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Immunomodulatory tetracyclines ameliorate DNBS-colitis: Impact on microRNA expression and microbiota composition

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Abstract

Objective

The use of immunomodulatory antibiotics to simultaneously target different factors involved in intestinal inflammatory conditions is an interesting but understudied pharmacological strategy. A great therapeutic potential has been obtained with minocycline and doxycycline in experimental colitis. Therefore, understanding the contribution of the different activities of immunomodulatory tetracyclines is crucial for the improvement and translation of their use into clinic.

Design

A comparative pharmacological study including tetracyclines and other antibiotic or immunomodulatory drugs was performed in 2,4-dinitrobenzene sulfonic acid (DNBS)-induced colitis in mice. The correlation between the therapeutic efficacy of each drug and changes in the gut microbiota composition, markers of barrier integrity, inflammatory mediators, microRNAs and TLRs was analysed to identify the main mechanisms of action.

Results

Tetracyclines counteracted most of the markers found altered in DNBS-colitis, which differed from effects of corticosteroid treatment. Of note, administration of tetracyclines led to increased mucosal protection, associated with up-regulated expression of CCL2, miR-142 and miR-375. All drugs with antibiotic activity ameliorated the progression of inflammation and reduced neutrophil-related genes, such as miR-223, despite their effects were not associated with restored intestinal dysbiosis. However, reduced bacterial richness was correlated with increased expression of TLR2 and TLR9 in antibiotic-treated groups and TLR6 was also up-regulated by

the immunomodulatory tetracyclines with higher efficacy (doxycycline, minocycline and tigecycline).

Conclusion

The anti-inflammatory effect of tetracyclines involves specific modifications in TLR and microRNA expression leading to an improved microbial-derived signalling and mucosal protection. These results support the potential of immunomodulatory tetracyclines to prevent inflammation-associated tissue damage in acute intestinal inflammation.

Abbreviations: IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; DNBS, 2, 4-dinitrobenzene sulfonic acid; NC, non-colitic; RFX, rifaximin; TTC, tetracycline; DXC, doxycycline; MNC, minocycline; TGC, tigecycline; DEX, dexamethasone; TLR, toll-like receptor; miR, micro-RNA

Keywords: Tetracyclines; Intestinal inflammation: immunomodulation; Microbiota; microRNA; TLR

1 Introduction

Patients suffering inflammatory bowel disease (IBD), either ulcerative colitis (UC) or Crohn's disease (CD), develop recurrent inflammatory flares in the intestinal tract in response to unknown antigens from gut microbiota. This triggers the production of reactive oxygen species, proteases and inflammatory mediators [1–3] that further alter intestinal protective barriers [4,5] and lead to tissue damage and fibrosis as the disease progresses [6]. Standard therapy for IBD includes aminosalicylates, glucocorticoids, immunosuppressant drugs and biologics. However, antibiotics are also frequently used in clinical practice in the treatment of IBD, particularly in fistulizing disease and post-operative management of CD [7]. Although the beneficial effect of antibiotics in IBD has been traditionally attributed to their antimicrobial properties and a reduction of the antigenic load would support the preventative effect of antibiotics [7,8], different studies have reported the ability of many antibiotics to modulate the immune response [9]. Therefore, a single compound that combines these properties would be useful to induce remission in intestinal inflammatory conditions, by simultaneously targeting the altered immune response and the microbial imbalance. Among antibiotics, different members of the tetracycline family, such minocycline (MNC) and doxycycline (DXC), have proven to benefit many inflammatory conditions [10] by exerting additional non-antibiotic effects [11,12]: immunomodulatory activity on different immune cell populations, direct inhibition of enzymes involved in the inflammatory process (such as matrix metalloproteinases and secretory phospholipase A2), anti-apoptotic, antiproliferative and antioxidant properties. Indeed, promising results have been obtained with MNC and DXC in experimental models of colitis [13–16], which encourage to further characterise this therapeutic opportunity. A preventative treatment with antibiotics will not be a feasible therapeutic strategy, considering the limitations in antibiotic usage, however, a short-term administration of immunomodulatory tetracyclines could significantly benefit the course of intestinal inflammation, a drug-reposition strategy devoid of important side effects, as it has been proved over 40 years of clinical use.

In addition, it is well reported that changes in microbiota-derived signals, recognised by PRRs such as TLRs, modulate the immune response and impact the intestinal inflammatory process [17–20]. MicroRNAs have emerged as important regulators of intestinal inflammation [21], fine-tuning the immune system and the mucosa barrier functions [22–24]. Of note, TLR-mediated signals modulate microRNA expression. Thus, variations in TLRs and miRNAs interconnect changes on the intestinal microbiota, the epithelial barrier and the immune response. Considering this, immunomodulatory drugs may modulate the impact of microbial signals on the inflammatory response by affecting TLRs and miRNA expression, microbiota-targeted strategies may result in an indirect immunomodulatory effect. Both TLRs and microRNAs have been found altered in IBD patients [17,20,21,25,26] and their evaluation could provide valuable information about the mechanisms of pharmacological strategies for intestinal inflammation.

Considering all the above, it is of utmost importance to identify whether the antibiotic activity provide additional benefit to the intestinal anti-inflammatory effect of tetracyclines. This would provide valuable information about the requirements for the pharmacological development of TTC-based compounds with improved activity in immune-related conditions such IBD. The aim of this study was to compare the effect of three tetracyclines (DXC, MNC and tigecycline (TGC)) with other antibiotic or immunomodulatory drugs in a 2,4-dinitrobenzene sulfonic acid (DNBS) model of experimental colitis in mice, which resembles many features of CD [27]. The impact of these treatments on intestinal microbiota was evaluated. In addition, their effects on several markers of the intestinal inflammatory process, including microRNA and TLR expression, were assessed.

2 Material and methods

2.1 Animals and drugs

All chemicals were obtained from Sigma-Aldrich Quimica S.L. (Madrid, Spain), unless otherwise stated. Drug doses used in mice were equivalent to the therapeutic doses used in humans. All animal studies were conducted according to the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the National Institute of Health. Male CD1 mice (30 g) were obtained from Janvier Labs (Saint-Berthevin Cedex, France). Mice were housed in a specific-pathogen–free animal facility at the University of Granada.

2.2 DNBS colitis induction and experimental design

DNBS colitis, a variant of the tri-nitrobenzene sulfonic acid (TNBS) method first described in rats [28], was induced as previously reported [29] with minor modifications. Briefly, a dose 4 mg of DNBS (in 100 µL of a EtOH:H_2O (1:1) solution) was injected with a 4 cm-polyethylene catheter in the distal of the colon of mice anaesthetised with 2% enflurane. Mice were then maintained for 15 min upside-down inside the anaesthetic chamber to avoid the loss of the DNBS dose. A non-colitic (NC) group followed the same procedure but mice were administered PBS instead of the DNBS solution. Six hours after colitis induction, to avoid a possible preventive effect, mice started their respective treatments by oral gavage, while non-treated mice received the vehicle only (200 µL of sterile water). Colitic mice were randomised into 7 groups: a DNBS control and 6 treated groups, which received: (1) rifaximin (RFX) (200 mg/kg/day), a non-absorbable antibiotic (2) tetracycline (TTC) (250 mg/kg/day), (3) DXC (25 mg/kg/day), (4) MNC (50 mg/kg/day), (5) TGC (25 mg/kg/day) and (6) dexamethasone (DEX) (2.4 mg/kg/day), as a reference immunosuppressant drug. The doses were equivalent to the therapeutic doses used in humans and according to previous studies [14,16]. These different treatments were given daily until the end of the study. Disease evolution was monitored by a daily measurement of body weight. After 6 days of treatment, mice were killed by cervical dislocation, the colon was then resected and stools were collected aseptically. Adherent tissue was removed and the colon was rinsed with ice-cold saline. Afterwards, the colonic segment was weighed and its length measured under a constant load (2 g). Representative whole gut specimens were taken from a region of the inflamed colon corresponding to the adjacent segment to the gross macroscopic damage (~1 cm from the distal end). Specimens were fixed in 4% buffered formaldehyde for the histological studies. Then, the remaining colonic tissue was minced, frozen in liquid nitrogen and stored for subsequent evaluations.

2.3 Histology

Sections (4 µm) of paraffin embedded histological samples were stained with haematoxylin and eosin combined with histochemical staining of mucins with alcian blue. Colonic microscopic damage was evaluated by a pathologist blinded to the experimental groups according to the criteria described in table 1.

Table 1 Criteria for scoring of full-thickness distal colon sections.

Mucosal epithelium and lamina propria
– Ulceration: none (0); mild surface (0–25%) (1); moderate (25–50%) (2); severe (50–75%) (3); extensive-full thickness (more 75%) (4). – Polymorphonuclear cell infiltrate – Mononuclear cell infiltrate and fibrosis – Edema and dilation of lacteals
Crypts
– Mitotic Activity: lower third (0); mild mid third (1); moderate mid third (2); upper third (3) – Dilations – Goblet cell depletion
Submucosa
– Polymorphonuclear cell infiltrate – Mononuclear cell infiltrate – Oedema – Vascularity
Muscular layer
– Polymorphonuclear cell infiltrate – Mononuclear cell infiltrate – Oedema – Infiltration in the serosa

Scoring scale: 0, none; 1 slight; 2, mild; 3, moderate; 4, severe. Maximum score: 59.

2.4 RNA extraction and gene expression analysis

Total RNA, including both microRNAs and mRNAs, were isolated using a miRNeasy mini Kit (Qiagen, Hilden, Germany). A total of 500 ng of RNA were reverse transcribed using the miScript II RT kit from Qiagen (Qiagen,

Hilden, Germany). RT-qPCR of microRNAs was performed using the QuantiTect SYBR Green PCR Master Mix with miScript Universal Primers and the specific miRNA primer sequences (Qiagen, Hilden, Germany). For mRNA expression, RT-qPCR was performed using KAPA SYBR® FAST qPCR Master Mix (KapaBiosystems, Inc., Wilmington, MA, USA). Detection was performed on optical-grade 48 well plates in an EcoTM Real-Time PCR System (Illumina Inc., San Diego, CA, USA). The small nucleolar RNA C/D box 95 (SNORD95) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured to normalize microRNA and mRNA expression (−ΔCt), respectively. For the gene expression profile of the DNBS model of colitis, the mean −ΔCt value of NC control group was subtracted from individual -ΔCt values [$y = (-\Delta Ct) - (-\Delta Ct_{NC})$] to set the baseline expression at 0, and thus values shown (−ΔΔCt) represent logarithm base 2 expression levels. For the evaluation of the effect of the treatments, gene expression was calculated using normalized expression levels ($2^{-\Delta Ct}$) referred to the mean of NC control group to obtain the fold increase values ($2^{-\Delta Ct}/2^{-\Delta Ct_{NC}}$). SNORD95, miRNA and reverse universal primer for miRNA were sourced commercially (Qiagen, Hilden, Germany). Table 2 shows the remaining specific primer sequences (Sigma-Aldrich Quimica S.L., Madrid, Spain).

Table 2 RT-qPCR primer sequences.

Gene		Sequence 5′-3′	Annealing T (°C)
GAPDH	FW	5′-CCATCACCATCTTCCAGGAG	**60**
	RV	5′-CCTGCTTCACCACCTTCTTG	
MUC-1	FW	5′-GCAGTCCTCAGTGGCACCTC	**60**
	RV	5′-CACCGTGGGGCTACTGGAGAG	
MUC-2	FW	5′-GATAGGTGGCAGACAGGAGA	**60**
	RV	5′-GCTGACGAGTGGTTGGTGAATG	
MUC-3	FW	5′-CGTGGTCAACTGCGAGAATGG	**60**
	RV	5′-CGGCTCTATCTCTACGCTCTC	
TTF-3	FW	5′-CCTGGTTGCTGGGTCCTCTG	**60**
	RV	5′-GCCACGGTTGTTACACTGCTC	
ZO-1	FW	5′-GGGGCCTACACTGATCAAGA	**56**
	RV	5′-TGGAGATGAGGCTTCTGCTT	
OCCLUDIN	FW	5′-ACGGACCCTGACCACTATGA	**56**
	RV	5′-TCAGCAGCAGCCATGTACTC	
MMP-9	FW	5′-TGGGGGGCAACTCGGC	**60**
	RV	5′-GGAATGATCTAAGCCCAG	
TLR2	FW	5′-CCAGACACTGGGGGTAACATG	**60**
	RV	5′CGGATCGACTTTAGACTTTGGG	
TLR4	FW	5′-GCCTTTCAGGGAATTAAGCTCC	**60**
	RV	5′-AGATCAACCGATGGACGTGTAA	
TLR6	FW	5′-GACTCTCCCACAACAGGATACG	**60**
	RV	5′-TCAGGTTGCCAAATTCCTTACAC	
TLR7	FW	5′-TCTTACCCTTACCATCAACCACA	**60**
	RV	5′-CCCCAGTAGAACAGGTACACA	
TLR9	FW	5′-ACTCCGACTTCGTCCACCT	**61**

	RV	5′-GGCTCAATGGTCATGTGGCA	
CXCL2	FW	5′-CAGTTAGCCTTGCCTTTGTTCAG	**62**
	RV	5′-CAGTGAGCTGCGCTGTCCAATG	
CCL2	FW	5′-CAGCTGGGGACAGAATGGGG	**62**
	RV	5′-GAGCTCTCTGGTACTCTTTTG	
TNFα	FW	5′-AACTAGTGGTGCCAGCCGAT	**56**
	RV	5′-CTTCACAGAGCAATGACTCC	
IL-1β	FW	5′-TGATGAGAATGACCTCTTCT	**55**
	RV	5′-CTTCTTCAAAGATGAAGGAAA	
IL-6	FW	5′-TAGTCCTTCCTACCCCAATTTCC	**60**
	RV	5′-TTGGTCCTTAGCCACTCCTTC	
miR-9-5p	FW	5′-UCUUUGGUUAUCUAGCUGUAUGA	**55**
miR-29c-3p	FW	5′-UAGCACCAUUUGAAAUCGGUUA	**55**
miR-142-3p	FW	5′-UGUAGUGUUUCCUACUUUAUGGA	**55**
miR-143-3p	FW	5′-UGAGAUGAAGCACUGUAGCUC	**55**
miR-145a-5p	FW	5′GUCCAGUUUUCCCAGGAAUCCCU	**55**
miR-146a-5p	FW	5′-UGAGAACUGAAUUCCAUGGGUU	**55**
miR-150-5p	FW	5′-UCUCCCAACCCUUGUACCAGUG	**55**
miR-155-5p	FW	5′-UUAAUGCUAAUUGUGAUAGGGGU	**55**
miR-203-3p	FW	5′-GUGAAAUGUUUAGGACCACUAG	**55**
miR-221-3p	FW	5′-AGCUACAUUGUCUGCUGGGUUUC	**55**
miR-223-p	FW	5′-UGUCAGUUUGUCAAAUACCCCA	**55**
miR-375-3p	FW	5′-UUUGUUCGUUCGGCUCGCGUGA	**55**
miR-483-3p	FW	5′-UCACUCCUCCCCUCCCGUCUU	**55**

2.5 Bacterial DNA pyrosequencing and analysis

Conventional phenol:chloroform extraction and ethanol purification were used to isolate DNA from faecal samples. 16S rRNA gene was amplified by PCR using primers targeting regions flanking the variable regions V1 to V3 of the bacterial 16S rRNA gene (V1-3). Sequence recovery and integrity was analysed the 454/Roche GS Titanium technology (Roche Diagnostics, Basel, Switzerland). The amplification of a 600 bp sequence in the variable region V1-V3 of the 16S rRNA gene was performed using bar-coded primers. The PCR was performed in a total volume of 15 µL for each sample containing the universal 27F and Bif16S-F primers (10 µmol/L) at a 9:1 ratio, respectively, and the bar-coded universal reverse primer 534R (10 µmol/L) in addition to dNTP mix (10 mmol/L), FastStart 10× buffer with 18 mmol/L of $MgCl_2$, FastStart HiFi polymerase (5 U in 1 mL), and 2 µL of genomic DNA. The dNTP mix, FastStart 10× buffer with $MgCl_2$, and FastStart HiFi polymerase were included in a FastStart High Fidelity PCR System, dNTP Pack (Roche Applied Science, Penzberg, Germany). PCR conditions were as follows: 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 56 °C for 30 s, and 72 °C for 5 min, and a final step at 4 °C. After PCR, amplicons were further purified using AMPure XP beads (Beckman Coulter, Ltd., High Wycombe, UK) to remove smaller fragments. DNA concentration and quality were measured using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Finally, the PCR amplicons were combined in equimolar ratios to create a DNA pool (10^9 DNA molecules) that was used for clonal amplification (emPCR) and pyrosequencing according to the manufacturer's instructions.

The reads obtained from 16S ribosomal DNA sequencing were scored for quality, and any poor quality and short reads were removed. Sequences were trimmed to remove barcodes, primers, chimeras, plasmids, mitochondrial

DNA and any non-16S bacterial reads and sequences <150 bp. The MG-RAST metagenomics analysis server [30] was used to analyse the sequences and make taxonomic assignments with Ribosomal Database Project (RDP). Operational taxonomic units (OTUs) were obtained with minimum e-value of 1e-5, minimum alignment length of 15 bp and minimum identity threshold was set at 95%. The relative abundance of OTUs for each sample was calculated on the output file and used for subsequent analysis, including the determination of ecological parameters indicative of α- and β-diversity, determined using Statistical Analysis of Metagenomic Profiles (STAMP) software package version 2.1.3 [31].

2.6 Statistics

Statistical significance was evaluated using one-way analysis of variance (ANOVA) and *post hoc* Tukey's Multiple Comparison tests. Survival curves were analysed with the Gehan-Breslow-Wilcoxon test. Non-parametric data were analyzed using the Mann-Whitney *U* test. All statistical analyses were carried out with the GraphPad 5.0 software package (GraphPad Software, Inc., La Jolla, CA, USA), with statistical significance set at $P<0.05$.

3 Results

3.1 Immunomodulatory tetracyclines ameliorate DNBS-colitis

DNBS instillation induced a severe damage in the colonic tissue. This damage is considered completely established in this model at day 3 and then progresses into fibrosis, necrosis and colonic obstruction [27,28]. Thus, colitic mice experienced a severe weight loss and high mortality rate during the 6-day experimental period (Fig. 1A and B). All antibiotic-treated colitic groups showed reduced weight loss, especially the mice treated with tetracyclines. Among them, the immunomodulatory tetracyclines DXC, MNC and TGC induced a clear amelioration of the colonic inflammatory process, with milder body weight loss evidenced from the beginning of the treatment. Of note, dexamethasone (DEX) treatment did not induce any beneficial effect in terms of weight evolution in comparison with the DNBS control group (Fig. 1A). The effects of the treatments were also evidenced on survival rates, since they increased in all groups treated with tetracyclines. Statistical differences on survival rate were found for colitic mice treated with MNC and DXC, whereas neither DEX nor RFX were able to reduce the high mortality rate caused by the DNBS-induced acute inflammation (Fig. 1B).

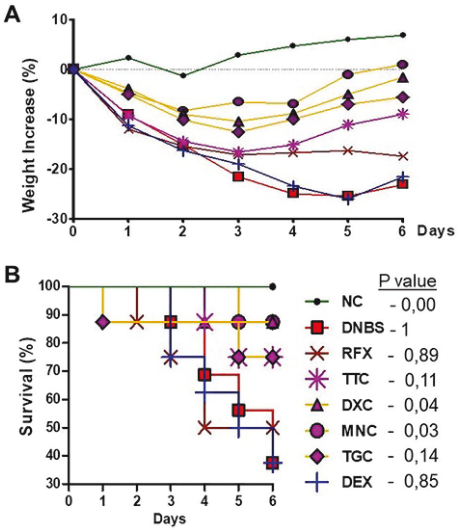

Fig. 1 Comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in DNBS-colitis. NC: Non-colitic group, DNBS: DNBS-colitic group. (A) Animal body weigh evolution and (B) Survival curves (%) of the different groups during the 6-days experimental period and their P values *vs.* DNBS control group.

The severe colonic damage was characterised by the shortening and thickening of the large intestine and thus quantified macroscopically by the colonic weight/length ratio. Only the four groups of colitic mice treated with tetracyclines showed a significant reduction compared to untreated colitic mice (Fig. 2A). The microscopic evaluation of the colonic samples showed extensive necrosis and ulceration affecting almost the entire surface of the colon in the DNBS control group. An average microscopic damage score of 28.6 was obtained for DNBS control group, indicating a severe colonic damage (Fig. 2B and C). The mucosal architecture was greatly affected and goblet cells were depleted from their mucin content. The inflammatory process involved all the intestinal layers, with intense leukocyte infiltration and thickening of the submucosa and muscularis mucosa. No differences regarding these parameters

were observed between the DNBS control mice and those treated with RFX or DEX. However, a smaller area of the colonic surface appeared damaged in mice treated with tetracyclines, being lesions in these animals less severe: the mucosal layer mostly conserved the crypt architecture with goblet cells replenished of their mucin content (Fig. 2C). As a result, the microscopic score values were significantly reduced, especially in the groups treated with immunomodulatory tetracyclines DXC, MNC and TGC (Fig. 2B).

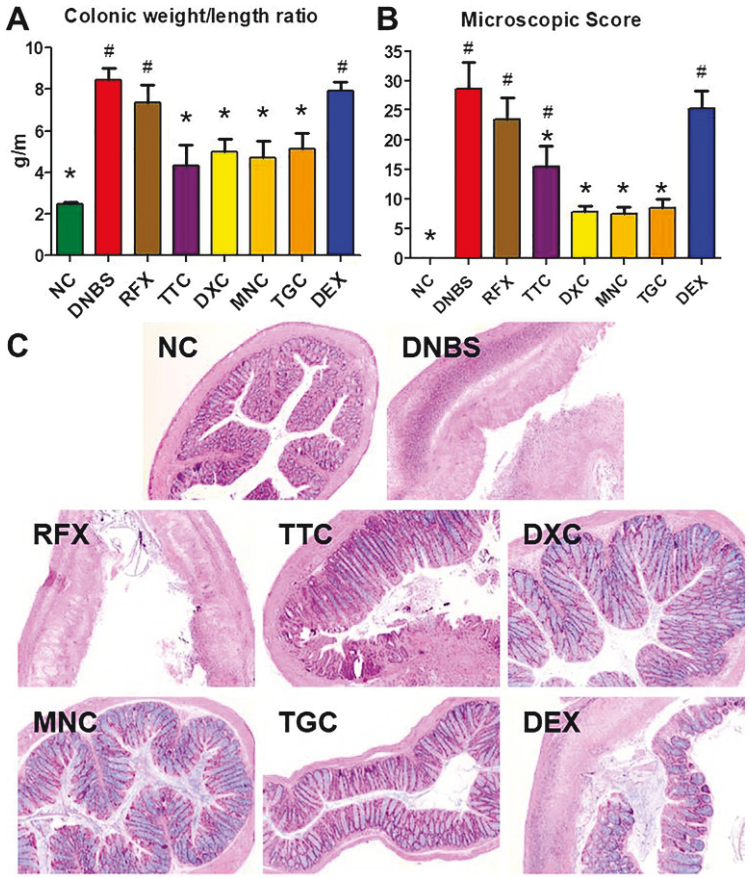

Fig. 2 Comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in DNBS mouse colitis. NC: Non-colitic group, DNBS: DNBS-colitic group. (A) Colonic weight/lenght ratio. (B) Microscopic damage score assigned according to the criteria described in Table 1. (C) Representative histological sections of colonic mucosa of the different experimental groups stained with haematoxylin, eosin and alcian blue (40× magnification). Data are expressed as mean ± SEM. $^{*}P < 0.05$ *vs.* DNBS control group. $^{\#}P < 0.05$ *vs.* NC control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2 Impact on intestinal microbiota composition

In order to characterise the modifications in the intestinal microbiota composition, 16S ribosomal DNA was isolated from stools and analysed by pyrosequencing. No differences were observed among the different ecological parameters related with richness (Margalef and Chao1), evenness (Simpson and Pielou) or diversity (Shannon). Surprisingly, DNBS control group showed a trend towards an increase in richness and diversity in comparison with the NC group, an effect that was generally reverted by antibiotics (Table 3). Analysis of β-diversity showed microbiota composition differed among the experimental groups. Hierarchical clustering analysis based on order-level composition separates DNBS-colitic mice from healthy controls, whereas antibiotic-treated mice are spread between them (Fig. 3A). Higher differences were observed in the composition of lower taxonomic groups, as illustrated in the PCA plot built from the dissimilarity analysis at genus level (Fig. 3B). The PC1 is associated with differences between antibiotic-treated mice and the other groups, which account for 42.7% of variability, whereas the PC2 (26.2% of the variability observed) mainly explains de differences between healthy and DNBS control mice. Therefore, 3 main clusters were identified: NC control, DNBS control, and the antibiotic-treated cluster. No distinctive effect was observed in the

microbiota composition of DEX treated mice, positioned between DNBS and NC clusters. Finally, no differential patterns were identified among antibiotic-treated groups, which may indicate they have a similar overall impact on microbial communities.

Table 3 Comparison of α-diversity measures of intestinal microbiota between Non-colitic group (NC) (n = 5), DNBS-colitic group (DNBS) (n = 6) and rifaximin (RFX) (n = 3), tetracycline (TTC) (n = 3), doxycycline (DXC) (n = 4), minocycline (MNC) (n = 4), tigecycline (TGC) (n = 4) and dexamethasone (DEX) (n = 3) treated groups in the DNBS model of mouse colitis. Data are expressed as means ± SEM.

INDEX	Margalef	Chao1	1-Simpson	Shannon	Pielou
NC	**7.6** ± 1.38	**105.3** ± 24.6	**0.64** ± 0.12	**1.89** ± 0.38	**0.46** ± 0.08
DNBS	**9.0** ± 0.65	**121.3** ± 14.3	**0.88** ± 0.01	**2.69** ± 0.11	**0.63** ± 0.03
RFX	*__6.0__ ± 1.00	**93.2** ± 12.9	**0.83** ± 0.03	**2.34** ± 0.14	**0.59** ± 0.02
TTC	**7.8** ± 2.47	**109.1** ± 38.8	**0.83** ± 0.06	**2.31** ± 0.48	**0.55** ± 0.08
DXC	**7.4** ± 0.60	**86.2** ± 13.8	**0.87** ± 0.01	**2.53** ± 0.13	**0.61** ± 0.02
MNC	**8.8** ± 1.32	**126.8** ± 24.6	**0.85** ± 0.03	**2.58** ± 0.20	**0.60** ± 0.04
TGC	**7.0** ± 2.21	**101.4** ± 25.5	**0.83** ± 0.05	**2.26** ± 0.40	**0.56** ± 0.06
DEX	**9.9** ± 1.69	**155.2** ± 14.0	**0.87** ± 0.01	**2.67** ± 0.22	**0.60** ± 0.03

* $P < 0.05$ *vs.* DNBS control group.

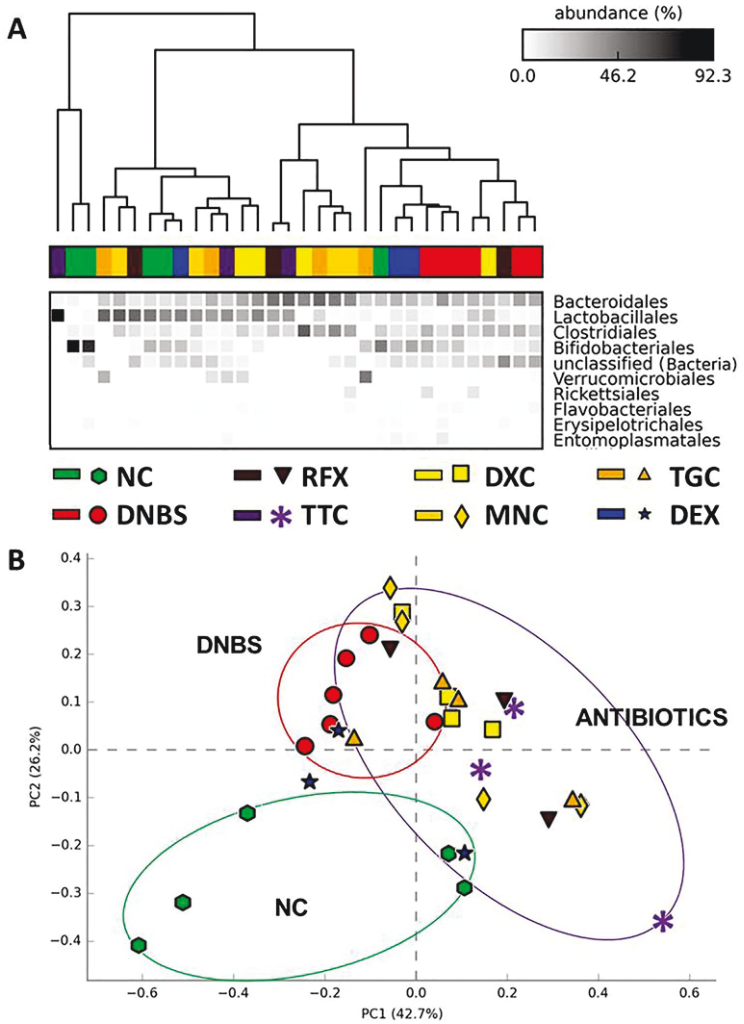

Fig. 3 Comparison of microbiota composition based on β-diversity analysis between Non-colitic group (NC) (n = 5), DNBS-colitic group (DNBS) (n = 6) and rifaximin (RFX) (n = 3), tetracycline (TTC) (n = 3), doxycycline (DXC) (n = 4), minocycline (MNC) (n = 4), tigecycline (TGC) (n = 4) and dexamethasone (DEX) (n = 3) treated groups in the DNBS model of mouse colitis. (A) Heatmap with relative abundance of the 10 most abundant orders, including hierarchical clustering of samples based on order level composition analysed with the method of minimum variance of Ward (B) PCA plot representation based on the ordination of the distance matrix built with a dissimilarity analysis at genus level using the taxon-based Bray-Curtis complementary algorithm. Green ellipse includes NC samples, red ellipse includes DNBS samples and purple ellipse includes sample from antibiotic-treated groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

When considering the abundance of the most predominant Phyla (*Firmicutes*, *Bacteroidetes* and *Actinobacteria*), a significant and pronounced decrease in the proportion of reads of *Actinobacteria* was observed in antibiotic-treated colitic groups, which also decreased in untreated and DEX-treated colitic mice compared to NC control group (Fig. 4 A1). DNBS-induced colitis was associated with a significant increase in *Bacteroidetes* abundance compared to NC control group, which was not significantly modified by any of the treatments (Fig. 4 B1). By contrast, although the Phylum *Firmicutes* was not altered in DNBS control group compared to NC mice, increased abundance was generally observed with antibiotic treatments (Fig. 4 C1). Further analysis showed that changes found in the Phylum *Actinobacteria* were mainly associated with *Bifidobacterium pseudolongum*. This species was significantly reduced in the DNBS control group and further decreased by antibiotics but not by DEX treatment (Fig. 4 A2). Similarly, *Bacteroides acidifaciens* was the main species involved in the increase in the Phylum *Bacteroidetes* observed in colitic mice (Fig. 4 B2). Despite no differences were observed between NC and DNBS groups in *Firmicutes* abundance, among this Phylum, *Clostridia* class was significantly increased in DNBS control mice (Fig. 4 C2). Differences within the *Clostridia* class were

more prominent on deeper taxonomic levels. For instance, DNBS control mice had a marked increase in *Ruminococcus*, which were reduced in all treated groups (Fig. 4 C3). DNBS control mice showed a significant increase in *Clostridium disporicum* (Fig. 4 C4), which generally go undetected in NC and antibiotic treated groups but not in DEX-treated mice. In addition, the genus *Blautia* was significantly increased in DNBS control mice and, whereas it was significantly reduced by RFX, DEX and TTC treatments, it remained high in DXC, MNC and TGC groups (Fig. 4 C5), interestingly, the treatments that exerted the highest therapeutic activity.

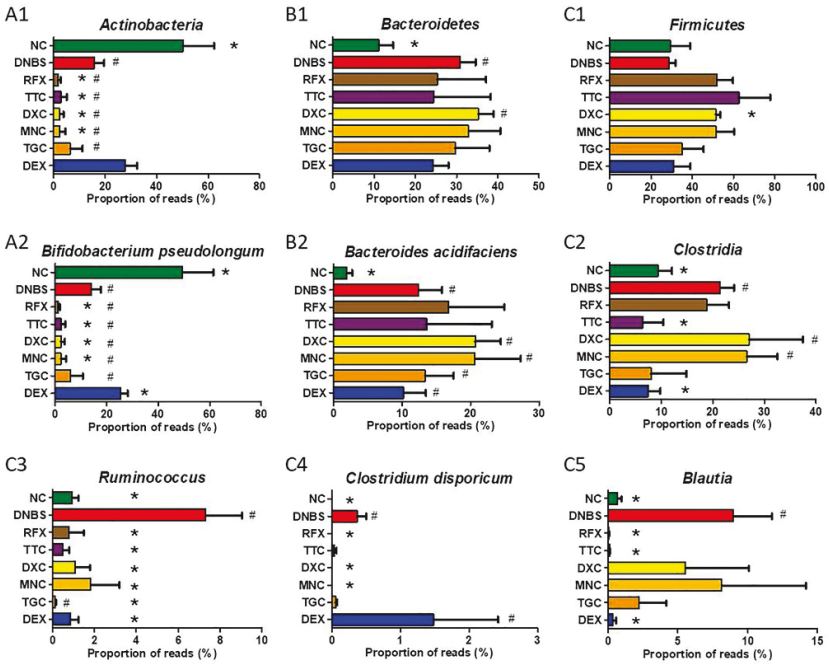

Fig. 4 Comparison of microbiota composition between Non-colitic group (NC) (n = 5), DNBS-colitic group (DNBS) (n = 6) and rifaximin (RFX) (n = 3), tetracycline (TTC) (n = 3), doxycycline (DXC) (n = 4), minocycline (MNC) (n = 4), tigecycline (TGC) (n = 4) and dexamethasone (DEX) (n = 3) treated groups in the DNBS model of mouse colitis. Relative abundance of various taxonomic groups. Data are expressed as mean ± SEM. $^{*}P < 0.05$ *vs.* DNBS control group. $^{\#}P < 0.05$ *vs.* NC control group.

3.3 Impact on gene expression

In order to characterise DNBS-colitis, gene expression profile was performed focused on markers involved in barrier integrity, inflammatory mediators (MMPs, cytokines and chemokines), microRNAs and TLRs (Fig. 5). Six days after colitis induction, minor changes were observed in the expression of mucins and tight junction components, being MUC-3, TTF-3 and Occludin the most down-regulated genes in DNBS-colitis. By contrast, the inflammatory mediators evaluated were significantly up-regulated in the DNBS group with the exception of CCL2, whose expression was reduced compared to healthy controls. TLR-7 up-regulation in DNBS-colitic mice was the only significant difference observed among the TLRs evaluated. Finally, although changes in microRNAs are generally subtle despite being biologically relevant, colitic mice showed a significant up-regulated expression of miR-9 and miR-223, whereas the expression of miR-142, miR-143 and miR-150 was down-regulated compared to NC group (Fig. 5).

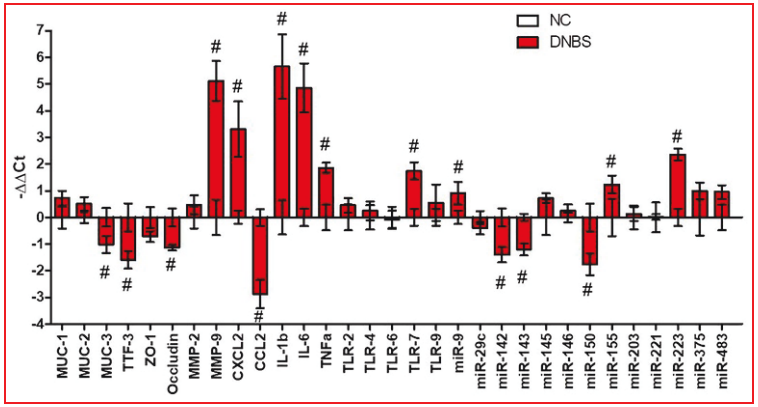

Fig. 5 Gene expression profile of the DNBS model of mouse colitis quantified by real-time PCR. NC: Non-colitic group, DNBS: DNBS-colitic group. Relative expression represented in logarithm base 2 as −ΔΔCt values, with NC mean being set as baseline for each gene. Data are expressed as mean ± SEM. #$P < 0.05$ *vs.* NC control group.

In general, an up-regulated expression of the barrier function markers MUC-3, TFF-3, ZO-1 and occluding was found in groups treated with tetracyclines when evaluating the effects of the treatments on gene expression (Fig. 6). A down-regulated expression of the inflammatory mediators MMP-9, CXCL2, IL-1β and IL-6 was found in all group treated with tetracyclines (Fig. 7). By contrast, minor effects were observed with RFX and DEX treatments among these markers. Regarding TNFα expression, only a small and non-significant down-regulation was observed with the immunomodulatory tetracyclines DXC, MNC and TGC; whereas RFX, TTC and DEX, with reduced therapeutic effect, had significantly down-regulated levels compared to DNBS control. Of note, the down-regulated expression of CCL2 in DNBS control mice was restored and further up-regulated above basal levels in the groups treated with tetracyclines. Among the different TLRs evaluated, TLR6 was significantly up-regulated by MNC, DXC and TGC treatments, while none of the other treatments modified its expression. Although significant differences were not always reached, all the groups treated with antibiotics showed increased TLR2 expression, whereas TLR9 was up-regulated in mice receiving tetracyclines, but not with RFX or DEX (Fig. 7). Regarding microRNA expression, the treatment with tetracyclines or DEX significantly ameliorated the up-regulation of miR-9 in DNBS control mice (Fig. 8). By contrast, miR-223 expression, also increased in the DNBS group, was reduced by all antibiotics but not by DEX treatment. Finally, the effect of tetracyclines was associated with the increase of miR-375, downregulated by DNBS-induced colitis. A similar effect was observed for miR-142 expression, where only the restored expression achieved with DXC, MNC and TGC reached statistically significant differences (Fig. 8).

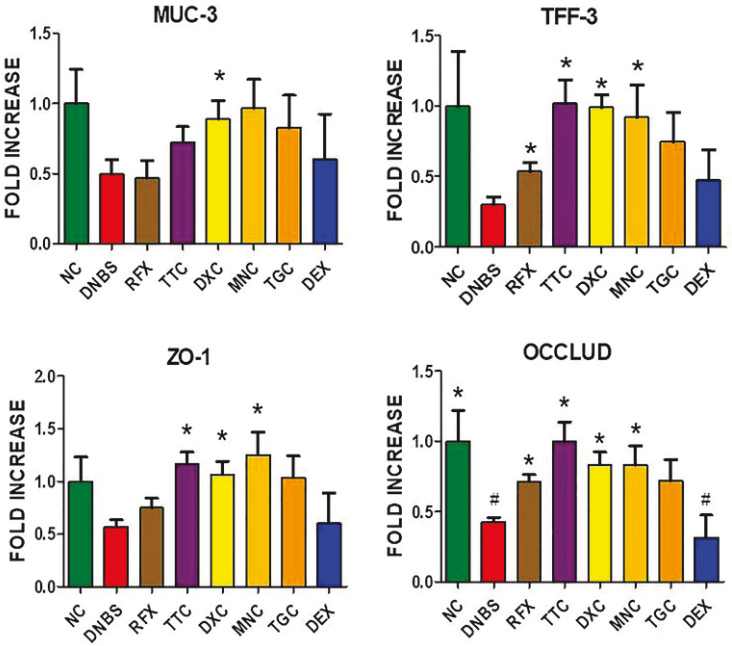

Fig. 6 Comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in the DNBS model of mouse colitis. NC: Non-colitic group, DNBS: DNBS-colitic group. Colon mRNA expression of the indicated genes was quantified by real-time PCR. Fold increase was calculated *vs.* NC group. Data are expressed as mean ± SEM. $^{*}P < 0.05$ *vs.* DNBS control group. $\#P < 0.05$ *vs.* NC control group.

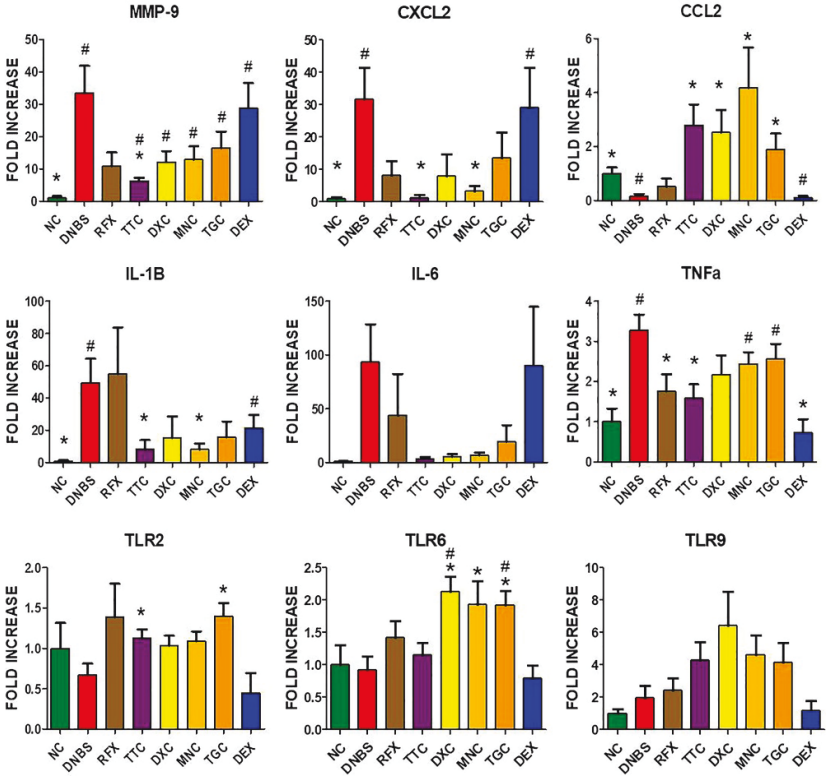

Fig. 7 Comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in the DNBS model of mouse colitis. NC: Non-colitic group, DNBS: DNBS-colitic group. Colon mRNA expression of the indicated genes was quantified by real-time PCR. Fold increase was calculated *vs.* NC group. Data are expressed as mean ± SEM. $^{*}P < 0.05$ *vs.* DNBS control group. $^{\#}P < 0.05$ *vs.* NC control group.

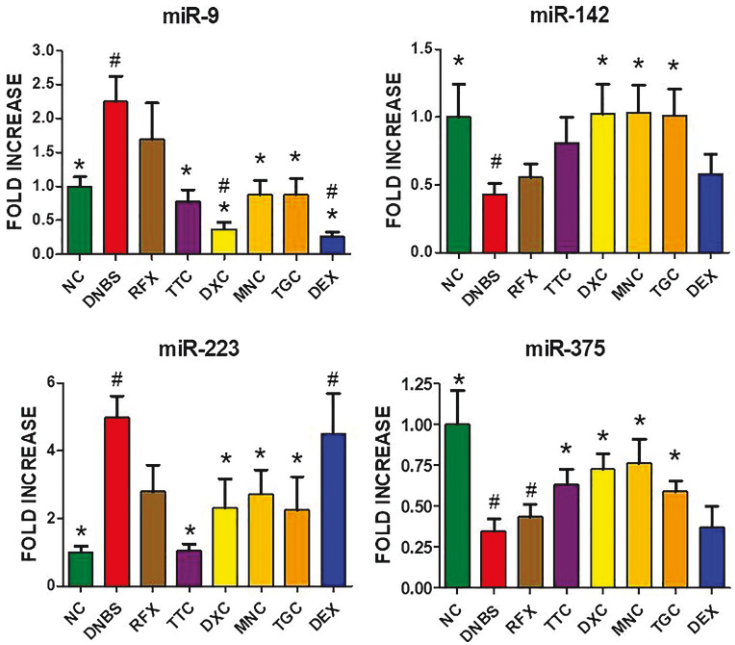

Fig. 8 Comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in the DNBS model of mouse colitis. NC: Non-colitic group, DNBS: DNBS-colitic group. Colon expression of the indicated microRNAs was quantified by real-time PCR. Fold increase was calculated *vs.* NC group. Data are expressed as mean ± SEM. $^{*}P < 0.05$ *vs.* DNBS control group. $^{\#}P < 0.05$ *vs.* NC control group.

3.4 Intercorrelation between gene expression, microbial changes and therapeutic efficacy

A correlation matrix was generated to illustrate how the parameters evaluated correlated with therapeutic efficacy and to identify associations between microRNA and TLR expression with microbial changes. This matrix compiled all the data produced in our comparative pharmacological study, including four measures to score DNBS colitis: the area under the curve (AUC) of the weight loss evolution (%), the mortality rate at the end of the study, the colonic weight/length ratio and the histological score (Fig. 9). As expected, barrier function markers negatively correlated with disease scores, whereas most of the inflammatory mediators showed the opposite pattern, with a positive correlation with disease scores and negative correlation with intestinal barrier genes. However, TNFα showed no correlation with either groups and CCL2 had a divergent effect than the other inflammatory mediators, being negatively correlated with these and colitic score measures (Fig. 9A). Regarding microRNA expression, miR-223 fell within the “inflammatory cathegory”, with positive correlation with disease scores, whereas miR-142 and miR-375 correlated negatively with colitis activity. MiR-9 and TLR expression showed a weak and non-significant correlation despite being miR-9 positively associated with inflammation whereas the three TLRs showed a negative correlation. The strongest correlations found between microRNAs and TLR were the negative association of miR-223 – TLR2 and the positive correlation of miR-142 with the three TLRs evaluated, particularly with TLR6 (Fig. 9B). Abundance of the bacterial taxa with the biggest differences between treated groups did not correlate with disease scores, however, microbial diversity (Shannon and Pielou indexes) positively correlated with the inflammatory scores and the abundance of *Bacteroidetes* and *B. acidifaciens*, as well as *Clostridia* and *Blautia*, but not *Firmicutes* (their corresponding Phylum). By contrast, the abundance of *Actinobacteria* (and *B. pseudolongum*) negatively correlated with *Firmicutes* and *Bacteroidetes*, and also showed a minor negative correlation with microbial diversity (Fig. 9C). Finally, the negative correlation of *B. pseudolongum* with TLR9 and TLR6 is noteworthy, whereas the gene expression of these receptors positively correlated with *B. acidifaciens* abundance. Interestingly, TLR2 showed a strong and significant negative correlation with bacterial richness, and bacterial diversity was positively correlated with miR-223 but negatively correlated with miR-142 and miR-375. Despite TNFα expression showed very weak correlation with disease scores and other genes evaluated, interesting associations were found with microbiota measures: TNFα expression negatively correlated with *B. pseudolongum* whereas it showed a positive correlation with microbial diversity, *B. acidifaciens* and *Blautia* abundance (the strongest and only significant correlation observed for this cytokine) (Fig. 9D).

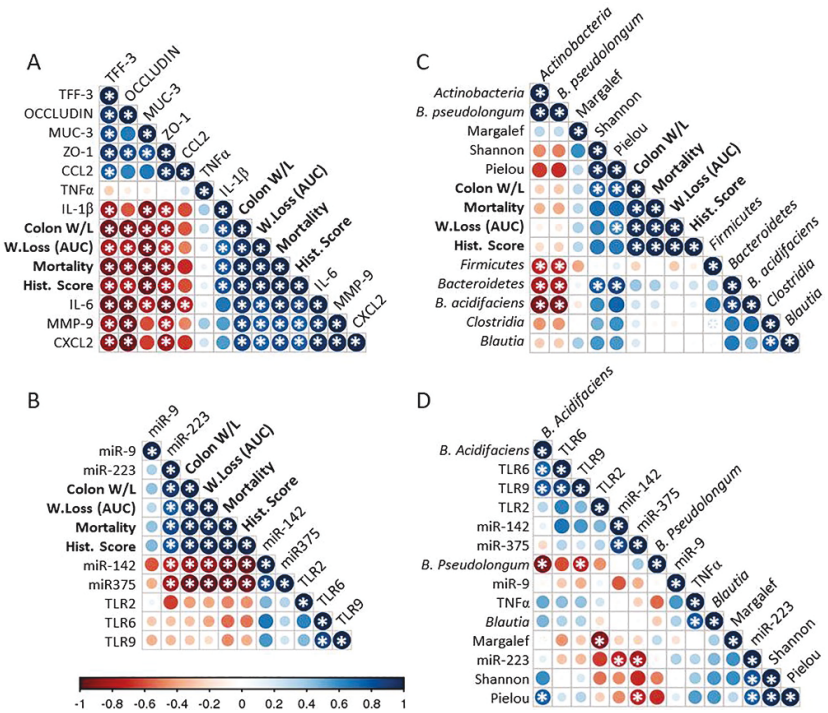

Fig. 9 Association between measures of colitis activity, gene expression and intestinal microbiota composition within the comparative pharmacological study. A correlation analysis was performed by Pearson correlation test using all data generated in the present study. Strength of the association is denoted by both the colour and the size of the bubble (darker colour and bigger size meaning higher correlation; red, negative; blue, positive). White asterisks indicate statistically significant association ($^{*}p < 0.05$). (A) Disease scores and gene expression of markers of barrier function and inflammatory mediators. (B) Disease scores and TLR and microRNA expression. (C) Disease scores and measures of α-diversity and abundance of intestinal microbiota. (D) Gene expression and measures of α-diversity and abundance of intestinal microbiota. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4 Discussion

Following on reports describing the intestinal anti-inflammatory activity of two immunomodulatory tetracyclines, DXC and MNC [13–16], we aimed to evaluate the contribution of their pharmacological, antibiotic and immunomodoulatory activities to their beneficial effects on intestinal inflammation. TTC is considered as the prototype of this antibiotic family, and it was also previously reported to provide some therapeutic benefit in TNBS-induced colitis in rats [14]. This finding suggested that the antibiotic activity might be involved in the intestinal anti-inflammatory effect of tetracyclines in this model of colitis, although the contribution of non-antibiotic properties, also retained by this TTC compound to a lesser extent, cannot be excluded. Hence, RFX, a gastrointestinal-selective antibiotic belonging to a different family [32], was used to overcome this limitation. In addition, TGC, a third generation MNC derivate that retains many of its non-antibiotic properties [33], and dexamethasone, one of the most potent and widely used corticoids, were included in this comparative pharmacological study.

All the antibiotics promoted body weight recovery, which suggests a beneficial effect of the antibiotic action itself, likely by reducing the bacterial load and the exacerbated antigenic stimulation [34,35]. The reduced bacterial diversity in antibiotic-treated groups might reflect this effect. Curiously, the only microbial indicator correlated with disease scores was the microbial diversity, which was higher in colitic mice, in contrast with the trend described for IBD patients [36]. However, extreme conditions, such as fasting, have been reported to increase colonic microbial diversity [37]. Thus, the severe impact of this acute inflammatory process in mice wellbeing, which extremely reduced food intake, might create the optimal conditions for bacterial overgrowth and increase diversity.

Regarding the modifications in the composition of the microbiota, which could influence the inflammatory process, we observed antibiotics further accentuated some of the changes observed in colitic mice. This finding leads us to hypothesise that the antibiotic action could indeed not reverse the dysbiotic process, but favour the adaptability of the microbiota to the changing environment by reducing the bacterial load, thus creating the biological niche for the new dominant species to grow faster in these novel conditions. This could be the case of the reduction of *Bifidobacterium* observed in colitic mice and potentiated by all the antibiotics. Although *Bifidobacterium* has been associated

with active IBD in humans [38], these variations should not be always regarded as detrimental simply by association. An interesting finding was the increase in the genus *Blautia* in DNBS control and colitic mice treated with the immunomodulatory tetracyclines DXC, MNC and TGC, which, strikingly, were the most effective treatments in our study. This genus has been associated to a beneficial anti-inflammatory effect in various intestinal conditions [39], most likely linked to its metabolitc ability to degrade non-digestible fibre and produce short chain fatty acids (SCFA) [40]. In this regard, metabolic evaluation of the microbiota would provide more accurate functional information than the taxonomic characterization. Despite high initial variation at genus level in NC group (PCA plot), the influence of extreme conditions such as colitis or antibiotic intake impacted the microbiota in particular patterns, leading to a more similar composition in these groups. Associating these modifications with changes in metabolic pathways would significantly improve our understanding of the intestinal ecosystem and how the effect of the treatments on it could influence the intestinal inflammatory response.

However, considering that all antibiotics had a similar overall impact on the gut microbiota composition but only tetracyclines decreased mortality, colonic weight/length ratio and the microscopic score, it would be reasonable to ascribe these beneficial effects to the systemic antibiotic action provided by tetracyclines but not RFX, and the additional immunomodulatory properties of tetracyclines. Indeed, none of the effects on the abundance of the different taxonomic groups correlated with therapeutic efficacy. Thus, considering all the above and the severity and briefness of the process, changes in microbiota composition do not seems to have a major contribution to the therapeutic effect so far. A longer study might be required to evaluate the consequences of microbiota changes.

DNBS-colitis is characterised by the generation of an intense oxidative stress and the initial inflammatory reaction rapidly progresses into tissue remodelling and fibrosis [6,27]. Therefore, the previously reported antioxidant properties of tetracyclines and their well-known ability to inhibit matrix metalloproteinases (MMP) expression and activity [12] could contribute to ameliorate tissue damage: DXC is the most potent MMP inhibitor among these tetracyclines, whereas MNC stands out for its antioxidant activity, which it is likely shared by TGC due to their common tetracyclic structure [11,41]. The intestinal anti-inflammatory effects of tetracyclines were supported by the gene-expression results: inflammatory cytokines, the neutrophil chemoattractant CXCL2 and MMP-9, a matrix-degrading enzyme most abundantly expressed in the inflamed gut [2], were down-regulated in colitic mice treated with tetracyclines and associated with therapeutic efficacy. Consistent with these findings, tetracyclines also down-regulated the expression of miR-223, a granulocytic lineage microRNA whose expression increases as granulocytes mature [42,43]. Interestingly, miR-223 was the most up-regulated miRNA in this model of colitis, which further highlights the crucial role played by neutrophils in DNBS-induced inflammation and the ability of tetracyclines to reduce inflammation-associated tissue damage. MiR-9, linked to the activation of NFκB pathway [44], was also increased in DNBS-colitis and down-regulated by all tetracyclines. Furthermore, the anti-inflammatory action of DEX was manifested in TNFα, IL-1β and miR-9 down-regulation, but the fact that no significant protective effect was obtained with this corticoid in this setting indicates that interfering with inflammatory stimulation alone was not sufficient to achieve therapeutic benefit. By contrast, the antibiotic effect provided by RFX resulted in the amelioration of TNFα, MMP-9, CXCL2 and miR-223 expression levels, being most of these markers associated with neutrophil-mediated actions and positively correlated with disease scores.

It is worth noting that tetracyclines increased the expression of the monocyte chemoattractant protein CCL2, which was down-regulated in colitic animals, and therefore it was negatively correlated with disease scores and the other inflammatory mediators. Previous reports showed alternatively activated macrophages are necessary to control inflammation in this model of colitis and prevent the formation of fibrotic tissue [45–47], an immune response that mediates the therapeutic effects of helminths in experimental colitis [48]. In this regard, by increasing the presence of macrophages in the colonic tissue, tetracyclines might be promoting the resolution of the inflammatory process, preventing the progression of inflammation into fibrosis and the associated loss of function. Consistent with this effect, miR-142 and miR-375 were also up-regulated in TTC-treated mice and grouped with barrier function markers associated with therapeutic effect. MiR-142 and CCL2 have been described to elicit Th2 polarising effects [49,50], thus counterbalancing the detrimental immune response found in chronic inflammatory disorders and displaying direct protective effects on the mucosa. Indeed, a restored mucosal architecture and mucin staining were found histologically, especially in mice receiving immunomodulatory tetracyclines, as well as increased expression of mucins and tight junction proteins. Furthermore, improved goblet cell function in mice treated with tetracyclines is also indicated by increased levels of TFF-3 [51] and miR-375, which has been reported to inhibit KLF5, an antagonist of the goblet cell differentiation factor KLF4 [22,24].

In contrast with the effects of the above mentioned markers, TLR expression showed a weak correlation with the therapeutic efficacy, which may result from their role integrating microbial signals with host pathways and highlights the complexity of their regulation in inflammatory conditions. Interestingly, TLR6 up-regulation by DXC, MNC and TGC, the three tetracyclines with the highest therapeutic benefit in our study, was the sharpest difference among all the markers evaluated. Similarly, TLR9 up-regulation in the groups treated with tetracyclines could also add to their beneficial effect. It has been reported that $TLR9^{-/-}$ mice have delayed wound repair in experimental colitis [25] and TLR9 agonists are in clinical evaluation for the treatment of IBD [52]. Of note, both TLR6 and TLR9 correlated with the altered balance between *B. pseudolongum* (*Actinobacteria*) and *B. acidifaciens* (*Bacteroidetes*), and both TLRs positively correlated with some of the markers of the “anti-inflammatory group”, such as miR-142, suggesting these TLRs may pave the way for changes in microbial signals from these groups to impact protective mucosal responses. Conversely, bacterial richness (Margalef index) was the strongest and the only significant association between microbiota diversity measures and TLRs: TLR2 expression was negatively correlated bacterial richness, and it also showed an inverse correlation with miR-223 expression and other pro-inflammatory genes related with neutrophils actions. Thus, TLR2-signals may confer protection by interfering with inflammation-associated tissue damage. Of note, the TLR2/TLR6 pair is essential for the immunoregulatory signalling of lactic acid bacteria [19], widely known for their health benefits. Moreover, genetic variants of TLR2 and TLR6 have been associated with a deficient innate

immune response to bacteria in IBD patients, resulting in more extensive disease localization in UC and colonic CD [53]. Therefore, our results, together with the current knowledge, indicate that TLR up-regulation by tetracyclines promotes the recovery of intestinal homeostasis [54].

The causality of the correlation between these changes and modifications in microbiota composition still needs to be determined. In this regard, a longer study would help to ascribe a protective or detrimental role to the antibiotic impact on microbiota in intestinal inflammatory conditions. While preventive antibiotic treatment benefit intestinal inflammation due to reduced antigenic load and avoiding some of the complications of IBD [55], here we reported a potent anti-inflammatory effect of treatment when it was applied after colitis induction. In addition to inducing antibiotic resistance, antibiotic exposure is causal factor for dysbiosis and associated health risks, such as *C. difficile* infection [56]. Thus, a long-term antibiotic treatment as preventive or maintaining therapy does not seem appropriate in clinic. However, a short-term administration could allow achieving remission whilst reducing the impact on the microbial ecosystem, a more suitable setting for antibiotics in IBD. Of note, the use of tetracyclines in particular has not been associated with increased *C. difficile* infection [57], thus becoming the uppermost candidate among antibiotics for the treatment of intestinal inflammation. Regarding their immunomodulatory activity, additional *in vitro* studies are required to confirm whether the changes described here, e.g. in TLR and microRNA expression, are a direct effect of tetracyclines in particular cell populations. However, considering the complexity of intestinal inflammation and the fact that multiple mechanisms have been described to contribute to the immunomodulatory effect of tetracyclines [11,12], the results observed in vivo are likely better explained by the convergence of several pathways and the subsequent changes in the overall inflammatory milieu. Nonetheless, mechanistic as well as comparative pharmacological studies would improve our understanding of how the different activities of multi-target drugs contribute to the positive outcome, which will lead to design better therapeutic strategies in the future [58,59].

In summary, this study confirms previous observations about the benefits of tetracyclines in intestinal inflammation and provides important clues about the mechanism of action of tetracyclines. The protection provided by the antibiotic activity seems to be relevant in this acute model of colitis. However, the impact of tetracyclines on the intestinal microbiota did not restore the dysbiotic process initiated in this acute setting. Immunomodulatory tetracyclines have demonstrated a prompt effect, driving an improvement in the epithelial barrier integrity and reducing colitis-associated mortality and tissue damage, which correlated with changes in the expression of miRNAs and mediators related to mucosal protective pathways. In this regard, further investigations to achieve these protective responses would be of great interest, e.g. identifying the source and targets of microRNAs and taking advantage of their wide regulatory potential. Our findings support the idea that the activation of specific inflammatory pathways with tetracyclines, as opposed to the general inhibition of the immune response caused by immunosupressants such as dexamethasone, could in fact constitute an advantage in the treatment of intestinal inflammation. This study also constitutes the first description of the intestinal anti-inflammatory activity of TGC, a third generation tetracycline, which reinforce the notion that immunomodulatory tetracyclines are a promising strategy for the treatment of acute inflammation and its devastating consequences [60,61]. The actions reported here add to the broad range of promising properties exerted by this safe and well-known family of compounds, offering an appealing drug-reposition strategy to manage intestinal inflammatory conditions and opening a door for drug development based on the structure of tetracyclines.

Author contributions

Garrido-Mesa J, Rodríguez-Nogales A, Algieri F, Vezza T, Rodriguez-Cabezas ME and Utrilla MP performed the experiments and contributed to the acquisition and analysis of data; Garrido-Mesa J, Rodríguez-Nogales A, Garcia F and Chueca N contributed to the taxonomic analysis and data interpretation; Garrido-Mesa J, Garrido-Mesa N and Gálvez J designed the experiments, performed the analysis of data and wrote the manuscript.

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The authors declare that they do not have any competing interests.

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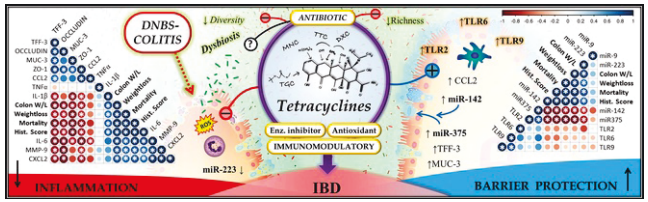

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