Chapter 6

Effect of redox modulation on intestinal disease using probiotic *Escherichia coli* Nissle 1917 producing antioxidant Pyrroloquinoline Quinone

6.1 Introduction

Innate immune system is the first line of defense against invading pathogens. This is a nonspecific response to pathogens and trauma unlike adaptive immune system, it does not confer long-lasting immunity to the host. The magnitude of innate immune response is controlled by activation and migration of immune cells (Wittmann et al., 2012). Inflammation is one of the first response of immune system to infection or injury, which is stimulated primarily by various factors released by injured cells, and hence it serves as physical barrier against the infected or inflamed tissue. This is followed by clearance of pathogen and cell debris and wound healing. However, inflammation is considered as critical player in several pathologies such as Inflammatory Bowel Disease (IBD), auto immune diseases, atherosclerosis, cancer, asthma, thyroiditis, Alzheimer's and Parkinson's disease (Coussens and Werb, 2002; Libby, 2002; Van Hove et., 2008).

Mammalian gastrointestinal tract is highly vulnerable to disease because it is continuously exposed to numerous bacterial species as well as food-borne environmental toxins. Inflammatory bowel disease (IBD) is characterized by acute and chronic inflammation of gastrointestinal tract with multifactorial etiology (Xavier and Podolsky, 2007). It includes ulcerative colitis (UC) and Crohn's disease (CD). IBD is inflammatory condition which is incurable. Surgery can cure UC if colon is removed. Recent data suggest the prevalence of IBD is constantly increasing and that 2.5 to 3 million people are affected by IBD in Europe with around 4.6 to 5.6 bn Euros/year direct health care cost (Burisch *et al*., 2013). IBD was thought to be uncommon for Asian countries particularly India (Ouyang *et al*., 2006; Bandyopadhyay, 2012). A growing number of reports suggests that IBD cases are increasing, which may be attributed to increased awareness and improved diagnostics, but may also reflect life style changes.

Medical treatment of IBD is individualized depending upon type, distribution, genetics and severity of disease. Anti-inflammatory steroids, TNF-α inhibitors, immunosuppressant and antibiotics are generally used for treatment. Fecal bacteriotherapy (FBT) is relatively new treatment option,

which has been used to treat IBD in several studies. Probiotics including several strains of *E. coli*, *Lactobacillus* and *Bifidobacteria* have also shown to prevent and improve intestinal inflammation and disease in IBD and related murine models, and currently many of them are under clinical trials. Murine models have been extensively used to demonstrate the physiology of disease and efficacy of various therapies aimed against IBD. These studies indicate that changes in host recognition and response to bacteria as well as alteration of microbiota communities are hallmarks of IBD development.

NADPH oxidases are multimeric protein complexes involved primarily in ROS generation and innate immune response (Babior, 2004). Members of this family, Nox2 and Nox1/Duox2 are expressed in neutrophils/macrophages and the intestinal mucosa, respectively. Clinical observations point to increased oxidative stress in IBD, although Nox2 deficiency leads in up to 40% of patients deficient in Nox2 (CGD) to the intestinal disease reminiscent of IBD and Nox2 KO mice show exacerbated disease in an IBD model (Campbell et al., 2014). DUOX is source of non-phagocytic ROS in intestinal epithelial cells and it is believed that DUOX dependent Hydrogen peroxide generation followed by hypothiocyanate formation in mucosal fluids, is microbicide and part of robust antimicrobial defense network in mammalian epithelial cells (Kim et al., 2013). DUOX mediated production of H_2O_2 has also been shown to prevent *Helicobacter* infection in mice (Grasberger et al., 2013). Additionally, severe host defense defect against pathogens was observed in DUOX deficient *Drosophila* gut (Ha et al., 2005a; 2005b).

H_2O_2 is non-radical Reactive oxygen species which is produced by family of NADPH oxidases, Xanthine oxidases and 5-lipoxigenases (Demiryurek and Wadsworth, 1999; Pritsos, 2000; Bedard and Krause 2007). DUOX enzymes are directly capable of generating H_2O_2, whereas NOXes 1-5 generate superoxide which is converted to H_2O_2 spontaneously or by superoxide dismutase (Rada and Leto, 2008).

NADPH oxidase mutant and specific gene knockout mice strains may provide a valuable platform for development of murine IBD, infection and inflammatory models which mimic the disease physiology.

Several *Lactobacillus* strains have been shown to ameliorate disease and inflammation in murine models of experimental colitis. Recently it was shown that *Lactobacillus johnsonii* (NCC 533) produces significant amount of H_2O_2*in vitro*. Deleting specific putative genes significantly diminish H_2O_2 production (Hertzberger et al., 2014). H_2O_2 is believed to be important in pathogen resistance, however little is known about its possible role is disease progression/amelioration during experimental colitis.

E. coli Nissle 1917 (EcN) is a well-established probiotic bacteria and partially ameliorates oxidative stress in experimental colitis and IBD (Grabig et al., 2006; Schultz and Habil, 2008). Modified probiotic EcN capable of secreting an antioxidant (Pyrroloquinoline Quinone; PQQ) was developed (Chapter 2). PQQ is one of the most efficient antioxidants and has the capability to influence mammalian cell signaling pathways, most importantly those related to induction of mitochondrial biogenesis (Rucker et al., 2009). *In Vitro* cell free experiments suggest that PQQ scavenges singlet oxygen (Mukai et al., 2011), superoxide anions and hydroxyl radicals (Urakami et al., 1997). It also suppresses peroxynitrite formation by NO donors preventing neurotoxicity (Zhang and Rosenberg, 2002). Recent reports suggest that EcN secreting PQQ significantly ameliorates chronic ethanol and rotenone induced oxidative stress in colonic and hepatic tissues respectively and moreover improves mitochondrial and antioxidant status in ageing rats (Chapters 4). Further investigation need to be done in order to evaluate its efficacy against oxidative stress and inflammation in murine IBD models.

The current work demonstrates the effect of probiotic EcN strains producing antioxidant, PQQ, in disease outcome using different models of experimental colitis in mice.

6.2 Methods and Materials

6.2.1 Bacterial strains and plasmids

E. coli Nissle 1917 was generous gift from Dr. Rer. Nat. Ulrich Sonnenborn, Ardeypharm GmbH, Loerfeldstrabe 20, Herdecke (Germany). They were grown in Luria-Bertani medium at 37 ^{0}C shaking at 150 rpm. GFP-expressing *Citrobacter rodentium* strain DBS100 (formerly *C. freundii* biotype 4280) was kindly provided by Dr. Bruce Vallance (University of British Columbia). The bacterial stocks were made in LB broth containing 20 % glycerol. All experiments in this chapter were performed in Prof. Ulla Knaus laboratory, UCD Conway institute, University College Dublin, Dubln-4, Ireland.

6.2.2 Animals

C57 BL/6 female and male mice obtained from Jackson laboratory and Taconic biosciences were bred and maintained in UCD Biomedical Facility, University College Dublin, Ireland. Details of mice used are stated in relevant experiments.

6.2.3 Colonization of *E. coli* Nissle 1917 in C57 BL/6 mouse gut

C57 BL/6 female mouse 6 weeks old were treated with antibiotics (Streptomycin; 5 g/l for 24 h; Bottle fed). After, removal of antibiotics, probiotic *E. coli* Nissle 1917 strain harboring pUC18-*gfp* plasmid (EcN-pUC18-*gfp*) mice were fed for consecutive 3 days followed by daily fecal bacterial enumeration of the same till 9 days. GFP positive colonies grown on Ampicillin plates were identified as EcN-pUC18-*gfp*.

6.2.4 DSS treatment and probiotic ingestion

DSS was purchased from TdB consultancy AB, Uppsala, Sweden. Mice were maintained in same cages till 10 days for pre-conditioning. DSS (3 %) was dissolved in sterile water and bottle fed to mice for 6 days **(Figure 6.1)**. DSS water was replaced at 3rd day of DSS treatment.6-8 weeks young C57 BL/6 black female mice from Jackson laboratory were bottle fed 3 % DSS for 6 days. EcN strains (5x 10^{9} CFU per mouse) were administered as co-treatment (from day 0 to 8) or post-treatment (from day 9 to 14) daily.

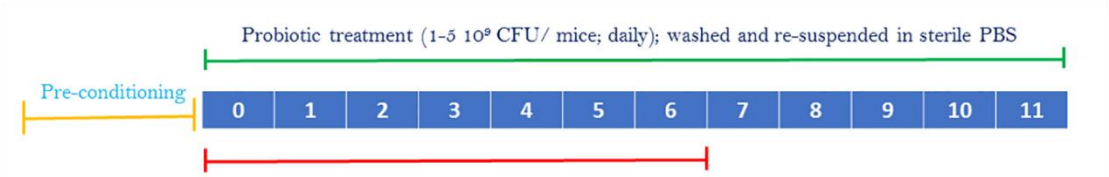

Figure 6.1 Schematic diagram representing DSS and EcN strains treatment.

6.2.5 Histological scoring for DSS experiment

Colonic sections were fixed in Buffered formalin. 0.5 micron paraffin fixed sections were stained with hematoxylin and eosin as described in Chapter 2. The level of colon inflammation was assessed based on a scale from 0 to 4. Scoring was done in a blinded fashion, and the histology scores used were as follows (Saunders et al., 2010).

A. Severity of inflammatory cell infiltration

score 0: none

score 1: slightly dispersed cell infiltrate

score 2: moderately increased cell infiltrates forming occasional cell foci

score 3: severely large areas of cell infiltrates causing loss of tissue architecture

B. Extent of injury

score 0: none

score 1: mucosal

score 2: mucosal and submucosal

score 3: transmural

C. Crypt damage

score 0: none

score 1: basal one-third damaged

score 2: basal two-thirds damaged

score 3: only surface epithelium intact

score 4: loss of entire crypt and epithelium

6.2.6 *C. rodentium* infection

For experimental procedures, *C. rodentium* was grown for 6-8 h on MacConkey agar containing 35 µg/ml chloramphenicol in aerobic conditions at 37 ºC. Further, a bacterial colony was harvested and inoculated in 10 ml of LB broth. The inoculum was incubated at 37 ºC, in aerobic conditions with shaking (200 rpm) for 12 h. Then the bacterial suspension was centrifuged 3500 g for 10 min at room temperature. OD of 1 corresponds to 10^9 CFU per ml. The pellet obtained was re-suspended in sterile PBS to a final concentration of $5x10^9$ CFU/ml. C57BL/6 black male mice were orally gavaged roughly $2x10^9$ CFU using sterile gavage needle.

During inoculation with *C. rodentium* DBS100, mice were kept in a CAT2 isolator. *C. rodentium* infected mice were all the time kept in the isolator and safety measures were employed in order to prevent an outbreak with this natural murine pathogen. All carcasses from infected mice were double-bagged and autoclaved at the end of experiment. EcN strains were gavaged pre- and post-Citrobacter infection as depicted in **Figure 6.2**.

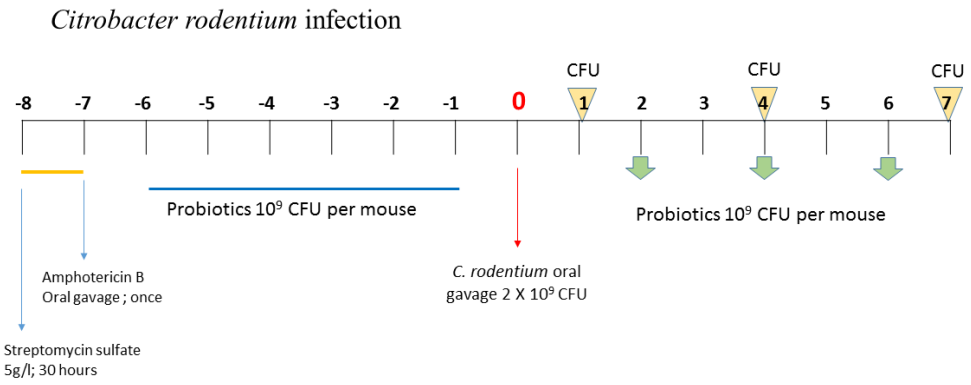

Figure 6.2 A schematic diagram of procedures operated during *C. rodentium* infection experiment on mice.

6.2.7 Bacterial enumeration

Fecal pellet, the cecal apex, 2 cm of terminal ileum and 2 cm of distal colon were placed each in 1.5 ml tubes and homogenized using a micropestle in sterile PBS. The homogenates were gently re-suspended in up to 1 ml of

sterile PBS and serial ten-fold dilutions (up to 10^{-8}) were made. All serial dilutions were spread on MacConkey agar plates containing chloramphenicol (35 µg/ml) and were incubated at 37 ºC overnight in aerobic conditions. Colonies that had a round shape with a red center and white rim were identified as *C. rodentium*. Bacterial numbers (colony forming units) were adjusted according to the tissue/feces weight (in grams), and expressed as CFU/g.

6.2.8 *In Vitro C. rodentium* growth inhibition assay

Freshly grown *E. coli* Nissle strains were re-inoculated in LB broth for 12 h (overnight) at 37 ^{0}C in aerobic condition (shaking 200 rpm). Next day, C. rodentium was inoculated from overnight grown MacConkey agar containing 35 µg/ml chloramphenicol, in to LB broth for 2-3 h at 37 ^{0}C in aerobic condition (shaking 200 rpm). 0.5 ml of grown *C. rodentium* was mixed with equal volume of *E. coli* Nissle 1917 strains and their supernatant (0.2 micron filtered) separately and incubated at 37 ^{0}C in 1 ml micro centrifuge tube for 4 hours. After 4 h, supernatant from each tube after serial dilutions were plated on LB agar plates containing 35 µg/ml chloramphenicol and left overnight in aerobic conditions at 37 ºC. Next day, *C. rodentium* colonies were counted manually and represented as CFU/ml.

6.2.9 mRNA extraction and qPCR analysis

Total RNA were extracted using RNeasy Kit (Qaigen) as per manufacturer's protocol. The extracted RNA were PCR amplified to cDNA using high capacity cDNA reverse transcription kit (Thermo Fisher Scientific) as per manufacturer's protocol. 100 ng cDNA was used for quantitative PCR per reaction. Amplification was performed using the ABI Prism 7900 machine with the TaqMan PCR gene expression and master mix system for mouse. All probes used were standard for mouse and can be found on Thermo Fischer Scientific website. The amplification was performed as per standard procedure using MicroAmp Fast Optical 96-Well Reaction Plates. *hprt* gene was used as endogenous control.

6.2.10 Protein extraction and Western blotting

The colonic tissues were disrupted on ice in RIPA lysis buffer (100-120 µl) containing protease inhibitors (Complete Mini from Roche). For Duox 1/2 detection, cells were lysed in RIPA buffer supplemented with complete Mini inhibitors (Roche), phosphatase inhibitor tablet (Roche), 200µM sodium orthovanadate, 200 µM PMSF, 50 mM β-glycerophosphate, 1 µM DFP, 1 µM microcystin. Samples were kept on ice 15 minutes. Tissue debris was separated from the proteins by centrifugation at 17,500 rpm for 10 minutes at 4°C. Protein concentrations were determined using the BCA assay kit. A BSA standard curve (1, 2, 4, 6 and eight µg/µl) was used for protein quantification. 20-30µg of protein was mixed with ice-cold 4x Laemmli buffer and loaded onto a 7 % denaturing polyacrylamide gel (no boiling). The proteins were transferred to nitrocellulose membranes (1.2 hours, 300 mA) and further blocked for 1 hour with 5% dry milk/TBST or 5%BSA/TBST. Primary antibody (homemade rabbit antibody 7536 bleed #6-8) used at 1:2000 in antibody diluent was added over night at 4°C. The next day, after washing with TBS-T (5X 10 min), the specific secondary HRP labeled antibodies was added for 1 hour in a 1:10000 dilution. Membranes were thoroughly washed in TBST. The membranes were then incubated with the substrate (Pierce ECL 1:1) and finally exposed to autoradiography film.

6.2.11 Statistical analysis

The statistical significance of the values has been determined by one way ANOVA using GraphPad Prism Version 5.0 (GraphPad software, Inc.). The results were considered significant at $p \leq 0.05$.

6.3 Results

6.3.1 Colonization of *E. coli* Nissle 1917 in mouse C57 BL/6 mouse gut

EcN-pUC18-*gfp* was orally gavaged for 3 consecutive days post antibiotics treatment. At day 9, mice fecal content had approximately 10^3 to 10^4 of EcN-pUC18-*gfp* bacteria (**Figure 6.3**). This indicates that probiotic EcN is capable of colonizing he mouse.

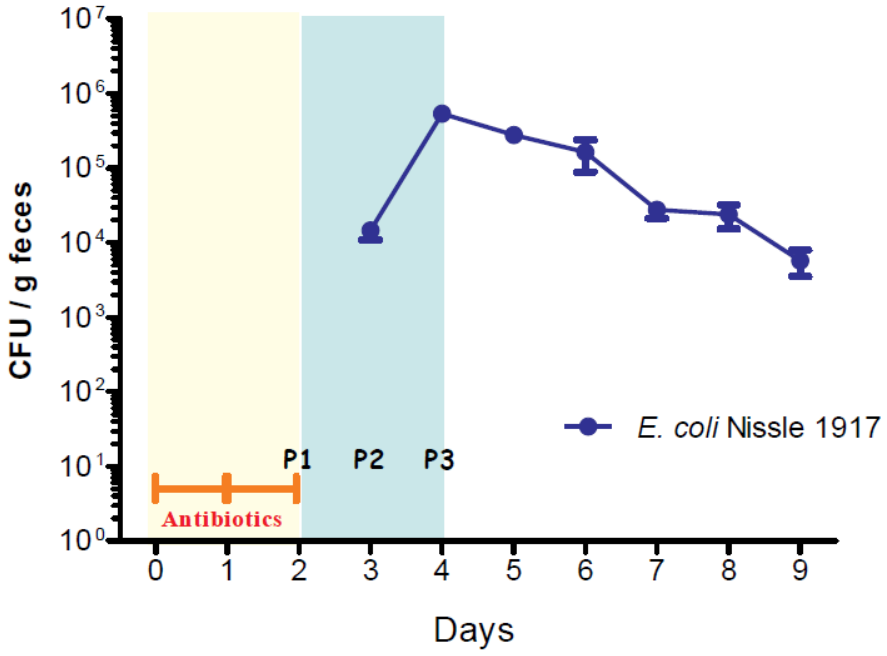

Figure 6.3 Colonization of probiotic EcN in mouse intestine. All values are represented as mean ± SEM (6 animals each group). P1, P2 and P3 represents consecutive probiotic treatment for 3 days.

6.3.2 Evaluating the effect of co- and post-treatment of *E. coli* Nissle 1917 producing antioxidant PQQ in DSS induced experimental colitis

DSS treatment significantly affected the body weight profile. The DSS treated mice exhibited decrease in body weight after day 6 with the minimum weight at day 9. Day 9 was considered here as disease peak which was also evident from DAI scores. As expected, co-treatment of EcN-wt strain significantly ameliorated the DSS phenotype, which is reflected from bodyweight profile and DAI scores. Interestingly, co-treatment of EcN-1 strain producing antioxidant exhibited exaggeration of DSS phenotype. Treated

mice showed significant reduction in body weight profile and increase in DAI score, when compared to only DSS treated (**Figure 6.4 A, B & C**). Moreover, DSS treated mice had shortened colon length, which was significantly increased in mice co-treated with EcN-wt, whereas mice co-treated with EcN-pqq (producing PQQ) exhibited more severe phenotype with more shortened colon length compared to DSS treated group **(Figure 6.5 A, B & C)**. EcN-wt co-treated mice exhibited significantly reduced spleen weight compared to DSS only treated mice. These ameliorative effects of EcN-wt were translated in colonic histology scores. Mice co-treated with EcN-wt had lowered colonic histology scores compared to DSS only and EcN-1 treated mice groups. Altogether, EcN-1 strain producing antioxidant PQQ did not ameliorate DSS induced colitis as seen in EcN-wt, however, EcN-1 co-treatment resulted in disease exacerbation.

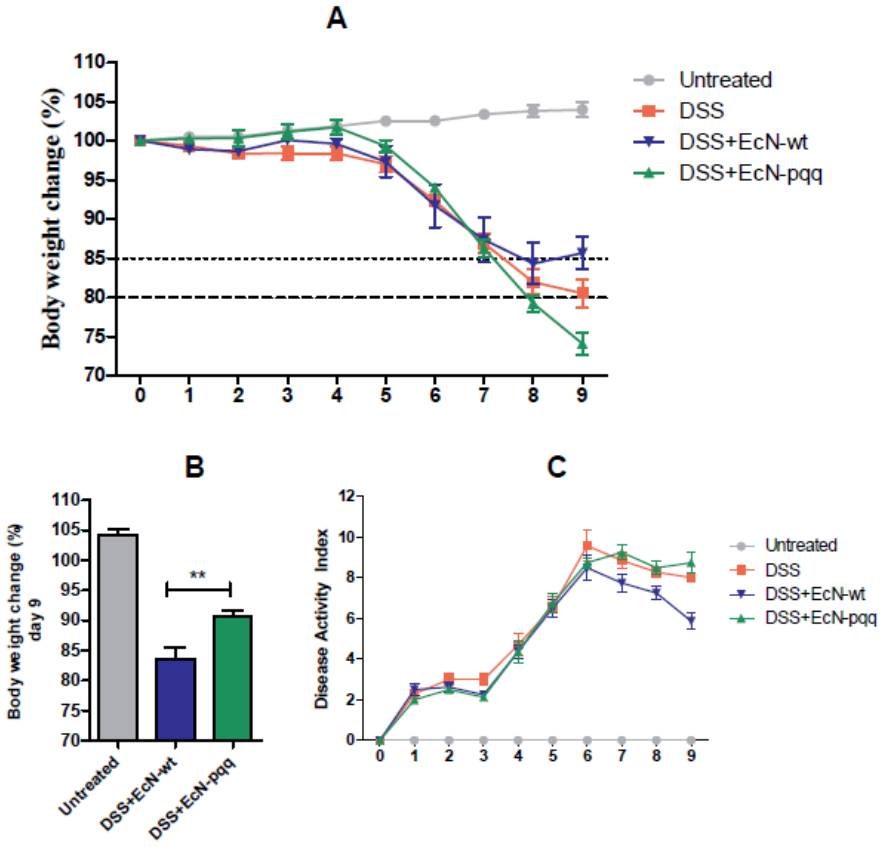

Figure 6.4 Body weight and disease score of mice treated with EcN strains in DSS-induced colitis. **p<0.01 represents significant difference between the groups.

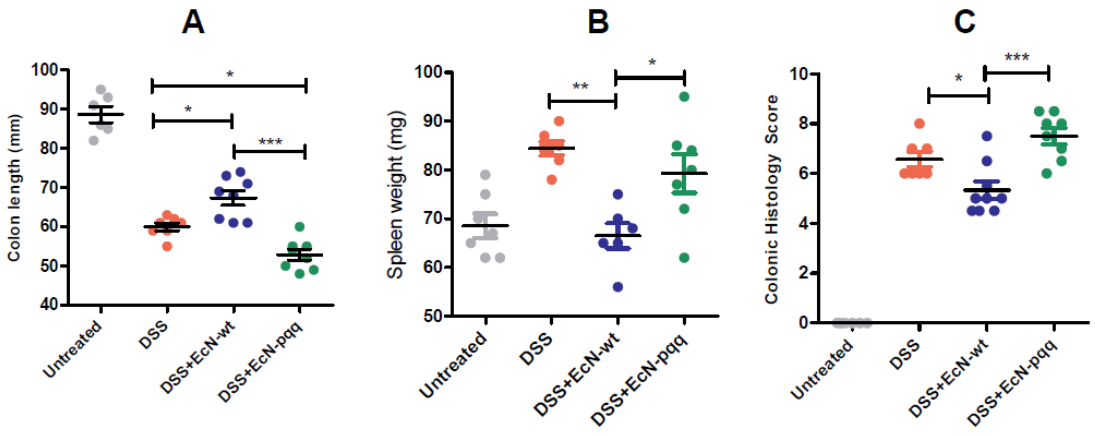

Figure 6.5 Colon length (A), spleen weight (B) and colonic histology scores (C) of mice treated with EcN strains in DSS-induced colitis. All values are expressed as mean ± SEM (8 animals each group). *p<0.05, **p<0.01 and ***p<0.001 represents significant difference between the groups.

Interestingly, when probiotic treatment was given daily starting from recovery phase (day 9 to day 16), Mice treated with EcN-1 (producing antioxidant PQQ) showed significantly increased rate of disease recovery as compared to EcN-wt as evident by body weight change and colon length (**Figure 6.6**).

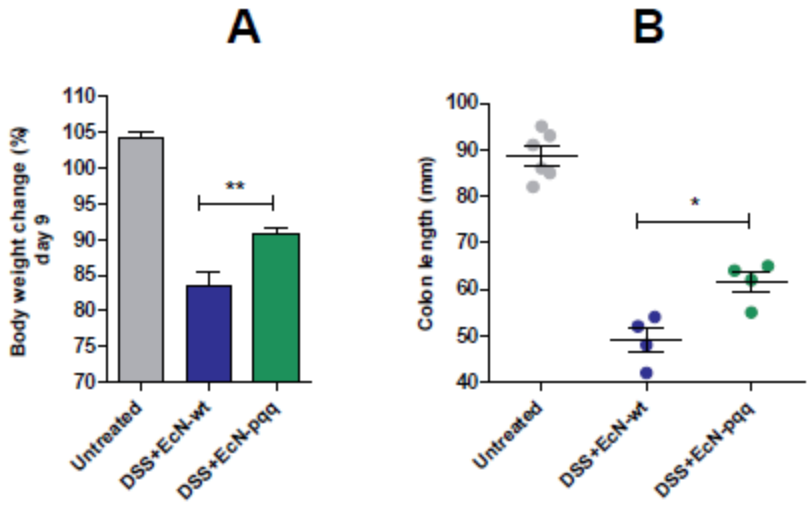

Figure 6.6 Effect of post-treatment of EcN-wt and EcN-1 in DSS induced murine colitis. All values are expressed as mean ± SEM (6-8 animals each group). *p<0.05 and **p<0.01 represents significant difference between the groups.

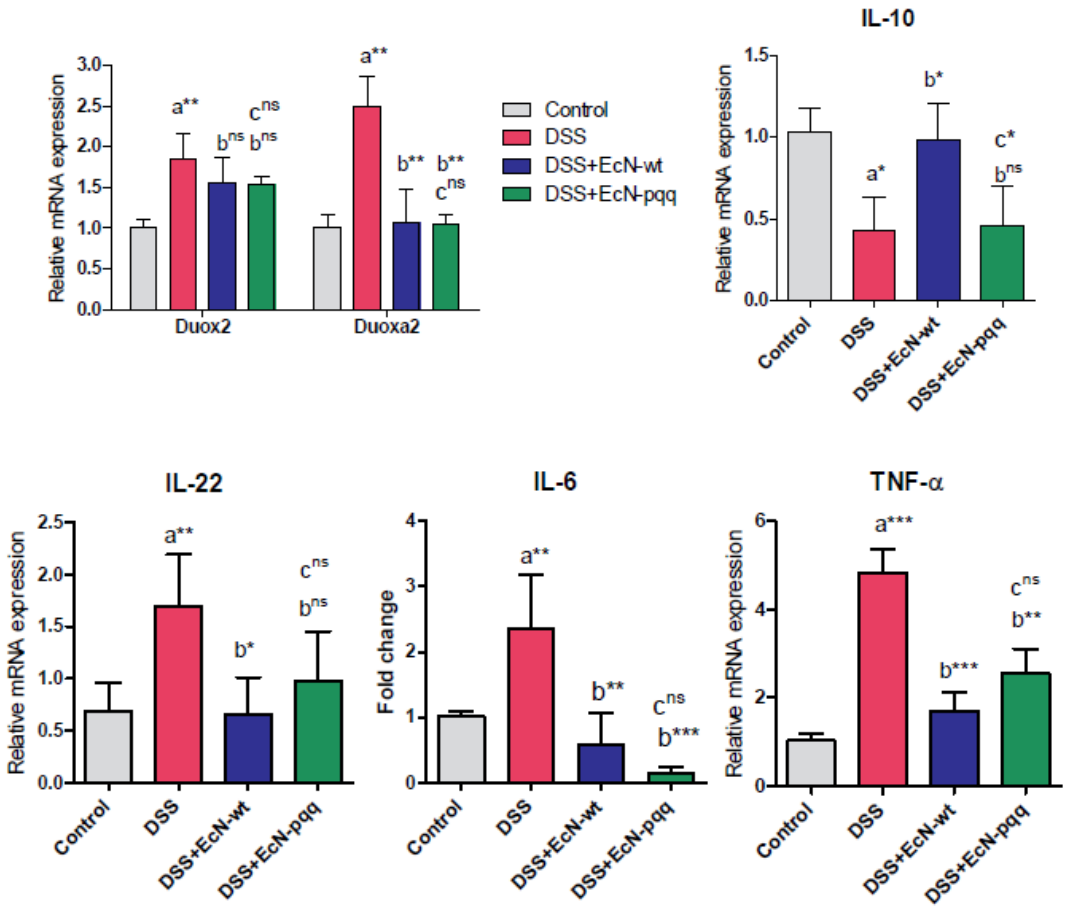

Figure 6.7 mRNA expression of cytokines from colon of mice treated with EcN strains in DS-induced colitis. All values are expressed as mean ± SEM (6 animals each group). *p<0.05, **p<0.01 and ***p<0.001 represents significant difference between the groups. a compares with control, b compares with DSS and c compares with DSS+EcN-wt groups.

DSS induced colitis causes immense inflammation marked by tremendous increase in inflammatory cytokines. As expected DSS treatment induces colonic mRNA expression of IL-6, IL-1β, TNF-α and IL-22 **(Figure 6.7)**. Moreover, it reduces anti-inflammatory cytokines such as IL-10. Treatment of antioxidant (PQQ) producing EcN strain showed significant decrease in IL_6 and TNF-α, but not IL-22. Also, the EcN-pqq treated mice did not showed any restoration of IL-10 expression in colonic tissue. On the other hand, EcN-wt strain was more effective, it not only reduced the levels of IL-6, TNF-α and IL-22, but also significantly increased the levels of IL-10 in treated mice compared to only DSS treated.

Moreover, treatment of DSS also induced expression of Duox2 and Duoxa2 gene expression. Treatment of both the strains did not alter the Duox2 expression, but significantly reduced Duoxa2 expression when compared to only DSS treated mice.

Western blotting DUOX 1&2 proteins with homemade primary antibodies revealed that DSS induced colitis expression in significantly increased in colon **(Figure 6.8)**. EcN-wt and EcN-pqq treatment did not alter the DUOX 1&2 protein expression.

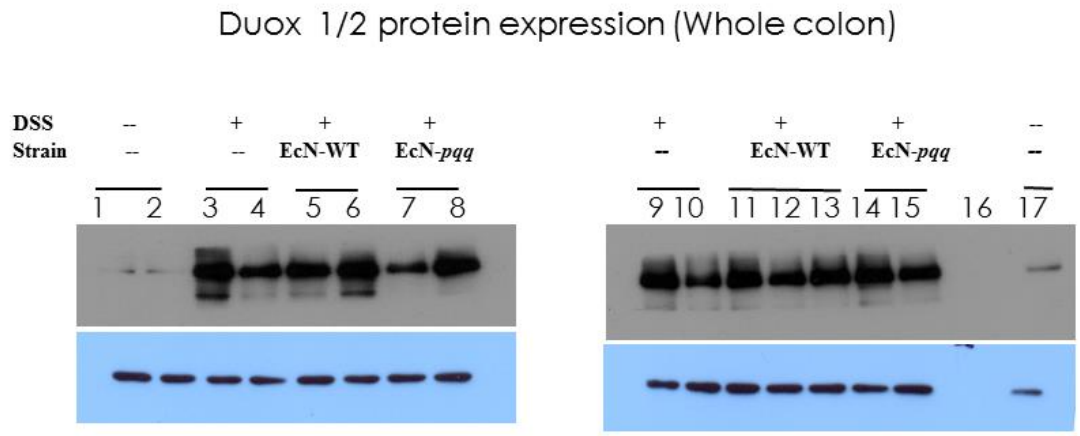

Figure 6.8 DUOX 1&2 protein expression in whole colon of mice treated with EcN strains in DSS-induced colitis. Upper lanes are DUOX proteins and lower lanes represent actin as endogenous controls. 15 µg proteins were loaded per well. Lane 16 in empty for loading control.

6.3.3 Evaluating the effect of co- and post-treatment of *E. coli* Nissle 1917 producing antioxidant PQQ in murine Citrobacter rodentium infection model

Mice treated with EcN-wt showed significant reduction in *C. rodentium* colonization in colon and caecum, and number sin feces at day 7 post infection **(Figure 6.9A, B, C & D)**. *C. rodentium* infection resulted in significantly reduced colon length and increased spleen weight as a result of local inflammation and systemic translocation and infection, respectively **(Figure 6.10A & B)**. Pre- and co-treatment of EcN-wt resulted in decrease of *C. rodentium* numbers in caecum, colon and feces. Also, treatment of EcN-wt

significantly ameliorated changes in colon length and the treated mice had reduced spleen weight as compared to non-treated mice. However, mice treated with PQQ producing EcN did not show any significant reduction in *C. rodentium* load, and no ameliorative effect on colonic inflammation (colon length) and systemic bacterial translocation (spleen weight).

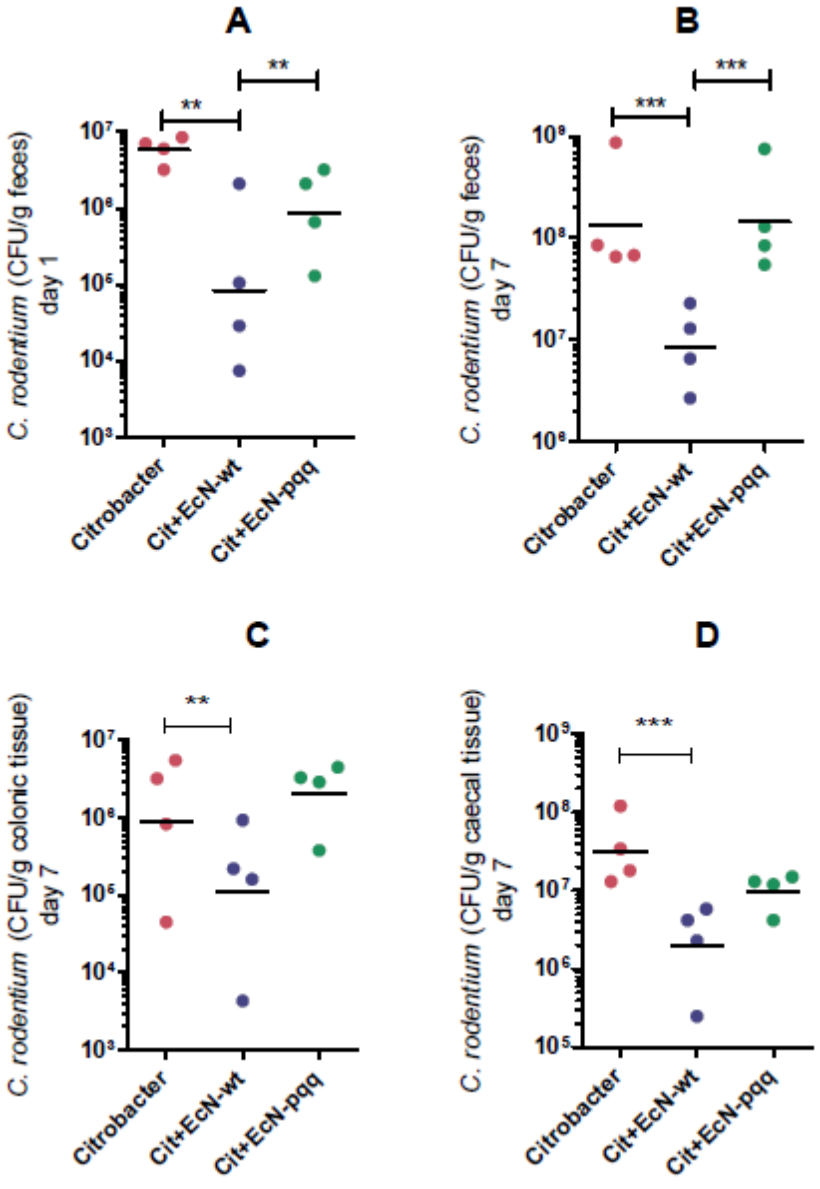

Figure 6.9 *C. rodentium* shedding in feces (A & B) and colonization in colon (C) & Caecum (D) after infection in mice treated with EcN strains. All values are expressed as mean ± SEM (4 animals each group). **p<0.01 and ***p<0.001 represents significant difference between the groups.

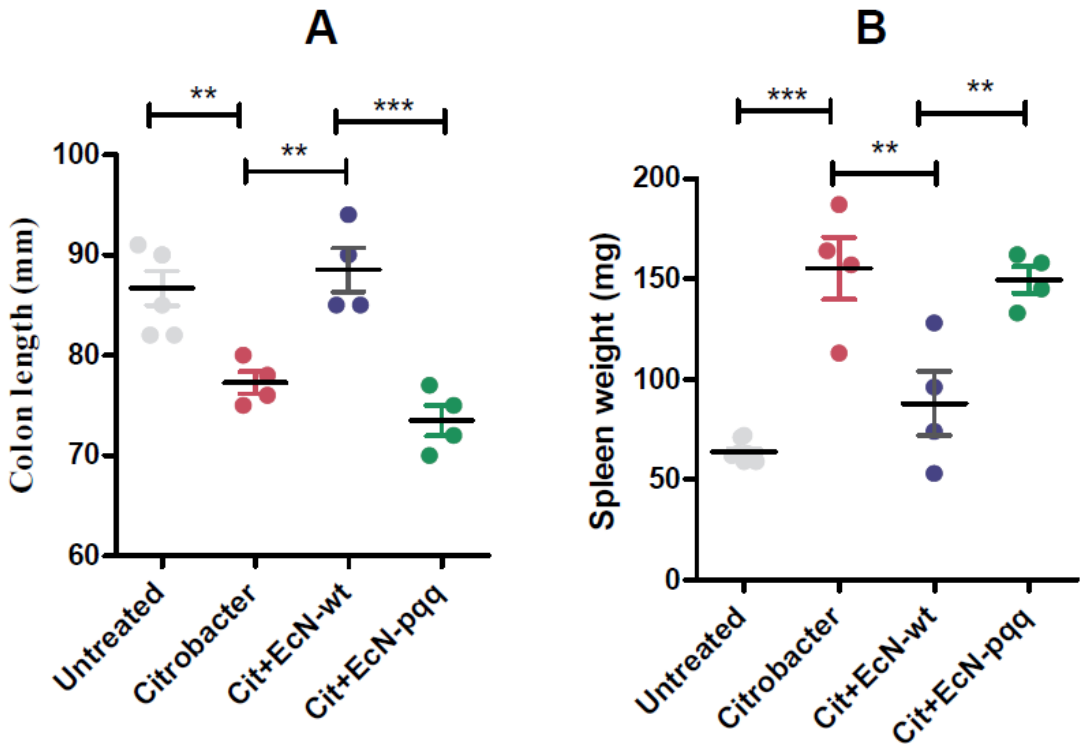

Figure 6.10 Colon length (A) and spleen weight (B) of mice infected with *C. rodentium* and treated with EcN strains. All values are expressed as mean ± SEM (4-6 animals each group). **p<0.01 and ***p<0.001 represents significant difference between the groups.

C. rodentium infection significantly reduced colonic expression of IL-10 and increased expression of IL-6 and TNF-α gene expression **(Figure 6.11)**. Treatment of PQQ secreting EcN-pqq strain did not showed any ameliorative effect on gene expression of IL-10, IL-6 and TNF-α. However, treatment of EcN-wt strain significantly increased IL-10 gene expression and decreased IL-6 and TNF-α genes expression when compared to only *C. rodentium* treated mice.

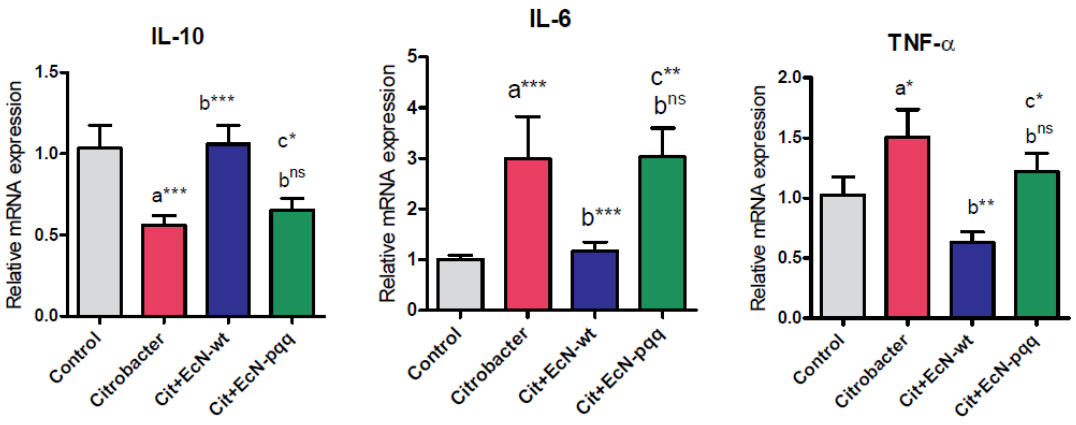

Figure 6.11 mRNA expression of colonic cytokines in mice infected with *C. rodentium* and treated with EcN strains. All values are expressed as mean ± SEM (4 animals each group). **p<0.01 and ***p<0.001 represents significant difference between the groups. *p<0.05, **p<0.01 and ***p<0.001 represents significant difference between the groups. a compares with control, b compares with Citrobacter and c compares with Cit+EcN-wt groups.

6.3.4 In vitro *C. rodentium* growth inhibition by wild type and PQQ producing *E. coli* Nissle 1917

Co-culturing EcN-wt and PQQ producing EcN resulted in significant growth inhibition of *C. rodentium* **(Figure 6.12)**. Supernatant from both strains failed to inhibit *C. rodentium* growth *in vitro.* Moreover, there was no significant difference in growth inhibition by EcN-wt when compared to PQQ producing EcN.

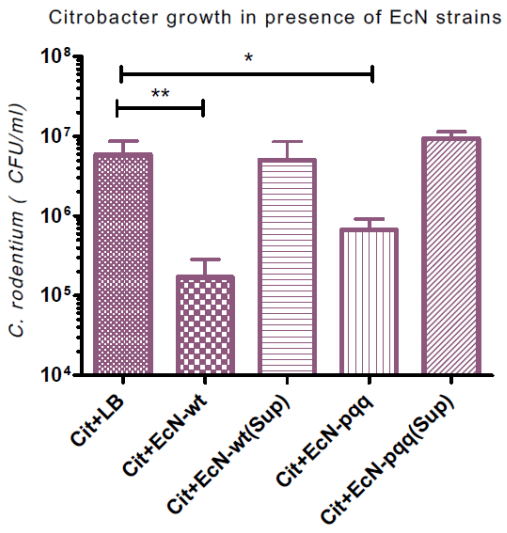

Figure 6.12 *In Vitro C. rodentium* growth inhibition by EcN-wt and EcN-*pqq*. All values are represented as mean ± SD from 3 individual experiments. **p<0.01 represents significant difference between the groups.

6.4 Discussion

The development of colitis is regulated by variety of immune cells along with colonic epithelial cells (Kader and Caser, 2013; Saleh et al., 2011; Matsushita et al., 2013). Inflammation and generation of ROS are prominent in IBD. DSS induced colitis model is the most common murine model used to investigate the pathogenesis of colitis and different factors affecting colitis. Histological and molecular changes seen in mouse DSS-induced colitis model are similar to human Ulcerative colitis and Crohn's disease (Perse and cerar, 2012). Recruitment of immune cells especially neutrophils is one of major factors which results in massive increase in ROS in the colon. Additionally, epithelial cells in colon and ilium also produces significant amount of ROS in form of H_2O_2 by Dual oxidases present in these cells (Rada and Leto, 2008). Colonic biopsies from human IBD patients have also been shown to express higher amount of DUOX protein (Rabinoiwtz, 2015). Both neutrophils (producing superoxide) and gut epithelial cells (producing H_2O_2) are considered as one of the primary innate immune response triggered after stimulation by bacterial ligands in the intestine (Grandvaux et al., 2007; Grasberger, 2010; 2013). But, uncontrolled and chronic production of ROS leads to several deleterious changes at molecular and cellular level which leads to pathologies observed in cases of IBD. Several previous studies have shown the amelioration of colitis using antioxidants (Korkina et al., 2003; Oz et al., 2005; Siddiqui et al., 2006; Sánchez-Fidalgo et al., 2012; Knight et al., 2012; Dashdorj et al., 2013).

The present study was carried out to understand the impact on disease outcome if majority of ROS is quenched by in situ secreted strong antioxidant. Hence, we exploited probiotic *E. coli* Nissle producing PQQ as a vehicle which is capable of producing and secreting PQQ in the colon (described in previous chapters). To our surprise, antioxidant (PQQ) producing EcN co-treatment in DSS-induced colitis model exuberated the disease phenotype (Body weight loss, DAI, colon length, spleen weight, colonic histology and cytokines expression in colon), whereas, wild type EcN ameliorated. However, when PQQ producing EcN was given during the start of recovery phase (day 9 to day 14), treated mice recovered fast as compared to wild type EcN (body

weight & colon length). This is in contradiction to some of the earlier reports demonstration ameliorative effect of antioxidants in DSS colitis model (Korkina et al., 2003; Oz et al., 2005; Siddiqui et al., 2006; Sánchez-Fidalgo et al., 2012; Knight et al., 2012; Dashdorj et al., 2013). We hypothesize that the different outcome in our experimental setup might be because PQQ is very strong antioxidant which may have been resulted in scavenging of wound healing signals mediated via H_2O_2 generated by epithelial cells DUOX-2 in response to inflammation and injury in DSS colitis (Wood, 2012; Bryan et al., 2012; Cordeiro and Jacinto, 2013; Razzell, 2013; De Deken et al., 2014). Whereas, in the case of post-treatment, where ROS and inflammation start diminishing, PQQ plays beneficial role which might be attributed to its mitochondrial biogenesis and cell proliferative functions (Baurley et al., 2011; Harris et al., 2013; Zhang et al., 2014; Zhang et al., 2015). DUOX-2 generated H_2O_2 mediated wound healing signaling is very crucial for wound healing response (Deken et al., 2014) and inhibition of this response could be the reason behind the outcome of present study. Interestingly, when DSS-colitis mice were co-treated with H_2O_2 producing probiotic lactobacilli, the treated mice showed significant amelioration n disease as compared to non H_2O_2 producing isotype (mutant strain) (unpublished lab work). This results strongly supports our hypothesis that DUOX-2 generated H_2O_2 wound healing signaling is important in maintaining cellular homeostasis during injury and inflammation as seen in DSS-colitis model. To understand and investigate the role of ROS (H_2O_2) in colonic pathologies, we used *C. rodentium* infection model. *C. rodentium* is murine pathogen and its pathology in mouse resembles to human EPEC and EHEC pathologies (Collins et al., 2014). We obtained similar results as DSS-induced colitis, wherein EcN wild type strain was found ameliorative whereas PQQ producing strain exuberated the infection or did not showed any protective effect.

Concluding all the findings, it can be stated that redox modulating the intestinal disease by quenching intestinal ROS results in more severe pathology. However, more detailed mouse studies must be carried out in order to understand the role of PQQ and other antioxidants in the pathogenesis of several intestinal disease like IBD and IBS.