1. Introduction

Inflammatory Bowel Disease (IBD) is a multifactorial chronic inflammatory disease of the gastrointestinal tract, characterized by cycles of acute flares, recovery and remission phases. Treatments for accelerating tissue restitution and prolonging remission are scarce, but altering the microbiota composition to promote intestinal homeostasis is considered a safe, economic and promising approach. Although probiotic bacteria have not yet fulfilled fully their promise in clinical trials, understanding the mechanism of how they exert beneficial effects will permit devising improved therapeutic strategies. Here we probe if one of the defining features of lactobacilli, the ability to generate nanomolar H$_2$O$_2$, contributes to their beneficial role in colitis. H$_2$O$_2$ generation by wild type L. johnsonii was modified by either deleting or overexpressing the enzymatic H$_2$O$_2$ source(s) followed by orally administering the bacteria before and during DSS colitis. Boosting luminal H$_2$O$_2$ concentrations within a physiological range accelerated recovery from colitis, while significantly exceeding this H$_2$O$_2$ level triggered bacteraemia. This study supports a role for increasing H$_2$O$_2$ within the physiological range at the epithelial barrier, independently of the enzymatic source and/or delivery mechanism, for inducing recovery and remission in IBD.

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**Keywords:**
- Inflammatory bowel disease
- Lactobacilli
- Hydrogen peroxide
- Mucosal healing
- Tissue restitution

**ABSTRACT**

Inflammatory bowel disease (IBD) is a multifactorial chronic inflammatory disease of the gastrointestinal tract, characterized by cycles of acute flares, recovery and remission phases. Treatments for accelerating tissue restitution and prolonging remission are scarce, but altering the microbiota composition to promote intestinal homeostasis is considered a safe, economic and promising approach. Although probiotic bacteria have not yet fulfilled fully their promise in clinical trials, understanding the mechanism of how they exert beneficial effects will permit devising improved therapeutic strategies. Here we probe if one of the defining features of lactobacilli, the ability to generate nanomolar H$_2$O$_2$, contributes to their beneficial role in colitis. H$_2$O$_2$ generation by wild type L. johnsonii was modified by either deleting or overexpressing the enzymatic H$_2$O$_2$ source(s) followed by orally administering the bacteria before and during DSS colitis. Boosting luminal H$_2$O$_2$ concentrations within a physiological range accelerated recovery from colitis, while significantly exceeding this H$_2$O$_2$ level triggered bacteraemia. This study supports a role for increasing H$_2$O$_2$ within the physiological range at the epithelial barrier, independently of the enzymatic source and/or delivery mechanism, for inducing recovery and remission in IBD.
The perceived redox imbalance in IBD prompted the use of antioxidants to improve or prevent pathology [20]. Antioxidants such as S-adenosyl methionine, green tea polyphenols [21], resveratrol [22], curcumin [23,24], quercetin [25,26], MitoQ [27], pyrroloquinoline quinone (PQQ) [28] and N-acetyl cysteine (NAC) [29–32] have been used to ameliorate colitis in murine models. A short trial with UC patients showed improved response and remission rates in patient groups receiving a combination of mesalamine and NAC, as compared to an UC patient group receiving mesalamine and placebo [33]. However, a proper conclusion cannot be drawn due to the small group size and age heterogeneity in both groups. Treatment with antioxidants such as Vitamin E, Vitamin C, Vitamin C, fish oil or β-carotene reduced oxidative damage markers, but disease activity remained unaltered in IBD patients [34–36]. However, there is very limited information on the efficacy of antioxidants in clinical trials with IBD patients [20].

Probiotic strains have also been used as therapeutic intervention in experimental colitis and human clinical trials. Lactobacilli and bifidobacteria are the main probiotic strains and a non-exhaustive list of their effects in murine colitis models (TNBS, DSS) is summarized by Martin and colleagues [37]. Various Lactobacillus strains, bifidobacteria, combination products (VSL3), Bacillus [38,39], Escherichia coli Nissle 1917 [40,41] and Saccharomyces [42–44] have shown benefit in colitis models to a varying degree. In clinical trials lactobacilli and bifidobacteria were moderately effective in ameliorating UC symptoms, while in CD only synbiotics proved beneficial [45]. Certain probiotics seem to improve clinical symptoms in other IBD associated pathologies such as cholangitis and pouchitis. It is often difficult to compare reports utilizing probiotic bacteria as strains, preparation and their dispensation differ across studies.

Physiological benefits linked to lactobacilli in the intestine include strengthening of epithelial junction complexes, elevating the antioxidant status, decreasing pro-inflammatory cytokines, increasing anti-inflammatory cytokine release, and modulating regulatory T cells and macrophages [46–53], but the underlying molecular mechanisms conveying these benefits in gut inflammation remain unknown. The host-protective role of lactobacilli was apparent in mice with Nox1–4 inactivation in the intestinal epithelium, which overcompensated the loss of epithelial ROS by significantly increasing the abundance of lactobacilli throughout the intestine. Extensive colonization by L. reuteri and L. murinus protected these mice from Citrobacter rodentium and Listeria monocytogenes infection by increasing colonization resistance and downregulating the locus of enterocyte effacement (LEE) pathogenicity island [17]. This study connected host protection in infection conclusively with lactobacilli-derived H2O2 by utilizing wild type L. johnsonii and a deletion strain (L. johnsonii Δnfr) characterized by markedly reduced H2O2 production. In light of these results and the increased risk of children with loss-of-function NOX1 or DUOX2 variants for developing pancolitis at an early age, sufficient H2O2 production at the barrier is likely required for intestinal homeostasis. Here, we probed the role of H2O2 generated in the intestinal lumen in DSS-induced colitis by utilizing L. johnsonii strains with varying capacity for H2O2 generation.

2. Material and methods

2.1. Mice

C57Bl6/J (Jackson Lab., USA) wild type mice were used for colitis models with antioxidant administration, while C57Bl6/N (Taconic, Germany) wild type mice between 6 and 8 weeks of age were used for all Lactobacillus colitis studies. Both mouse strains were kept in IVC cages in a specific pathogen free (SPF) facility with maintained temperature (21 °C), humidity (55%), 12 h light/dark cycle and were continuously bred in the same room of the facility. They received ad libitum irradiated chow (Teklad Global) and sterilized filtered water (0.2 μm filter). Experimental groups were randomly assigned by combining age and gender matched littermates of several in-house breeders. Male and female mice were used. All animal experiments were carried out in compliance with EU Directive 86/609/EEC, were approved by the Institutional Animal Research Ethics Committee and were authorized by Irish Regulatory Authority.

2.2. Lactobacillus strains and culture conditions

Lactobacillus johnsonii NCC533, here denoted as wild type (WT), obtained from Nestec culture collections was cultured in MRS media (Oxoid, Thermo Scientific) under static/anaerobic conditions at 37 °C. L. johnsonii deletion strain NCC9360 (Δnfr, Δnox), here designated DEL, was constructed using strain NCC9359 [58]. Supplementary Methods contain detailed information on strain construction. To construct the nfr overexpression strain plasmid pDP1019 [54] was used to transform wild type NCC533 strain. The nfr overexpressing strain is designated OE. Strains DEL and OE were grown in MRS media supplemented with 5 µg/ml erythromycin and 5 µg/ml chloramphenicol respectively. See Fig. S2A, Table S2 and S3 for additional information.

2.3. Lactobacilli colonization

Intestinal colonization by lactobacilli was analysed by plating on selective MRS media. Briefly, forestomach content, cecal content and feces were suspended in sterile Dulbecco’s Phosphate-Buffered Saline (Thermo Fischer, Ireland) and plated on MRS agar plates after serial dilutions and incubated at 37 °C for 12 h in anaerobic conditions. Next day, colonies (colony forming units, CFU) were counted and represented as CFU per unit weight of the content. Presence of bacteria in blood was analysed by plating mouse serum on Luria-Bertani agar plates (Thermo Scientific, Ireland) incubated at 21% O2 / 37 °C. Next day, CFU were counted and represented as CFU per ml of serum.

2.4. Hydrogen peroxide detection

Lactobacillus strains were grown in LAPtg medium (20 g/l glucose, 10 g/l yeast extract, 10 g/l Bacto Peptone, 10 g/l Bacto Tryptone and 1 g/l Tween 80). H2O2 was determined in the supernatant of bacteria grown at different conditions as follows. Aerobic: Bacteria were grown in LAPtg medium at 37 °C for 24 h in the presence of 21% oxygen (shaking 200 rpm). Anaerobic: Bacteria were grown in LAPtg medium at 37 °C for 24 h in the absence of oxygen (15 ml fully filled pre-saturated tubes incubated in anaerobic chamber). Microaerophilic: Bacteria were grown in LAPtg medium pre-saturated with 3% O2 and incubated at 37 °C for 24 h in a growth chamber maintaining 3% O2. To determine the H2O2 production by varying bacterial cell numbers (1011–1013cells), overnight growing lactobacilli were split and suspended in volume/cell number adjusted pre-warmed LAPtg media followed by 1 h incubation at 37 °C and 200 rpm (21% O2). Amplex UltraRed reagent (Molecular Probes, A36006) was used for H2O2 measurements as per manufacturer’s protocol and related to an H2O2 standard curve. Fluorescence was recorded using a BioTek Synergy plate reader (λex = 530 nm ± 9; λem = 590 nm ± 9; sensitivity 60%). Before recording fluorescence, 0.5 µl of 25 U/ml catalase (C40 from bovine liver; Sigma, Ireland) was added to control wells when indicated.

2.5. Analysis of lipid peroxidation

Malondialdehyde (MDA) was analysed in colonic tissue lysate as a marker for lipid peroxidation as described earlier [55]. Briefly, colonic tissue was homogenized in ice cold 1.15% potassium chloride (Sigma, Ireland). 100 µl of homogenate was mixed with 2-thiobarbituric acid (TBA) reagent containing 15% trichloroacetic acid (Thermo Fischer, Ireland) and 0.8% TBA (Sigma, Ireland) in 0.25 N hydrochloric acid solution. The mixture was incubated for 20 min at 95 °C. The cooled mixture was centrifuged at 3000 g for 10 min. Absorbance of the supernatant was measured at 532 nm against reagent control; results are
presented in arbitrary values.

2.6. Dextran Sodium Sulfate (DSS) induced colitis

Before starting the DSS treatment, mice on experiment were conditioned for 14 days in their cages. DSS (40 kDa, TdB consultancy, Sweden) was dissolved in sterile filter water. 2.5% or 3% DSS was given to mice for 6 consecutive days followed by sterile filter water up to days 9, 11 or 16 as indicated. DSS was prepared fresh at day 3.

2.7. Treatments

2.7.1. Antioxidant treatment

N-acetyl cysteine (NAC; Sigma, Ireland) (1 g/100 ml, adjusted to pH 7.0) and pyrroloquinoline quinone (PQQ; World-way-biotech, China) (0.4 mg/100 ml) were supplied in drinking water with or without DSS (3%). After removal of the DSS solution at day 6, mice were supplied with sterile water containing antioxidants. Untreated mice received sterile water throughout.

2.7.2. Lactobacillus administration

*Lactobacillus* strains grown in MRS media (with or without antibiotics), washed with PBS and re-suspended in PBS were orally gavaged once/day for 7 consecutive days before starting the DSS treatment. Oral gavage of indicated lactobacilli was continued daily until experiments were terminated (11 days, 16 days). Lactobacilli were administered at varying cell number. Regular dose consisted of $10^9$ CFU/mouse/day (denoted as $1 \times$), high dose consisted of $10^{10}$ CFU/mouse/day (denoted as $10 \times$ ) and low dose consisted of $10^8$ CFU/mouse/day (denoted as $0.1 \times$ ) (See Fig. S2). Control mice (untreated, DSS only) received daily gavage of PBS instead of bacteria.

2.8. Disease Activity Index (DAI)

DAI was calculated for each mouse daily. A 12 point scoring system was designed based on earlier publication [56]. Body weight loss, fecal consistency and visible blood in feces were collectively scored as DAI. See Table S1 for detailed information.

2.9. Clinical score

In order to score general health of mice, a 9 point health scoring system was designed and approved by the designated Veterinarian. Body weight loss, fecal consistency, behavior and appearance were collectively plotted as clinical score. See Table S1 for detailed information.

2.10. Colon histology and scoring

Mouse colons were fixed in neutral buffered formalin for 48 h and embedded in paraffin. 5-micron thick sections were stained with Hematoxylin & Eosin as per standard procedures. Sections were visualized with a transmission light microscope (Nikon E80i), images were captured with a $10 \times$ objective and analysed by blind scoring. An arbitrary combined score from severity of inflammatory cell infiltration to extent of injury and crypt damage was generated using a 12 point scoring system adapted from Saunders and colleagues [56]. This system accounts for mild to moderate histopathological findings of colitis in mouse colon.

2.11. Statistical analysis

Results are shown as mean ± SEM for in vivo studies, and mean ± SD for in vitro studies. Analysis was performed using one-way ANOVA with Bonferroni’s multiple comparison test using GraphPad PRISM® (La Jolla, CA, USA). The Mann-Whitney non-parametric test was used for DAI, clinical score and histological scores. Survival curves were generated using Kaplan-Meir and analysed by Log-rank (Mantel-Cox) test. $P$ value < 0.05 was considered as significant.

3. Results

3.1. Oral antioxidants do not prevent DSS-induced colitis in mice

Several studies used NAC (150 mg/kg in water [29]; 40 mM in water [30]; intrarectal [31,32]; intraperitoneal [57]) in various rodent colitis models and observed moderate protection, while PQQ has never been studied in dextran sodium sulfate (DSS) or other common colitis models. DSS is a sulphated polysaccharide that causes disruption of tight junctions and increases gut permeability (leaky gut), resulting in an increased inflammatory response to the translocated microbiota. As a result, a cascade of inflammatory reactions occurs involving ROS, Hsp27, NF-kB and interleukins [58–62]. The DSS model is mainly used to study epithelial barrier function, innate immune responses, and the influence of dysbiosis on the host response. To assess whether a high dose of oral antioxidants NAC or PQQ can ameliorate acute colitis, wild type mice were exposed to 3% DSS with or without NAC or PQQ in the drinking water for 6 days. Oral compound uptake in drinking water can only be estimated; in our conditions a high dose of approximately 60 mg NAC and 0.024 mg PQQ per day per mouse was administered (intake -6 ml water/day) [63]. After removal of DSS at day 6, mice continued to receive either water alone or water supplemented with NAC or PQQ until day 8 to evaluate the early recovery phase (Fig. S1A).

As expected DSS treatment resulted in significant body weight loss (Fig. S1B), increased disease activity (Fig. S1C), reduced colon length due to inflammation (Fig. S1D) and damaged colon crypts (Fig. S1E, F). Supplementation of drinking water with high dose NAC or PQQ did not prevent colitis, but rather exacerbated disease, leading to increased body weight loss and heightened inflammation (Fig. S1B, D). The body weight loss was so pronounced that the experimental endpoint (≥ 20% body weight loss) was reached at day 8 for NAC (3 of 8 mice) and PQQ (4 of 8 mice). Exogenous antioxidants at high concentrations may not only impede redox signalling, but may act as pro-oxidants and cause oxidative damage [64]. In order to determine any underlying harmful effect of NAC and PQQ administration at indicated doses, we subjected mice to the same concentration of NAC or PQQ in drinking water for 9 days without inducing colitis. No adverse effect of these treatments was detected (Fig. S1G-I). Thus, altering the luminal intestinal environment and epithelial redox signalling with high concentrations of oral redox active compounds did not protect wild type mice from DSS colitis, but rather exacerbated disease.

3.2. Creation of a *Lactobacillus johnsonii* double deletion mutant with abolished H$_2$O$_2$ production

Altering the commensal communities in the intestine can have beneficial or detrimental effects. The beneficial effects of increasing probiotic species in the intestine is promoted for its health benefit in various disorders. Recommended probiotic strains are lactobacilli, bifidobacteria, *E. coli* Nissle and certain Clostridia species. Lactobacilli and certain bifidobacteria generate H$_2$O$_2$ in the presence of molecular oxygen, but the bacterial enzymes responsible for H$_2$O$_2$ production are largely uncharacterized. NADH-dependent flavin reductase (nfr) is the primary source of H$_2$O$_2$ in *L. johnsonii* NCC533, and deletion of this gene reduces H$_2$O$_2$ production in the presence of oxygen [54]. Upon genome-wide transcriptional response analysis of *L. johnsonii* to oxygen exposure, one of the highest up-regulated genes displayed strong homology to NADH oxidoreductases (*nax* locus) (data not shown). A double deletion mutant (∆*nfr* ∆*nox*) was generated to abolish H$_2$O$_2$ production in this strain. *L. johnsonii* double deletion mutant (∆*nfr* ∆*nox*) is herein denoted as DEL (Fig. S2A). Deletion of *nfr* and *nax* locus genes (DEL) led to significantly diminished H$_2$O$_2$ production.
we generated also H2O2 overproducing nfr (not shown). Complementation of the DEL strain with L. johnsonii NCC9359 Δnfr (63.8 ± 3.2 nmol H2O2/OD) (not shown). Complementation of the DEL strain with nfr recovered H2O2 production [58]. We generated also H2O2 overproducing lactobacilli by transforming L. johnsonii WT with a plasmid harbouring the nfr locus [54]. Overexpression of nfr (OE) in L. johnsonii WT increased H2O2 output 2-fold. Microaerophilic conditions reduced H2O2 production by the WT or OE strains by approximately 50% to 107.5 ± 17.5 nmol H2O2/OD (WT) and 258.0 ± 44.6 nmol H2O2/OD (OE) (Fig. 1B), while in anaerobic conditions H2O2 production was not detected in lactobacilli (Fig. 1C). The ability of lactobacilli to generate H2O2 in the intestine, albeit at reduced levels, is preserved by the oxygen gradient radiating from the host epithelium into the lumen. Estimates of physiological oxygenation in the loose mucus layer, the site of Lactobacillus colonization in the colon, range from 1 to 10 mm Hg [65] or 2-3% O2 [66]. Deletion of both, nfr and nox, decreased fitness of the bacteria (See growth rates Fig. S2B, C), therefore all L. johnsonii strains were grown in anaerobic conditions and adjusted by OD before use in colitis experiments.

3.3. Luminal H2O2 production by L. johnsonii accelerates recovery and restitution in DSS colitis

We reported recently Lactobacillus-mediated protection of mice in Citrobacter rodentium infection and conclusively associated this phenotype to H2O2 generation by lactobacilli [17]. To investigate the role of Lactobacillus-derived H2O2 in DSS colitis, mice were subjected to 2.5% DSS in drinking water for 6 days followed by sterile water until day 11 or day 16. Additionally, groups of mice received daily 109 CFU/day of L. johnsonii (WT), of the deletion mutant (DEL) or PBS by oral gavage for 7 days as prophylactic pre-treatment, which was then continued until the end of the experiment (Fig. 2A). While we did not observe apparent changes in the acute colitis phase, mice supplemented with H2O2-generating L. johnsonii WT recovered more rapidly with a significant improvement in body weight and disease index when compared to the DSS only group (Fig. 2B–E), while colonization with L. johnsonii DEL (no H2O2) provided no benefit. Accordingly, other inflammatory parameters such as colon length and spleen weight showed pronounced recovery towards the healthy physiological state when L. johnsonii WT was administered, but not when L. johnsonii DEL was used (Fig. 2F, G).

Histologically the colon of L. johnsonii WT treated mice resembled the crypt architecture of untreated mice gavaged with PBS (Fig. 2H, I). These results indicate that the production of H2O2 by lactobacilli improves significantly mucosal healing and restoration of tissue architecture in the recovery phase of colitis.

3.4. Optimal concentration of H2O2 is essential for accelerated recovery from colitis

Next, we asked whether enhancing production of H2O2 significantly above physiological levels will improve similarly, or even to a higher extent, the course of disease. To address this we used the L. johnsonii OE strain with increased H2O2 production (Fig. 1A, B). To produce increased H2O2 levels in the intestine, we administered orally either L. johnsonii WT at a 10-fold higher dose (1010 CFU/day; 10 × ) or the H2O2 overproducing strain (L. johnsonii OE) at normal dose (109 CFU/ day; 1 × ), and as control the DEL strain at high dose (1010 CFU/day; 10 × ) without inducing DSS colitis. To determine H2O2 production of these strains the conditions were adjusted for differences in end product accumulation and shortened to avoid proliferation. H2O2 generation of L. johnsonii WT 1 × (43.6 ± 3.4 nmol H2O2/h) was comparable to L. johnsonii OE 0.1 × (37.8 ± 9.0 nmol H2O2/h), while WT 10 × produced 35–40% more H2O2 than the OE strain at 1 × (Fig. 3A).

Colonization of mice with L. johnsonii WT (10 × ) or OE (1 × ) resulted in deterioration of their health at day 3 (Fig. 3B). We observed reduced mobility and alertness with symptoms of severe dehydration and all WT and OE mice were sacrificed between day 4 and 5 due to welfare considerations (Fig. 3C). In contrast, no mortality was observed in mice supplemented with L. johnsonii DEL (10 × ). As the symptoms accompanying mortality resembled generalized sepsis, blood was collected at the endpoint and bacteria in serum were enumerated by serial dilution. A significant number of bacterial colonies was detected in blood derived from L. johnsonii OE (1 × ) and WT (10 × ) treated mice, respectively (Fig. 3D), while no bacteria were found in blood collected from PBS treated or L. johnsonii DEL (10 × ) treated mice. To ensure efficient colonization was achieved, the abundance of total lactobacilli in feces, cecum and forestomach was determined. Colonization with all bacterial strains was achieved, albeit not uniformly as the colonization or/and fitness of the OE strain seemed reduced (Fig. 3E). The presence of significant Lactobacillus colonies in feces, cecum and forestomach of mice treated with the DEL strain did not cause adverse effects on health and survival, strongly suggesting that if H2O2 production exceeds a certain physiological range adverse effects can occur in mice.

To further establish that an optimal concentration of H2O2 is
beneficial in the colitis recovery phase, we supplemented mice before and during DSS exposure with a 10-fold lower dose of the \textit{L. johnsonii} OE strain (10^8 CFU/day, 0.1 ×; day −7 to day 11). No mortality or changes in clinical scores were observed until day 0 (data not shown), but similarly to \textit{L. johnsonii} WT (1 ×; Fig. 2) recovery and restitution was accelerated. A significant increase in body weight and colon length, a decreased disease index and improved colon histology demonstrated that the \textit{L. johnsonii} OE strain will improve tissue restitution and mucosal healing when the appropriate number of bacteria is administered (Fig. 4A–D). Thus, the health benefits of \textit{L. johnsonii} during the recovery from colitis are attributable to bacterially produced H2O2 generation.

4. Discussion

Current therapy for IBD comprises of anti-inflammatory compounds (e.g. mesalamine, corticosteroids), immunosuppressive agents (e.g. azathioprine), and biologics (e.g. anti-TNFα antibody) [67–69]. However, there is still a high unmet need for new and/or adjuvant IBD
therapies as response rates differ or patients lose response, and often severe adverse effects are associated with prolonged treatment. Microbiota modifiers and therapeutic approaches that focus on mucosal healing are promising avenues for prolonging remission. Probiotics, prebiotics, synbiotics and bacteria genetically modified to produce compounds beneficial for intestinal health are suitable approaches to address this opportunity, but improved knowledge of their mechanism of action will be necessary.

This study together with our recent report on Lactobacillus overgrowth in Nox1–4 inactivated mice (Cyba\textsuperscript{Tie-cm}) highlights the importance of bacterial-derived H\textsubscript{2}O\textsubscript{2} at the intestinal barrier and reveals that intestinal homeostasis is maintained and even improved by H\textsubscript{2}O\textsubscript{2} as long as concentrations do not exceed a certain physiological range. Although Lactobacillus species can produce antimicrobial peptides, bacteriocins, and several organic compounds, releasing H\textsubscript{2}O\textsubscript{2} seems to be central for antimicrobial and restorative processes. Adaptive overgrowth of lactobacilli, which accompanied epithelial NADPH oxidase inactivation [17], as well as supplementation of wild type hosts with up to 10\textsuperscript{9} lactobacilli per day was associated with protective and healing responses. In contrast, a 10-fold increase in bacterial cell numbers (10\textsuperscript{10}) or genetic manipulation to increase H\textsubscript{2}O\textsubscript{2} output from 10\textsuperscript{9} bacteria caused bacteraemia and led to high mortality in wild type mice.

Histopathology of the intestine in deceased mice did not reveal changes in crypt architecture, overt oxidative damage, or apparent remodelling.

The connection of ROS and lactobacilli is more complicated than often appreciated in the literature. A reported beneficial effect of lactobacilli was lowering oxidative tissue damage [2], but L. rhamnosus GG was reported to stimulate NOX1-mediated superoxide production by an unidentified mechanism in vivo [70]. Notably, all lactobacilli can generate continuously H\textsubscript{2}O\textsubscript{2} in concentrations comparable or even higher than the regulated H\textsubscript{2}O\textsubscript{2} production by the epithelial NOX1 or DUOX2 oxidases [17,54,71–73]. Moreover, L. reuteri-mediated increased mucus thickness and protection in DSS colitis [74] may be linked to H\textsubscript{2}O\textsubscript{2} production, which plays a role in goblet cell function [15,75]. Thus, many of the observed effects of lactobacilli might be caused by altering host epithelial signalling via oxidation of thiols after aquaporin-mediated entry of H\textsubscript{2}O\textsubscript{2} into intestinal epithelial cells [76], which can then positively influence host responses via redox sensitive pathways such as decreasing the inflammatory response, limiting and repairing tissue damage, fine tuning cell division and restricting intracellular pathogen viability [77–82]. Intracellular signalling mediators regulated by epithelial NADPH oxidases include PTEN and PTP-PEST phosphatases, certain kinases (e.g. ASK1, SRC), NF-\kappa B and NRF2, but which of these pathways (or others) may be predominantly triggered by lactobacilli-generated H\textsubscript{2}O\textsubscript{2} is unknown.

Lactobacilli are recognized for their ability to strengthen epithelial junctions, but in cell-based studies exposure of epithelial cells to excess H\textsubscript{2}O\textsubscript{2} led to junction protein reorganization and altered redox signalling, thereby increasing permeability. Alternatively, limited cytotoxicity affecting single epithelial cells or delayed repair after expulsion of senescent single cells may occur [83,84]. In both cases the barrier function will be compromised and bacterial translocation will take place as response rates differ or patients loose response, and often severe adverse effects are associated with prolonged treatment. Microbiota modifiers and therapeutic approaches that focus on mucosal healing are promising avenues for prolonging remission. Probiotics, prebiotics, synbiotics and bacteria genetically modified to produce compounds beneficial for intestinal health are suitable approaches to address this opportunity, but improved knowledge of their mechanism of action will be necessary.

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place as observed in this study. In contrast to humans, lactobacilli colonize in large numbers \((10^8 - 10^9 \text{ CFU/g tissue})\) [85] the squamous gastric epithelium of rodents, form biofilms and participate in food digestion. Although \(L.\ johnsonii\) NCC533 is a human gut isolate and did not co-evolve with the rodent host, stable colonization of \(10^7 \text{ CFU/g for 1–3 days}\) was detected in mice [86], suggesting that Lactobacillus translocation could even originate from the gastric compartment. Autotrophic lactobacilli are less prevalent in the human gut, but the increased use of probiotics even in severely ill and immunocompromised patients has increased the number of Lactobacillus-induced bacteraemia case reports [87–90]. \(L.\ rhamnosus\) and \(L.\ acidophilus\), both common ingredients of probiotic treatments, were the main species detected in patients’ organs or blood. Bacteraemia with extreme fever and/or liver abscess occurred mainly in patients with underlying medical conditions such as immunosuppression, diabetes mellitus, \(C.\ difficile\) infection, short gut syndrome, or severe ulcerative colitis [87–89,91,92] and was manageable with antibiotic therapy. A greater awareness of potential side effects of excessive use of probiotics seems warranted.

Physiological H\(_2\)O\(_2\) levels in the GIT are likely in the nanomolar range when output by epithelial cells (mostly NOX1 and DUOX2) is combined with H\(_2\)O\(_2\) generated by lactobacilli and certain strains of bifidobacteria and streptococci colonizing the mucus layer [71,72,93,94]. The availability of molecular oxygen will be a limiting factor for H\(_2\)O\(_2\) production independently of the enzymatic source. Intestinal brush border and mucus layer oxygenation depends on blood flow, the distance to the host epithelium and disease activity [65]. Accumulation and activation of neutrophils in the acute phase of colitis will reduce the oxygen concentration temporarily to hypoxic conditions as the oxidative burst by activated phagocytes will reduce oxygen availability considerably. Probiotic bacteria will not be able to generate H\(_2\)O\(_2\) in these conditions, not only due to lack of oxygen, but also as active inflammation triggers changes in mucus quantity and quality (particularly in UC), thereby decreasing mucus-associated attachment sites required for colonization. In accord, supplementation with \(L.\ johnsonii\) WT had no effect on the acute inflammatory phase, but the bacteria accelerated recovery and tissue restitution later in disease when H\(_2\)O\(_2\) production was regained by increased oxygen availability. Similar effects on recovery were reported for Ultrabiotique®, a mixture of four probiotics [95], suggesting that H\(_2\)O\(_2\) promotes by a yet unknown mechanism mucosal healing and tissue restitution during the colitis recovery phase. A universal role of nanomolar H\(_2\)O\(_2\) in the healing process, independently of its enzymatic source, is further supported by reports linking NOX1 and DUOX to intestinal and pulmonary mucosal wound repair [96–99].

5. Conclusions

The impact of epithelial H\(_2\)O\(_2\) on development and progression of colitis requires re-evaluation. The enzymatic ROS source including the timing, location, chemistry and concentration of the arising reactive species will determine beneficial or detrimental outcomes in inflammation. Preserving and boosting intestinal H\(_2\)O\(_2\) accelerates tissue restitution and increases the colonization resistance of the microbiota.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2016.02.003.

References

[1] D.C. Baumgart, W.J. Sandborn, Inflammatory bowel disease: clinical aspects and established and evolving therapies, Lancet 369 (2007) 1641–1657, http://dx.doi.org/10.1016/S0140-6736(07)60579-X.

[2] E. Distritti, L. Monaldi, P. Ricci, S. Fiorucci, Gut microbiota role in irritable bowel syndrome: new therapeutic strategies, World J. Gastroenterol. 22 (2016) 2219–2241, http://dx.doi.org/10.3748/wjg.v22.i17.2219.

[3] P. Biai, G. Leonardiuzzi, P.L. Oretti, G. Poli, Inflammatoty Bowel Disease: Mechanisms, Redox Considerations, and Therapeutic Targets, Mary Ann Liebert, Inc, 2013, http://dx.doi.org/10.1089/2012.4530.

[4] A. Bhattacharyya, R. Chattopadhyay, S. Mitra, C. Owyang, J.Y. Kao, J. Brault, M.J. Stasia, X.J. Li, Genetics and immunopathology of chronic granulomatous disease, Blood 116 (2010) 1570–1581, http://dx.doi.org/10.1182/blood-2010-01-264218.

[5] M. Barbato, G. Ragusa, F. Civitelli, A. Marcheggiano, G. Di Nardo, M. Iacobini, A. Rezaie, R.D. Parker, M. Abdollahi, Oxidative Stress and pathogenesis of inflammatory bowel disease in CGD reproduces the clinicopathological features of inflammatory bowel disease in mice by blocking STAT3 signaling pathway, Int. Immunopharmacol. 17 (2013) 314–320, http://dx.doi.org/10.1016/j.intimp.2013.06.020.

[6] C.J.S. Dombeli, V. Fattal, G. Geretti, F.T.M.C. Vicentini, R. Casagrande, M.M. Baracat, A.W. Verri, Quercetin-loaded microcapsules ameliorate experimental colitis in mice by anti-inflammatory and antioxidant mechanisms, J. Nat. Prod. 76 (2013) 200–208, http://dx.doi.org/10.1021/np300670w.

[7] R. Sotarauta, V. Nosova, F. Zaghari, M. Brouns, N. Vannini, Efficacy of quercetin gastronutritional intervention in prevention of ulcerative colitis in rats, Toxicol. 6 (2013) 9–12, http://dx.doi.org/10.4249/toxicon.2013-0002.

[8] S. Pande, A. Singh, P. Kumar, A. Chaudhari, G. Nareshkumar, Probiotic Escherichia coli CBF 16 producing pyrolylquinoline quinone (PQQ) ameliorates 1,2-dimethylhydrazine-induced oxidative damage in colon and liver of rats, Appl. Biochem. Biotechnol. 173 (2013) 775–786, http://dx.doi.org/10.1007/s12010-014-1669-2.

[9] I. Amrouche-Mekkib, B. Djerdjoui, N-acetylcycteine improves reduct status, mitochondrial dysfunction, mucin-depleted crypts and epithelial hyperplasia in dextran sulfate sodium-induced oxidative colitis in mice. Eur. J. Pharmacol. 691 (2012) 209–217, http://dx.doi.org/10.1016/j.ejphar.2012.06.014.

[10] A. Arzeti, M. Sans, J. Panés, F.J. Romero, J.M. Piqué, J.C. Fernández-Checa, Replenishment of glutathione levels improves mucosal response in experimental acute colitis, Lab. Invest. 80 (2000) 735–744, http://dx.doi.org/10.1038/sj.liv-2000-735.

[11] Y. You, J.-P. Fu, J. Meng, G.-D. Huang, Y.-H. Liu, Effect of N-acetylcysteine on the murine model of colitis induced by dextran sodium sulfate through up-regulating PON1 activity, Dig. Dis. Sci. 54 (2009) 1643–1650, http://dx.doi.org/10.1007/s10620-008-0656-9.

[12] A. Siddiqui, H. Ancha, D. Tedesco, S. Lightfoot, C.A. Stewart, R.F. Harty, K.A. Eaton, M. El-Zaatari, A.B. Shreiner, J.L. Merchant, C. Owyang, J.Y. Kao, Increased expression of DUOX2 is an epithelial response to mucosal dysbiosis required for immune homeostasis in mouse intestine, Gastroenterology 149 (2015) 1849–1859, http://dx.doi.org/10.1053/j.gastro.2015.07.062.

[13] G. Puri, T. Tsujikawa, A. Elkadri, C.H. Guo, D.J. Bartsch, F. Chain, S. Miquel, J.-P. Motta, N. Vergnolle, H. Sokol, P. Langella, M. Bras, A. Pouliet, S. Rakotobe, F. Ruemmele, U.G. Knaus, First ever description of probiotic Bacillus polyfermenticus on human intestinal microvascular endothel ands of mice, JCMGH.2015.06.005.

[14] L. Liu, Y.J. Liu, G.X. Liu, X. Chen, K.Y. Yang, X. Qie, H.K. Gan, X.L. Huang, H.T. Gan, Curcumin ameliorates dextran sodium sulfate-induced experimental colitis by blocking STAT3 signaling pathway, Int. Immunopharmacol. 17 (2013) 314–320, http://dx.doi.org/10.1016/j.intimp.2013.06.020.

[15] M.J. Stasia, G. Geretti, F.T.M.C. Vicentini, R. Casagrande, M.M. Baracat, A.W. Verri, Quercetin-loaded microcapsules ameliorate experimental colitis in mice by anti-inflammatory and antioxidant mechanisms, J. Nat. Prod. 76 (2013) 200–208, http://dx.doi.org/10.1021/np300670w.

[16] R. Hertzberger, D. Papkovsky, B. Bourke, U.G. Knaus, Defensive mutualism rescues bacterial responses in gut infection, Cell Host Microbe 19 (2016) 651–663, http://dx.doi.org/10.1016/j.chom.2016.04.007.

[17] S. Lipinski, A. Till, C. Sina, A. Arth, H. Grasberger, S. Schreiber, P. Rosentiel, DUOX2-derived reactive oxygen species are effectors of NOD2-mediated anti-bacterial responses, J. Cell Sci. 122 (2009) 3522–3530, http://dx.doi.org/10.1242/jcs.056969.

[18] H. Grasberger, M. El-Zaatari, D.T. Dang, J.L. Merchant, Dual oxidasues control release of hydrogen peroxide by the gastric epithelium to prevent helicobacter cells infection and inflammation in mice, Gastroenterology 145 (2013) 1045–1054, http://dx.doi.org/10.1053/j.gastro.2013.07.011.

[19] F. Cavalcanti, M. Iacobini, A.K. Singh et al. Mucosal Immunol. 8 (2015) 712–723, http://dx.doi.org/10.1016/j.mucim.2015.08.002.

[20] F. Cavalcanti, M. Iacobini, A.K. Singh et al. Mucosal Immunol. 8 (2015) 712–723, http://dx.doi.org/10.1016/j.mucim.2015.08.002.

[21] G. Puri, T. Tsujikawa, A. Elkadri, C.H. Guo, D.J. Bartsch, F. Chain, S. Miquel, J.-P. Motta, N. Vergnolle, H. Sokol, P. Langella, M. Bras, A. Pouliet, S. Rakotobe, F. Ruemmele, U.G. Knaus, First ever description of probiotic Bacillus polyfermenticus on human intestinal microvascular endothel ands of mice, JCMGH.2015.06.005.

[22] L. Liu, Y.J. Liu, G.X. Liu, X. Chen, K.Y. Yang, X. Qie, H.K. Gan, X.L. Huang, H.T. Gan, Curcumin ameliorates dextran sodium sulfate-induced experimental colitis by blocking STAT3 signaling pathway, Int. Immunopharmacol. 17 (2013) 314–320, http://dx.doi.org/10.1016/j.intimp.2013.06.020.

[23] G. Puri, T. Tsujikawa, A. Elkadri, C.H. Guo, D.J. Bartsch, F. Chain, S. Miquel, J.-P. Motta, N. Vergnolle, H. Sokol, P. Langella, M. Bras, A. Pouliet, S. Rakotobe, F. Ruemmele, U.G. Knaus, First ever description of probiotic Bacillus polyfermenticus on human intestinal microvascular endothel ands of mice, JCMGH.2015.06.005.
[84] M. Pentecost, G. Otto, J.A. Theriot, M.R. Amieva, Listeria monocytogenes invades the epithelial junctions at sites of cell extrusion, PLoS Pathog. 2 (2006) 0029–0040, http://dx.doi.org/10.1371/journal.ppat.0020003.

[85] H. Takahashi, Y. Nakano, T. Matsuoka, N. Kumaki, Y. Asami, Y. Koga, Role of indigenous lactobacilli in gastrin-mediated acid production in the mouse stomach, Appl. Environ. Microbiol. 77 (2011) 6964–6971, http://dx.doi.org/10.1128/AEM.05230-11.

[86] E. Denou, R.D. Pridmore, B. Berger, J.M. Pannf, F. Arigoni, H. Brüssow, Identification of genes associated with the long-gut-persistence phenotype of the probiotic Lactobacillus johnsonii strain NCC533 using a combination of genomics and transcriptome analysis, J. Bacteriol. 190 (2008) 3161–3168, http://dx.doi.org/10.1128/JB.01637-07.

[87] M.A. De Groote, D.N. Frank, E. Dowell, M.P. Glode, N.R. Pace, Lactobacillus rhamnosus GG bacteremia associated with probiotic use in a child with short gut syndrome, Pediatr. Infect. Dis. J. 24 (2005) 278–280 (doi:00006454-200503000-00022) (pii).

[88] M.K. Salminen, S. Tynkkynen, H. Rautelin, M. Saxelin, M. Vaara, P. Rusetu, S. Sarna, V. Valtonen, A. Järvinen, Lactobacillus bacteremia during a rapid increase in probiotic use of Lactobacillus rhamnosus GG in Finland, Clin. Infect. Dis. 35 (2002) 1155–1160, http://dx.doi.org/10.1086/343912.

[89] E. Vahabnezhad, A.B. Mochon, L.J. Wozniak, D.A. Ziring, Lactobacillus bacteremia associated with probiotic use in a pediatric patient with ulcerative colitis, J. Clin. Gastroenterol. 47 (2013) 437–439, http://dx.doi.org/10.1097/MCG.0b013e318279bb80.

[90] M. Sherid, S. Samo, S. Sulaiman, H. Husein, H. Sifuentes, S. Sridhar, Liver abscess and bacteremia caused by lactobacillus: role of probiotics? Case report and review of the literature, BMC Gastroenterol. 16 (2016) 138, http://dx.doi.org/10.1186/s12877-016-0552-y.

[91] M. Doufair, C. Eckert, L. Drieux, C. Amani-Moibeni, L. Bodin, M. Denis, J.D. Grange, G. Arlet, F. Barbut, Clostridium difficile bacteremia: report of two cases in French hospitals and comprehensive review of the literature, IDCases 8 (2017) 54–62, http://dx.doi.org/10.1016/j.idcr.2017.03.012.

[92] S.C. Trevelin, D. Carlos, M. Beretta, J.S. da Silva, F.Q. Cunha, Diabetes Mellitus and Septis, SHOCK 47 (2017) 276–287, http://dx.doi.org/10.1097/SHK.0000000000000778.

[93] C.D. Percione, K. Overweg, P.W.M. Hermans, J.N. Weiser, Inhibitory and bactericidal effects of hydrogen peroxide production by Streptococcus pneumoniae on other inhabitants of the upper respiratory tract, Infect. Immun. 68 (2000) 3990–3997, http://dx.doi.org/10.1128/IAI.68.7.3990-3997.2000.

[94] S. Kawasaki, T. Satoh, M. Todoroki, Y. Niimura, 8-type dihydroorotate dehydrogenase is purified as a H2O2-forming NADH oxidase from Bifidobacterium bifidum, Appl. Environ. Microbiol. 75 (2009) 629–636, http://dx.doi.org/10.1128/AEM.02111-08.

[95] U.V. Wesley, P.F. Bove, M. Hristova, S. McCarthy, A. Van Der Vliet, Airway epithelial cell migration and wound repair by ATP-mediated activation of dual oxidase 1, J. Biol. Chem. 282 (2007) 3213–3220, http://dx.doi.org/10.1074/jbc.M606533200.

[96] S.H. Gorissen, M. Hristova, A. Habibovic, L.M. Sipsey, Y.M.W. Janssen-Heininger, A. Van Der Vliet, Dual oxidase-1 is required for airway epithelial cell migration and bronchiolar reepithelialization after injury, Am. J. Respir. Cell Mol. Biol. 48 (2013) 337–345, http://dx.doi.org/10.1165/rcmb.2012-0393OC.