicillin (1 g/l), kanamycin (1 g/l), and metronidazole (1 g/l) in their drinking water.

2.7 Histology

Sections from the distal colon of each mouse were fixed in 10% buffered formalin and analysed using haematoxylin and eosin (H&E) staining. H&E stained sections of distal colon were blind scored to ascertain the extent of colitis using a previously established protocol[14]. A combined score of inflammatory cell infiltration and tissue damage was determined as follows: cell infiltration: score 0, occasional inflammatory cells in the lamina propria (LP); 1, increased infiltrate in the LP predominantly at the base of crypts; 2, confluence of inflammatory infiltrate extending into the mucosa; 3, transmural extension of infiltrate. Tissue damage: score 0, no mucosal damage; 1, partial (up to 50%) loss of crypts in large areas; 2, partial to total 50–100% loss of crypts in large areas, epithelium intact; 3, total loss of crypts in large areas and epithelium lost.

2.9 qRT-PCR

Total RNA was isolated from colonic sections using the RNeasy PlusMini kit (QIAGEN) and quantified using a Nanodrop 2000 UV-visible spectrophotometer. cDNA was prepared using 20–100 ng/ml total RNA by a RT-PCR using a high capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer's instructions. Real-time qPCR for miR-21 and IL-1β expression was performed on cDNA using Taqman probes. qPCR was performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using Kapa fast master mix high ROX (Kapa Biosystems). Fold changes in expression were calculated by the Delta Delta Ct method using mouse Rps18 or RNU6B as an endogenous control for mRNA expression.

2.10 16S sequencing and analysis

To determine the gut microbiota composition of WT and miR-21$^{-/-}$ mice, 16S rRNA sequence analysis was performed on fecal samples from each cohort. Fecal samples were snap frozen in liquid nitrogen and DNA extraction performed using MOBIO PowerLyser DNA extraction kit according to the manufacturers' instructions. The 16S rRNA gene (V3-V4 region) was PCR amplified following the Illumina 16S Sample Preparation Guide and resulting amplicons were sequenced on the Illumina MiSeq platform using v3 sequencing chemistry with 2x250pb paired-end reads. Sequences were further filtered on the basis of quality (removal of low quality nucleotides at the 3' end, and remove windows 20 nt with a low average quality) and length (removal of sequences with less than 200nt) with prinseq-lite, and joined using fastq-join (https://code.google.com/archive/p/ea-utils/)[15]. The sequences were clustered with 97% identity level (calculated at the operational taxonomic unit; OTUs) using closed-reference *usearch* v7.0 algorithm[16], using to assignment the cluster sequences the RDP's Classifier[17]. Alpha and Beta-diversity was determined using QIIME [18]. Data were statistically analyzed by Adonis for beta-diversity analysis. Statistical differences between multiple samples were estimated by Kruskal-Wallis and False discovery rate (FDR, qvalue) control based on the Benjamini-Hochberg procedure was used to correct for multiple testing with the R statistical package (https://www.r-project.org/).

2.11 Fecal sIgA detection

Fecal pellets were taken, weighed and homogenized in protease inhibition buffer. Homogenate was centrifuged at 16000g for 5 mins at 4 °C before IgA was measured using an IgA ELISA kit (BD Pharmingen) in accordance with the manufacturer's instructions.

2.12 MPO activity assay

Colon sections were snap frozen in liquid nitrogen before homogenization and MPO activity was assessed using a slightly modified version of a previously described assay[19]. The modifications are as follows: 3,3',5,5'-Tetramethylbenzidine (TMB) was used a substrate for the assay, and MPO activity was expressed in arbitrary units (AU).

2.13 Statistical Analysis

Numerical results are given as arithmetic means ± standard error of the means. Statistical differences were analysed by GraphPad Prism 5.0 statistical software [GraphPad Software Inc., San Diego, USA] to perform Student's *t*-tests or Chi squared test for Kaplan-Meier survival curve. *P*-values of less than 0.05 [$p \leq 0.05$] are considered statistically significant.

3. Results

3.1 miR-21 deletion is protective in DSS-induced colitis

We initially compared WT and miR-21$^{-/-}$ mice in an acute model of DSS induced colitis. It was evident that miR-21-deficient mice were significantly protected from DSS-induced colitis, as evidenced by significantly reduced weight loss [Fig. 1A], strikingly reduced levels of occult blood in the stool [Fig. 1B] and a reduction in diarrhoea score [Fig. 1C]. Indeed, miR-21$^{-/-}$ mice had a significantly reduced DAI score [Fig. 1D], indicative of reduced susceptibility to disease. The colons of miR-21$^{-/-}$ mice were also significantly longer [Fig. 1E]. MiR-21 expression was elevated in DSS treated WT mice when compared to control animals [Fig. 1F]. In a DSS-recovery model, in which mice received normal drinking water following 5 days treatment with 2.5% DSS, miR-21$^{-/-}$ mice again demonstrated protection from the DSS-induced colitis, recovering faster and showing reduced disease symptoms for each parameter [Fig. 2 A-D]. Whilst there was no significant difference in colon length between WT and miR-21$^{-/-}$ mice treated with DSS, there was a significant difference between WT control mice and WT mice treated with DSS which was absent between the miR-21$^{-/-}$ groups [Fig. 2E]. Histological analysis was performed to assess the inflammation in the colon of WT and miR-21$^{-/-}$ mice post-DSS treatment. Control mice displayed no inflammation whereas WT mice treated with DSS had significantly more inflamed colons than miR-21$^{-/-}$ mice as assessed by a cumulative histology score [Fig. 2F] generated from crypt damage score and inflammation scores [Fig. 2G and 2H]. Representative images show this difference in inflammation [Fig. 2I].

3.2 Co-housing confers protection to WT mice against DSS-induced colitis

To determine any influence of miR-21 expression on the composition and activities of the gut microbiota and subsequent development of DSS-induced colitis, WT and miR-21$^{-/-}$ mice were co-housed for four weeks prior to DSS treatment. Control mice co-housed with mice of the same genotype displayed a similar disease phenotype to that observed in previous experiments [Fig. 3A, B]. However, there was a slight reduction in disease severity in WT mice that had been co-housed with miR-21$^{-/-}$ (CH-WT) as observed by a reduced DAI score compared to WT housed with other WT mice [Fig. 3A, C]. Although there was no reduction in weight loss [Fig 3D], CH-WT had reduced diarrhoea and blood scores compared to WT controls [Fig. 3E, F]. Furthermore, the colon lengths of the CH-WT mice were longer than those of the WT controls [Fig. 3G]. Although subtle, these results suggested that natural transfer of the fecal microbiota from miR-21$^{-/-}$ mice offers some protection against the severity of DSS-induced colitis. As such, the miR-21$^{-/-}$ fecal microbiota appears to display a less colitogenic phenotype compared to WT fecal microbiota, and can protect against development of colitis in recipient WT mice. We next attempted to determine whether the miR-21$^{-/-}$ fecal microbiota could also influence the course of established colitis (Supplementary S1). To do this, WT mice were given DSS for 5 days prior to inoculation with either PBS (control) or a homogenate of miR-21$^{-/-}$ feces (prepared in the same way as for the GF colonization model). On the day of inoculation, DSS was removed and mice received normal drinking water *ad libitum* for a further 5 days. Mice were scored daily throughout the model, as before, for disease parameters and DAI generated. No difference in the rates of recovery between control and miR-21$^{-/-}$ fecal homogenate treated mice was observed, other

than reduced shortening of colons in miR-21$^{-/-}$ fecal homogenate treated mice (Supplementary S1E).

3.3 Colonisation of GF mice with miR-21 fecal microbiota offers protection against DSS-induced colitis

Having observed protection being partially transferred to WT mice via co-housing, we wished to explore whether or not this effect could be seen using a germ-free setting. GF mice were colonized with fecal homogenates from WT or miR-21$^{-/-}$ animals for 9 days before prior to DSS treatment. Upon DSS treatment, mice colonized with WT fecal homogenate exhibited significantly greater weight loss, significantly higher occult blood scores and higher diarrhoea scores [Fig. 4A-C]. This culminated in significantly higher DAI scores [Fig. 4D]. Overall colitis severity was assessed by colonoscopy, where scores were combined from multiple disease parameters [Fig. 4E]. Mice colonized with WT homogenate had a higher disease score than their miR-21$^{-/-}$ colonized counterparts [Fig. 4E] and this can be seen in representative images [Fig. 4F]. Interestingly, miR-21 expression is induced by DSS in the colons of GF mice [Fig. 4G].

3.4 Protection of miR-21$^{-/-}$ mice against DSS-colitis is lost after antibiotic treatment

In order to ascertain the extent to which the intestinal microbiota was influencing the disease severities between the different groups, WT and miR-21$^{-/-}$ mice were put on a course of oral antibiotics prior to DSS treatment. WT and miR-21$^{-/-}$ control mice had different weight loss levels, blood and diarrhoea scores as seen before, with miR-21$^{-/-}$ mice again showing reduced susceptibility to DSS-induced colitis [Fig. 5A-D]. However, interestingly, these differences between WT and miR-21$^{-/-}$ mice were lost following antibiotic pre-treatment, as can be seen in the DAI score (Fig. 5A-D). The difference in colon length between the WT and miR-21$^{-/-}$ mice was also lost following antibiotic pre-treatment [Fig. 5E]. Strikingly, miR-21$^{-/-}$ mice displayed a significantly greater loss of mobility than their WT counterparts upon pre-treatment with antibiotics, and this led to a significantly higher morbidity in this group [Fig. 5F and G].

3.5 Altered composition of the miR-21$^{-/-}$ microbiota

16S rRNA sequencing of fecal samples was employed to investigate the possibility of differences in the composition of the microbiota between WT and miR-21$^{-/-}$ mice. After quality filtering and length trimming, an average of 37313 (±7123 SD) 16S rRNA high-quality sequences were generated per sample. The average number of OTUs per sample was 355 (±16 SD). The top 25 most abundant OTUs were compared between the two groups with each OTU being given its phylum name followed by its genus name (e.g. *Actinobacterium*; *Bifidobacterium*) [Fig. 6A]. There were considerable differences between the two groups, with the miR-21$^{-/-}$ group displaying a higher proportion of *Actinobacterium*; *Bifidobacterium*, *Firmicutes*; *Coprococcus*,

Firmicutes; *Lactonifactor*, *Firmicutes*; *Oscilibacter*, *Firmicutes*; *Clostridium sensu stricto*, *Firmicutes*; *anaerosporobacter* and *Firmicutes*; *Catonella*, and a lower proportion of *Firmicutes*; *Clostridium XIVa*, *Firmicutes*; *Tannerella*, *Firmicutes*; *Dorea*, *Bacteroidetes*; *Barnesiella* and *Bacteroidetes*; *Prevotella*, relative to WT mice [Fig. 6A]. Beta diversity is represented by a Principal Coordinates Analysis (PCoA), performed using all 16S rRNA reads clustered at 97% similarity [Fig. 6B]. Clear phylogenetic separation between the WT and miR-21KO groups was evident (Anosim P value = 0.001). There were also several differences apparent at various taxonomic levels: at the phylum level, *Proteobacteria* (q=0.00079935) were present in higher proportions in the miR-21$^{-/-}$ microbiota. At the family level, the miR-21$^{-/-}$ samples contained higher proportions of *Bifidobacteriaceae* (q=0.01181564) and *Peptostreptococcaceae* (q=0.14586726), and reduced proportions of *Verrucomicrobiaceae* (q=0.07853655) and *Bacteroidadeae* (q=0.05746273). At the genus level, the WT samples group contains higher proportions of *Enterorhabdus* (q=0.00036569), and reduced proportions of *Odoribacter* (q=7.896e-05) and *Bifidobacterium* (q=0.00143734) [Fig. 6C-E].

4. Discussion

Over the past decade there has been an increasing appreciation of the crucial roles played by miRNAs in a wide variety of cellular processes. Among the biggest discoveries in that time was the role for miRNAs in inflammatory processes where they regulate both pro- and anti-inflammatory pathways[20]. MiR-21 is an extensively studied molecule with a known association with human IBD[6,8]. With this in mind, we generated a knock-out mouse to study the disease. Our initial finding that miR-21 was pathological in the DSS mouse model of colitis was surprising, as miR-21 is widely regarded as an anti-inflammatory mediator. These findings were supported by Shi *et al* who described the same phenotype and mechanistically suggesting that miR-21 influenced the disease by allowing the intestinal epithelial barrier to become more permeable via enhancing epithelial apoptosis and targeting the tight junction protein RhoB[6,8]. These results indicate to us that miR-21 overexpression in IBD is unlikely to be simply a bystander effect caused by an inflammatory milieu as has been suggested[21], but an active component of disease.

Another field of study that has exploded in recent years is the growing understanding that the gut microbiota greatly influences human health and disease. Microbial dysbiosis has been found to play a significant contribution in a number of DSS-induced colitis phenotypes exhibited by transgenic mice with alterations in immune function. One such study demonstrated that the inflammasome component NLRP6 was required for intact host-microbe interaction, and that the exacerbated colitis phenotype exhibited by NLRP6$^{-/-}$ mice in response to treatment with DSS could be transferred to wild-type mice by co-housing [22,23]. As miR-21 is a known regulator of the immunological activity of the bacterial sensor TLR4, we sought to ascertain the significance of the gut microbiota in our mice and we reasoned that this might be a crucial aspect of the disease phenotype. Firstly, we observed that following co-housing of mice, WT mice cohoused with miR-21$^{-/-}$ mice displayed a reduced colitis phenotype when compared to the WT control group, which was evident in fecal occult blood and in the diarrhea score. This indicated that mice lacking miR-21 have an altered microbiota compared to their WT counterparts, which offers protection against development of colitis and furthermore, that this protection is transferable. There is a sparse but growing literature regarding the potential of miRNAs to modulate the gut microbiota, but given the variety of roles in regulating bacterial

sensing it is likely that miRNA may play an indirect role. In one interesting study conducted by Lui *et al*, the authors demonstrated that host miRNA, including miR-21, are present in mouse feces and that they can be taken up by commensal bacteria to alter their gene expression. In addition, this study showed a significant microbial dysbiosis was present in *Dicer* knockout mice which lack all functional miRNAs, and that this dysbiosis could be rescued by re-introducing synthetic miRNA to the mice[11].

To further explore the protective microbiota observed in the miR-21$^{-/-}$ mice, we colonised GF mice with the fecal microbiota of WT or miR-21$^{-/-}$ mice prior to DSS challenge. In this experiment, mice colonised with a miR-21$^{-/-}$ fecal microbiota exhibited protection from DSS-induced colitis relative to the mice colonized with the WT microbiota. A colonoscopy confirmed that the miR-21$^{-/-}$ colonized mice displayed fewer signs of inflammation. Colonoscopy is the current gold standard in IBD diagnosis, and it is becoming increasingly utilised in studies modelling the disease[24]. It had been previously been reported that miR-21 expression was downregulated in the caecum of GF mice relative to conventional controls, implying that the caecal microbiota impacts on its expression[12]. In order to establish if miR-21 is induced in the DSS colitis model due to increased penetration of the epithelium by commensal bacteria and subsequently enhanced immune activation, miR-21 expression levels were compared in GF mice given DSS or water. It was observed that miR-21 expression remains enhanced in these mice upon DSS treatment. This was interesting and points to a mechanism of induction more related to the epithelial damage caused by DSS administration[25].

As a final confirmation that the microbiota of miR-21$^{-/-}$ mice confers protection against DSS-induced colitis, we determined the consequence of depleting this microbiota by administering antibiotics to mice prior to challenge with DSS. Both WT-depleted and miR-21$^{-/-}$-depleted mice displayed increased detectable fecal occult blood and higher diarrhea scores earlier in the experiment. However interestingly, the protective phenotype previously observed in the miR-21$^{-/-}$ mouse was lost following antibiotic treatment, as demonstrated in each of disease scores and overall DAI score. Antibiotic depletion of the microbiota has been shown to increase intestinal haemorrhaging and mortality in mice subsequently treated with DSS [26,27]. These effects have been demonstrated to be due to a weakened intestinal barrier and the absence of tonic microbial signals to the epithelium respectively. Our results demonstrate an increase in haemorrhaging (as measured by fecal occult blood) in keeping with these studies. Most striking of all the observations in this experiment was the highly significant increase in sickness behaviour (measured by loss of mobility) and associated mortality displayed in the miR-21$^{-/-}$ mice treated with antibiotics. This effect has previously been described in WT mice at slightly later time points, but interestingly we saw mortality as early as day 5 in the miR-21 $^{-/-}$ mice, indicating that in the absence of microbial signalling miR-21 appears to play a protective role in this disease model. This finding confirms that the microbiota of miR-21$^{-/-}$ mice is protective in DSS-induced colitis, and that the protection demonstrated by the miR-21$^{-/-}$ mice is at least in part, dependant on the presence of the microbiota.

Having shown that the microbiota of the miR-21$^{-/-}$ mouse determines the outcome of DSS-induced colitis, 16S rRNA sequencing was employed to compare the fecal microbial profiles of miR-21$^{-/-}$ and WT mice. The top 25 most abundant OTUs in WT and miR-21$^{-/-}$ mice were compared and there was several significant differences noted. Several of the observed differences pointed towards a potentially protective microbial signature in the miR-21$^{-/-}$ mouse, including a generally higher abundance of genera within the *Firmicute* phylum which correlates

with protection against IBD while there was a lesser abundance of *Bacteroidetes* and *Prevotella* which correlates with development of IBD[28,29]. The higher abundance in the gram positive probiotic genus A*ctinobacterium* and *Bifidobacterium* can also be interpreted as evidence of a protective microbiota as several studies have indicated a reduction in the severity of DSS-induced disease in mice treated with members of these genera[30-32]. In order to assess whether or not these populations shared similar species, beta diversity was assessed and found to be different between WT and miR-21$^{-/-}$ fecal bacterial populations. This supported the case for an altered, protective microbiota being present in the miR-21$^{-/-}$ intestinal compartment. We next performed a taxa analysis to compare the relative proportions of different bacterial groupings at various taxonomic levels. Surprisingly, at the phylum level there was an increased presence of *Proteobacteira* in the miR-21$^{-/-}$ mice which would run counter to the idea of a protective microbiota. This phylum of gram-negative bacteria generally increase in proportion in IBD with a commensurate decrease in *Firmicutes*[33,34]. Further down the taxa, at family level the fecal microbiota of the mice lacking miR-21 display an increased proportion of *Bifidobacteriaceae* and *Peptostreptococcaceae* relative to the WT microbiota, and at genus level an increase in *Bifidobacterium* and *Odoribacter,* all of which correlate with a healthy microbiota in various studies[35,36]. We examined the fecal microbiota as this is routinely performed in the field, and furthermore, as the fecal microbiota was implicated in the co-housing and GF colonization studies that we carried out. However, it should be noted that the fecal microbiota might be different to that at individual mucosal surfaces throughout the GI tract. This has been shown to be important in studies using microbial signatures as a biomarker for intestinal disease, and so further investigation may be required to elucidate if there are important differences in this model[37]. Overall, these data confirm that there is an altered microbiota in the miR-21$^{-/-}$ mouse, with some of these alterations correlating with studies that indicate protective associations with IBD and mouse models of colitis.

An interesting aspect of this work to consider is the potential alteration of other mediators present in the feces of miR-21$^{-/-}$ mice, which might impact the disease phenotype. These could include cytokines, antibodies, secreted epithelial factors or indeed other miRNA which have been demonstrated to be present in mouse feces and to have a transferable impact on disease outcomes[11]. Secretory IgA has been shown to be an important mediator of microbial composition, a factor in microbial transferability, and a marker for susceptibility in IBD models[38,39]. We measured sIgA levels in the feces of WT and miR-21$^{-/-}$ mice, and whilst there appeared to be reduced levels of sIgA in the feces of the miR-21$^{-/-}$ mice this was not statistically significant (Supplementary S2). We were unable to detect differences in fecal cytokines (data not shown). We also measured the inflammatory markers MPO activity and IL-1β expression in all mice following DSS-induced colitis, but found little consistent evidence that these mechanisms may be influencing our observed phenotype (Supplementary S3). This is somewhat in keeping with the counterintuitive nature of miR-21's established role as an anti-inflammatory molecule in immune signalling[21].

The mechanisms by which miR-21 may be influencing microbial composition have yet to be determined, but given that miR-21 has been demonstrated to be an important regulator of immune and epithelial barrier function it is likely that modulation of these important mediators of gut homeostasis is involved. For instance, the enhanced barrier integrity displayed in miR-21$^{-/-}$ mice demonstrated in previous studies via the increase in the expression of the miR-21 target RhoB may play a role in shaping the microbiota by creating a more favorable niche for colitis

protective bacteria[6,8]. MiRNA have also been reported to alter the expression of specific mucins in the gut, which may also have a bearing on the niche occupied by these bacteria[40,41]. It is also possible that miR-21 impacts the commensal bacteria more directly, by targeting secreted bactericidal mediators: for example, miR-21 has been shown to target *CAMP* and *DEFB4A* in humans[42]. T-cells play an important role in immune homeostasis in the gut, and have a crucial role in shaping the gut microbiota[43]. MiR-21 has been demonstrated to impact the differentiation and activation of different T-cell subsets via modulation of key genes such as IL-12p35 and Stat3. For instance, in different inflammatory contexts miR-21 has been shown to promote Th17 cells, negatively regulate Treg cells and promote memory T-cell activation[44-46]. In murine models of colitis, miR-21 deficiency has been shown to skew T-cells towards Th1 responses[47]. It would be interesting to explore the potential contribution of T-cell responses to shaping the microbiota in this system. These are a few of the possible mechanisms by which miR-21 may regulate the shape of the intestinal microbiota. It may be that one or all of them are involved in this system, and certainly it is likely that the altered barrier permeability demonstrated to be present in the miR-21$^{-/-}$ mouse by other groups is a factor. It is also possible that miR-21 may directly influence bacterial gene expression following secretion into the lumen in exosomes as has recently been demonstrated[11]. Clearly further studies are required to elucidate the pathways by which miR-21 may be exerting its influence on the microbiota.

Another interesting aspect of the findings presented here is the implications of microbial alteration in the divergent phenotype displayed by miR-21$^{-/-}$ mice when subjected to the T-cell-dependent TNBS colitis and T-cell transfer colitis models. It has been previously reported that miR-21 deletion exacerbates colitis in these models through aberrant skewing of T-cell responses. As stated previously, T-cells help shape the microbiota and the microbiota helps shape T-cell responses in inflammatory disease[43]. Accordingly, it would be interesting to explore the microbial contribution to disease onset and progression in this model, and whether or not microbial manipulation (e.g. antibiotic depletion) might affect these phenotypes[47].

There is a huge literature on miR-21's role in many forms of cancer. MiR-21 is upregulated in nearly all solid tumors and is a *bone fide* oncomiR, influencing many cancer related processes such as cell division, apoptosis and metastasis[21,48]. In the context of IBD, miR-21 is upregulated in colitis associated cancer (CAC) and has also been shown to have a negative role in the azoxymethane (AOM)/DSS model of CAC with miR-21$^{-/-}$ mice showing reduced susceptibility to the disease[7]. Our results are interesting in this regard, as disruption to the microbiota has been reported to be involved in carcinogenesis through various mechanisms including immune-microbiota crosstalk[49]. This is particularly relevant in the case of CAC as the breakdown of microbial tolerance and homeostasis in the gut leads to inflammation and subsequent tumorigenesis. It is therefore possible that miR-21's impact on the intestinal microbiome may contribute to disease pathogenesis alongside its direct role in tumor formation and persistence. In our model, we have seen that miR-21 deletion gives rise to a microbiota which correlates with healthy individuals and protects from disease initiation, but does not remedy established disease (Supplemental S1). Fecal microbial transplantation (FMT) therapy for IBD is in its infancy, with several studies reporting mixed efficacy for UC patients[50]. A recent large study has shown efficacy using a multi-donor approach to maximise bacterial diversity, without identifying any particularly beneficial microbes[51]. Future approaches for FMT and other microbial therapeutics may rely on a more detailed knowledge of which microbes are responsible for beneficial effects. Our findings are interesting in this context, as the mix of

microbes and other factors present in the miR-21$^{-/-}$ mice have prophylactic value but appear less therapeutically beneficial. A deeper exploration of the composition of the transferable microbiota could provide valuable insights to explain this difference and inform future work in this area. As mentioned earlier, it would be very interesting to explore the microbial contribution to miR-21$^{-/-}$ disease phenotypes in other models as this would further inform us of the potential efficacy of fecal transplant using a similar fecal bacterial signature.

MiRNA are currently being targeted for therapeutic use in inflammatory disease. A recent study by Wang *et al* has demonstrated that inhibition of the pro-inflammatory miRNA miR-223 can lessen disease severity in the DSS model of colitis[52]. It would be interesting to see whether miR-21 inhibition would have a similar outcome in the DSS model. MiR-21 inhibition is currently being developed for therapeutic use in a rare kidney disease, and has potential for use in IBD, though further work is required to tease out miR-21's role in the various aspects of the disease (immune, microbiota etc.) as highlighted by the divergence in its role in different models of disease[47,53]. Our study highlights the need to consider the impact on the microbiota of any study carried out to this effect, as this may have an important bearing on the function of any such therapeutic intervention.

In summary, our findings demonstrate that miR-21 expression is deleterious in the DSS model of IBD, and that the intestinal microbiota is crucial to the course of this disease progression. Indeed, our findings identify a novel role for miR-21 in regulating the composition of the intestinal microbiota and the resulting development of intestinal inflammation.

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Conflict of Interest

The authors have no competing interests to declare.

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Author Contributions

SCC conceived ideas and oversaw the research programme. DGJ carried out the research. SCC and DGJ analysed data and wrote the manuscript. MAW and CM performed experiments. MR analysed histology. CT and EE assisted with germfree experiments. RCR and PC assisted with

analysis of 16S data. LAON funded creation of the miR-$^{21-/-}$ strain and provided invaluable guidance during the course of the project.

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Figure Legends

Figure 1: Loss of miR-21 reduces susceptibility to DSS-induced colitis.
WT and miR-21$^{-/-}$ mice aged between 8 and 12 weeks were weighed on day 0 and given 2.5% DSS (w/v) *ad libitum* for 5 days. Weight (A), blood score (B) and diarrhea score (C) were determined daily throughout the experiment and combined to generate a disease activity index score (DAI) [D]. On day 5, the mice were sacrificed and their colons measured (E). miR-21 expression in WT mice post-DSS treatment was compared to water controls (F). (A-E) n=5 mice per group, mean values ± SEM are presented, P values were calculated using Unpaired T-test * p<0.05, ** p< 0.01, *** p<0.001; (F) miR-21 expression was assessed relative to RNU6B in mice treated with water, n=2, or 2.5% DSS for 5 days, n=4. Mean values ± SD are presented, P values were calculated using Unpaired T-test * p<0.05, ** p< 0.01.

Figure 2: miR-21-deficient mice are protected compared to WT mice in a DSS-induced colitis recovery model.
WT and miR-21$^{-/-}$ mice aged between 8 and 12 weeks were weighed on day 0 and given 2.5% DSS (w/v) a*d libitum* for 5 days followed by normal drinking water for a further 5 days. Control mice were given normal drinking water throughout. Weight (A), blood score (B) and diarrhea score (C) were determined daily throughout the experiment and combined to generate a disease activity index (DAI) score (D). On day 10, the mice were sacrificed and their colons measured (E). Colitis severity of H&E stained colonic tissues was assessed by combined histological score (F) of tissue disruption (crypt damage score; 0–3, according to the severity of mucosal and crypts damages) (G) and colon cellular infiltration (inflammation score; 0–3, according to the extent of inflammation throughout the intestinal wall) (H). Representative microscopic pictures of H&E stained colon sections (100x magnification) from water and 2.5% DSS treated WT and miR-21$^{-/-}$ mice are shown (I). Data represent mean values ± SEM of n=3 mice per group (n=2 in the miR-21$^{-/-}$ water control group). P values were calculated using Student's T-test, * p<0.05, ** p< 0.01.

Figure 3: WT mice cohoused with miR-21-deficient mice are protected from DSS-induced colitis.

WT and miR-21$^{-/-}$ mice aged between 4-6 weeks were cohoused for 4 weeks with either mice of the opposite genotype (e.g. WT mouse co-housed with miR-21$^{-/-}$ = CH-WT) or other mice of the same genotype (e.g. WT mouse co-housed with another WT = WT) before being weighed on day 0 and given 3.5% DSS (w/v) *ad libitum* for 7 days. Weight, diarrhea score and blood score was determined daily and combined to generate a disease activity index (DAI) score (A). Individual plots of selected groups DAI, weight loss, diarrhea score and blood score are shown (B-F). On day 7, the mice were sacrificed and their colons measured (G). Data represent mean values ± SEM of n=4 mice per group (except miR-21$^{-/-}$ control group where n=3). P values were calculated using Student's T-test, * p<0.05, ** p<0.01.

Figure 4: Colonisation of germ-free mice with the fecal microbiota of miR-21-deficient mice protects against DSS-induced colitis.

Germ-free (GF) Swiss-Webster mice were colonized with fecal preparations from WT or miR-21$^{-/-}$ mice by oral gavage. 9 days later, mice were given 3% DSS (w/v) *ad libitum* for 13 days. Weight (A), blood score (B) and diarrhea score (C) were determined daily and combined to generate a disease activity index (DAI) score (D). On day 13, the mice were anesthetized and underwent a colonoscopy using the ColoView system. Colons were blind scored for a number of clinical parameters that were combined to give a colitis severity score (E). Representative images are shown (F). Data are representative of two independent experiments, presented as mean values ± SEM of n=5 mice per group. P values were calculated for A-E using Student's T-test * $p<0.05$, **$p<0.01$. MiR-21 expression relative to RNU6B was assessed by qPCR in colonic tissue from GF mice given 1.5% DSS *ad libitum* for 10 days, where data represent mean values ± SD of n=3 mice per group, P values calculated using Student's T-test, * $p<0.05$

(G).

Figure 5: Protection of miR-21-deficient mice against DSS-colitis is lost after antibiotic treatment.

WT and miR-21$^{-/-}$ mice aged between 8 and 12 weeks were given a 4-way antibiotics mix *ad libitum* for 2 weeks. The mice were then weighed on day 0 and given 3% DSS (w/v) a*d libitum* for 7 days. Weight (A), blood score (B) and diarrhea score (C) were determined daily and combined to generate a disease activity index (DAI) score (D). The mice were sacrificed on day 8 and their colons measured (E). In addition, the mice were scored for mobility loss (F) and morbidity (G). Data represent mean values ± SEM pooled from two experiments of n=≥5 mice per group. P values were calculated using Student's T-test and Chi squared test for Kaplan-Meier survival curve, * $p<0.05$, ** $p< 0.01$, *** $p=<0.001$. Black asterisks compare WT vs. miR-21$^{-/-}$, except in panel G where they compare WT (ABX) vs. miR-21$^{-/-}$ (ABX).

Figure 6: 16 S rRNA analysis of WT and miR-21-deficient mice fecal microbiota.

The 16S rRNA reads were compared to the RDP database, and WT and miR-21$^{-/-}$ microbiota samples were compared for OTU analysis (A), beta diversity (B) and taxa analysis at phylum (C), family (D) and genus (E) levels. Data is representative of n=≥9.

Figure 1.

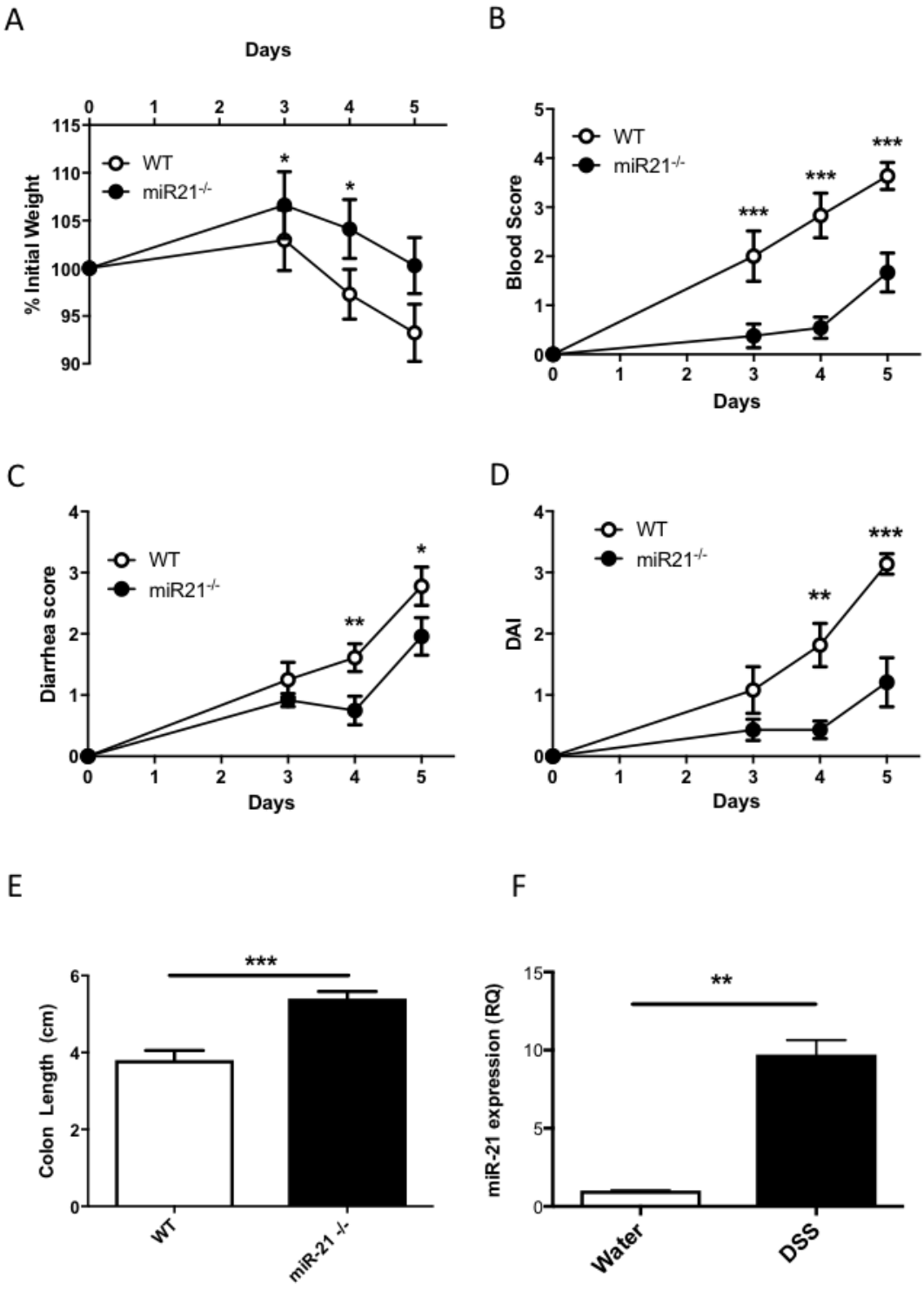
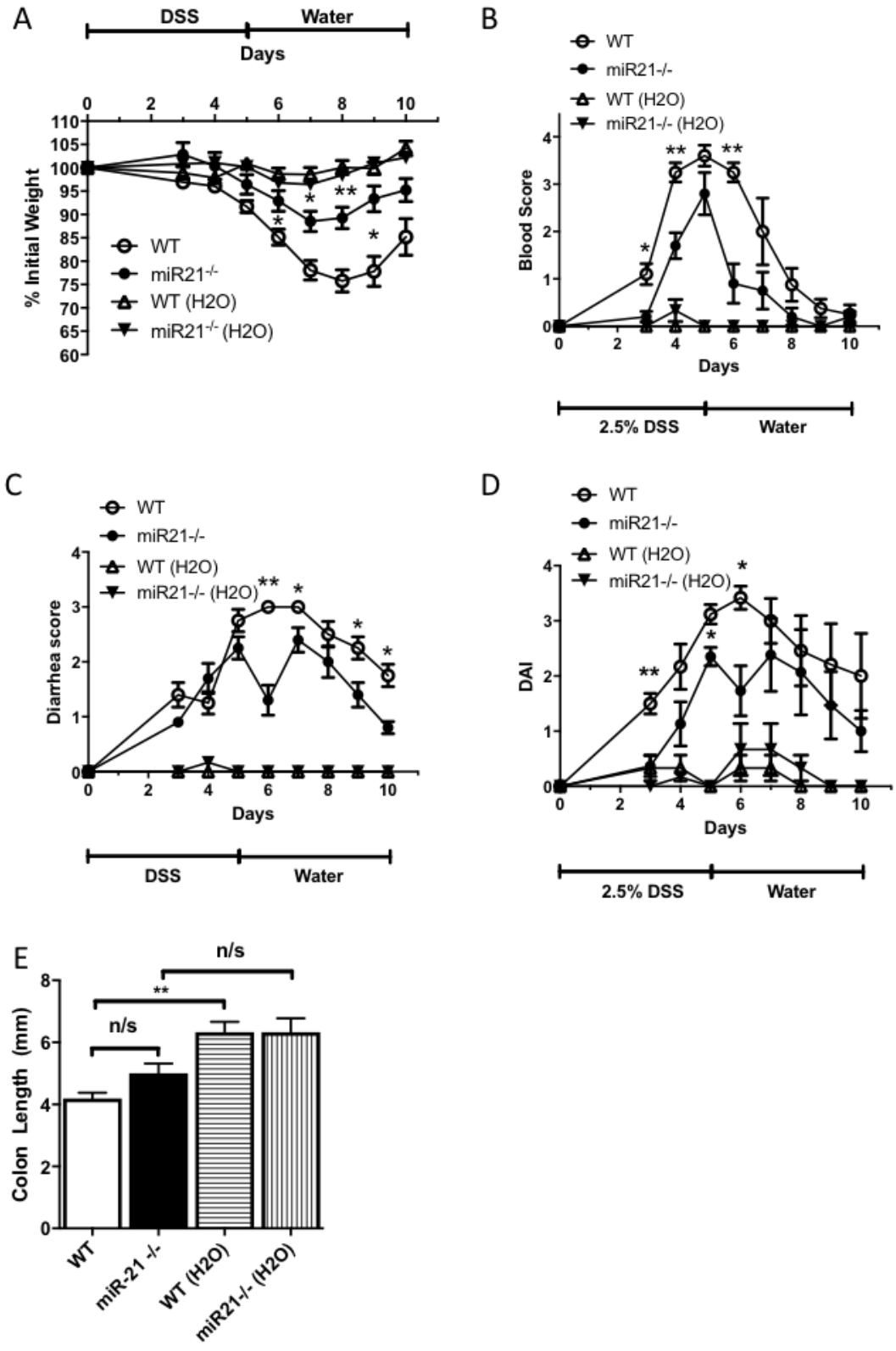


Figure 2.



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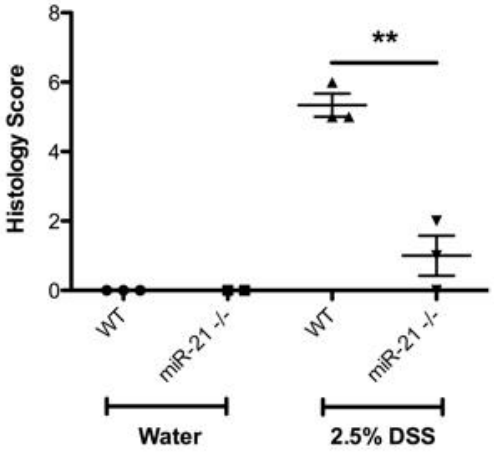

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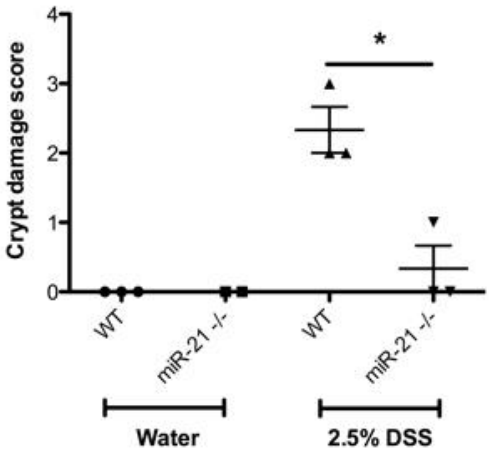

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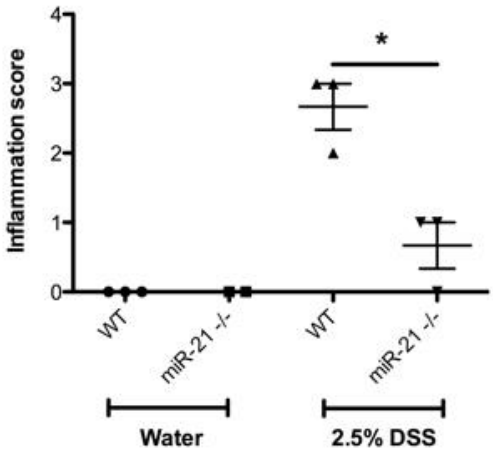

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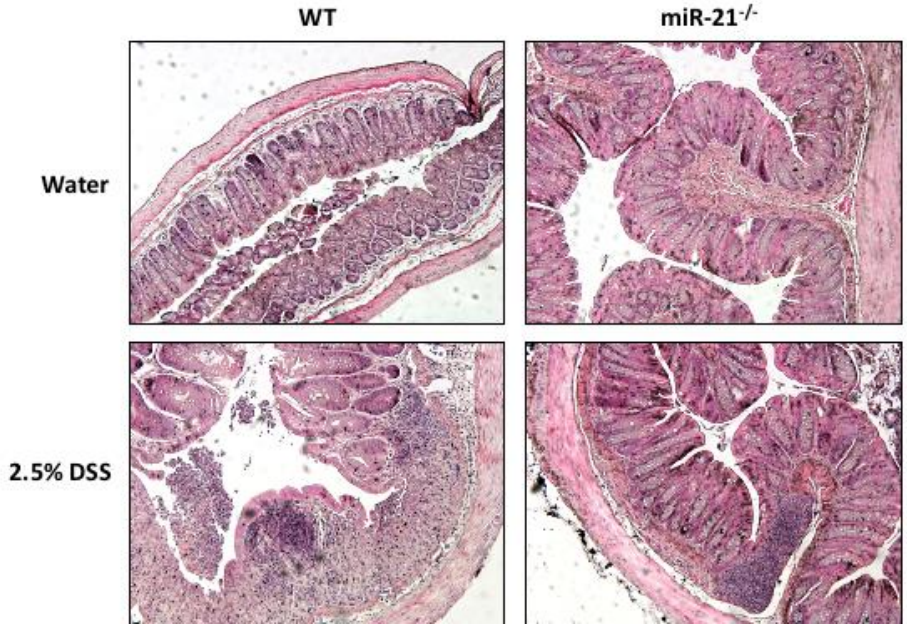

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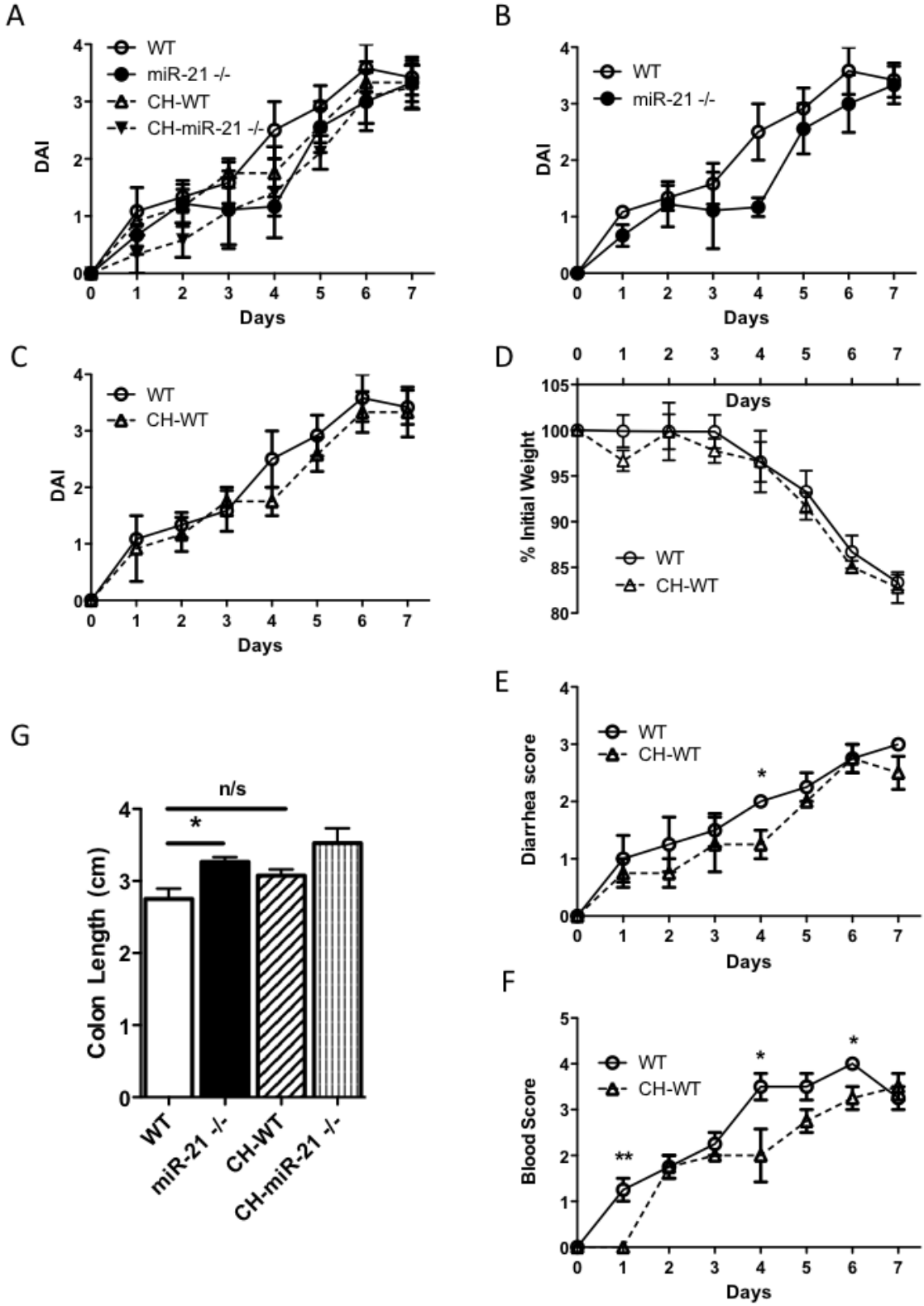

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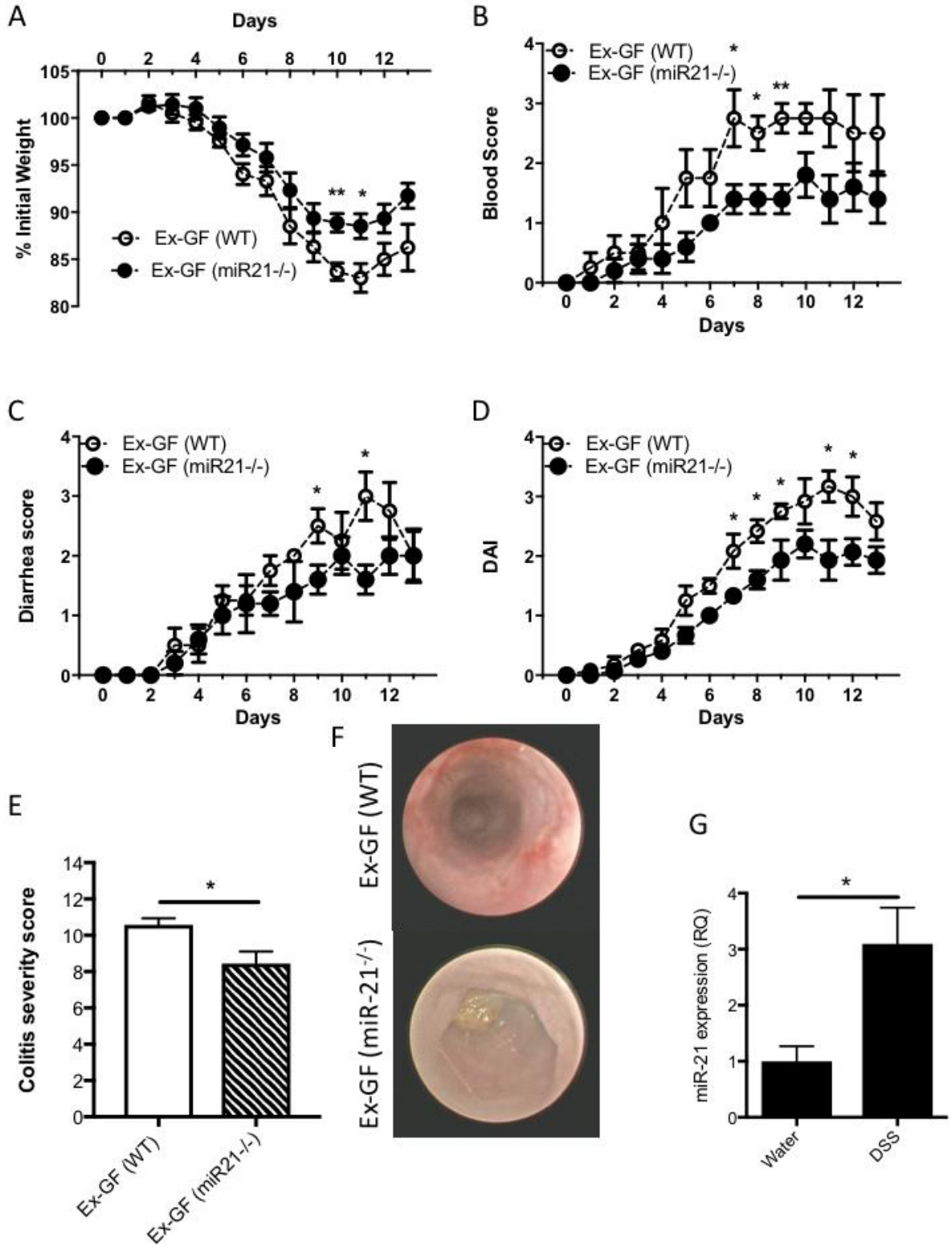

Figure 5.

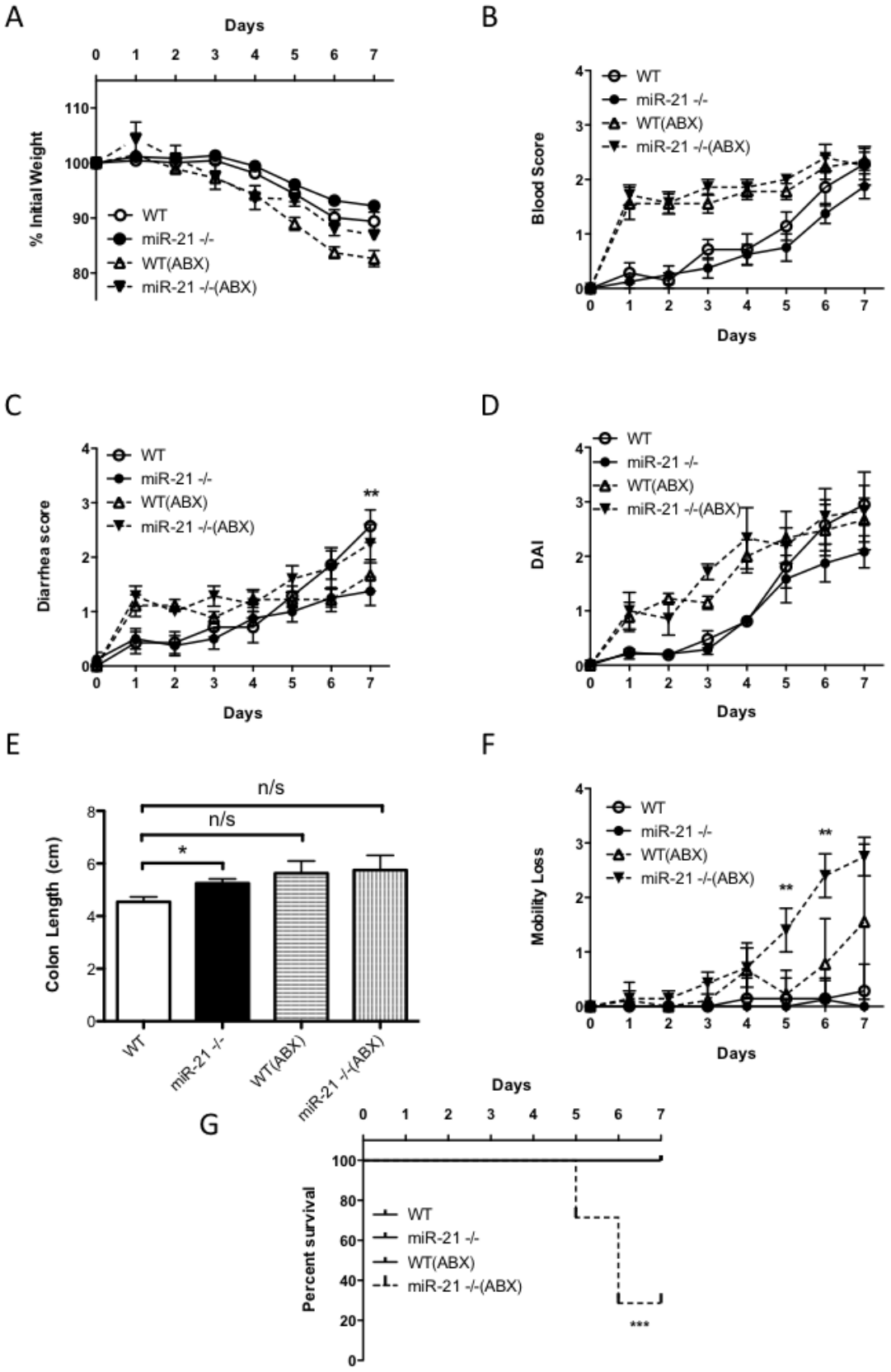

Figure 6.